R-RAS PROTEINS AND TGF-BETA IN CANCER

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To my infinitely supportive family and mentors

Thank you

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CHAPTER I

INTRODUCTION

Overview

Cancer is characterized by uncontrolled cell growth and the spread of these abnormal cells (Hanahan and Weinberg, 2000). Over time, carcinogens induce individual cells to acquire genetic and epigenetic changes in signaling pathways that regulate their growth and proliferation. These alterations change normal cells into malignant cells, a process that is referred to as "transformation". Transformed cells gain a distinct growth advantage over their neighbors and subsequently out-compete them for space and nutrients. These cells stop responding to natural mechanisms of growth regulation and acquire the ability to proliferate independent of their anchorage. Modified genes that can induce cellular transformation are known as oncogenes (Croce, 2008) and include various growth factors, receptor and non-receptor tyrosine kinases, transcription factors and regulatory GTPases.

Ras genes were originally identified as retroviral oncogenes in the 1960's and 70's following observations that viruses could induce tumor formation in mice and rats (Chang et al., 1982). The viral genes found to be responsible were called *ras*, for rat sarcoma, and turned out to be mutated versions of genes encoding enzymes with intrinsic GTPase activity. Ras research gained momemtum in the early 1980's with the discovery of activating Ras mutations in human tumors (Taparowsky et al., 1982). It was also during the early 1980's that transforming growth factor-beta (TGF-β) was identified as a polypeptide factor secreted from chemically or virally transformed fibroblasts with the potential to transform normal fibroblasts in classical *in vitro* assays (Moses et al., 1981). Soon after its discovery, it was shown that TGF-β could also act as an inhibitor of cell proliferation (Holley et al., 1985), thus establishing a dual role for TGF-β in the regulation of cell growth. Following these observations, it was demonstrated that a relationship

exists between Ras and TGF-β with respect to cellular transformation (Schwarz et al., 1988), and since then an increasing number of links between Ras and TGF-β-signaling have been identified in the context of cancer progression (Janda et al., 2002; Oft et al., 1996).

Most of the interactions between Ras and TGF-β that have been described previously have involved prototypic H-Ras, the founding member of the Ras superfamily. Since its discovery, the Ras superfamily has grown to over 170 small GTPases (Colicelli, 2004), however, only a small percentage of these have been shown to act as oncogenes. R-Ras and TC21(R-Ras2) are two highly homologous Ras-related GTPases with the potential to induce oncogenic transformation in mammalian cells, yet little is known of the mechanisms through which they act or whether they interact with TGF-β-signaling.

The focus of my research has been to investigate the transforming properties of R-Ras and TC21 using a murine epithelial model of tumorigenesis, and to determine whether TGF-β has a modulatory role in cells transformed by these oncogenes. My goal was to identify signaling mechanisms responsible for R-Ras and TC21-induced transformation, and to find mechanistic links between these oncoproteins and TGF-β-signaling. In chapter one, I will describe how R-Ras and TC21 signal under normal conditions and in cancer cells. I will go on to describe the TGF-β-signaling pathway and to illustrate various mechanisms that have been reported to link Ras and TGF-β. Focusing initially on TC21, chapter two will examine the transforming potential of TC21 in mammary epithelial cells relative to prototypic H-Ras. It will go on to identify downstream signaling pathways that mediate the transforming effects of oncogenic TC21. Chapter three will examine the transforming potential of R-Ras in EpH4 cells and how it compares with TC21, drawing emphasis on differences in signaling events that mediate R-Ras and TC21-transformation. In chapter four, I will describe the role of TGF-β-signaling in R-Ras- and TC21-transformed cells. I will go on to suggest a novel mechanism that links R-Ras- and TGF-β-signaling. In chapter five, I will conclude by discussing how my work is relevant to the field of

cancer biology, and by highlighting potential future experiments that will allow for better understanding of the roles played by R-Ras, TC21 and TGF-β in tumorigenesis.

The Development of Cancer

Cancer is a disease state where uncontrolled cell growth progressively impedes normal bodily functions and ultimately results in mortality (Hahn and Weinberg, 2002). The process through which a normal cell becomes a cancer cell is commonly referred to as transformation. There are several criteria that are used to define cellular transformation (Heeg et al., 2006). These include morphological changes, anchorage-independent growth, loss of contact-inhibition, and the ability to form tumors when transplanted into nude mice. Oncogenes encode proteins that have the potential to induce cellular transformation. These genes act in a dominant fashion, either through overexpression or activating mutations.

The *ras* gene has been the focus of great attention since it was shown to be activated by mutation in a significant percentage of human tumors (Bos et al., 1987). In addition to the activation of oncogenes, cellular transformation can also result from the inactivation of tumor suppressor genes such as *p53* (Somasundaram, 2000). Furthermore, cancer cells can acquire other aberrations that favor their growth in the complex environments of living tissues, including the ability to recruit blood vessels into the tumor mass (Goh et al., 2007), evade and overcome immune defenses (Yang, 2007) and invade and metastasize (Gupta et al., 2005). This work will focus on the ability of two Ras-related proteins to induce cellular transformation and the role of TGF-β in this process.

The Ras Superfamily of Small GTPases

Ras GTPases are signal-switch molecules that mediate signaling pathways responsible for a variety of cell functions including growth, adhesion, migration, cytoskeletal integrity, differentiation and survival (Takai et al., 2001). These proteins share significant homologies and

common motifs, making them nearly identical in tertiary structure. As enzymes with intrinsic GTPase activity, Ras proteins are characterized by their capacity to cycle between "active" guanine triphosphate (GTP)-bound and "inactive" guanine diphosphate (GDP)-bound conformations. Once GDP is exchanged for GTP, two key regions termed Switch I and Switch II undergo allosteric changes which alter the configuration of the so-called effector loop and enable interaction with downstream effectors. This cycle is illustrated schematically below in Figure 1.

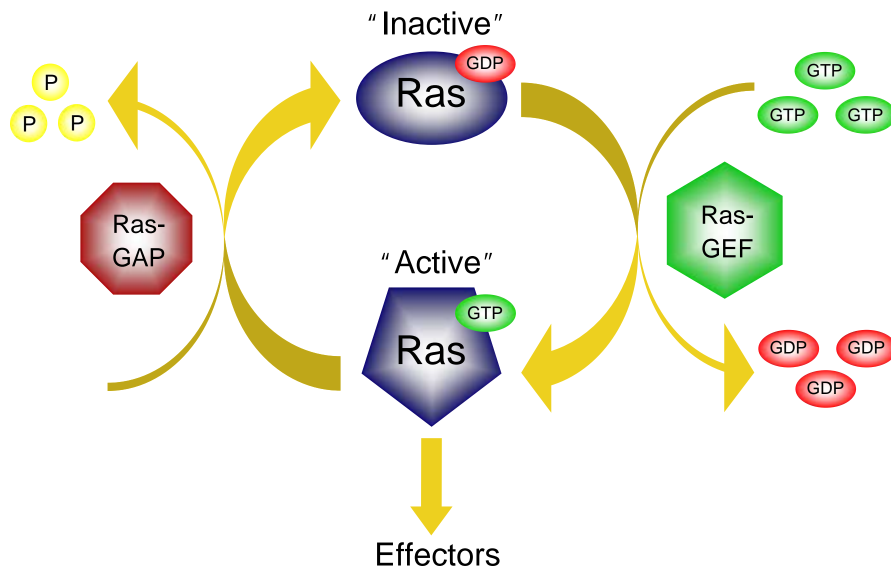


Figure 1. ***The GTPase cycle.***

The activation state of Ras is regulated by the activity of guanine nucleotide exchange factors (GEFs) which promote the exchange of GDP for GTP, and by GTPase activating proteins (GAPs) which catalyze the hydrolysis of GTP to GDP and induce an allosteric shift back to the "inactive" conformation. In addition to catalyzing the activation of GTPases, GEFs can also determine the signaling output of the activated GTPase by forming a scaffolding platform for

specific downstream effectors (Buchsbaum et al., 2002). GAPs act by inserting an "arginine finger" into the GTPase which stabilizes the transition state of the GTPase reaction, thereby dramatically increasing the hydrolysis rate (Ahmadian et al., 2003). While Ras effectors are defined as proteins with strong affinity for GTP-bound Ras and whose binding is impaired by mutations within the core effector domain, recent observations have shown that GDP-bound Ras can also interact with several effector proteins and modulate downstream signaling events. For example, Ras-GDP binds to the transcription factor Aiolos, thereby modulating the nuclear translocation of Aiolos and the expression of the anti-apoptotic protein Bcl-2 (Romero et al., 1999).

The normal function of Ras proteins requires them to be post-translationally modified (Konstantinopoulos et al., 2007). These modifications serve primarily to localize the proteins to the correct subcellular compartment, principally the inner face of the plasma membrane. Ras proteins that are mislocalized within the cell are inactive, most likely because they are unable to recruit their target enzymes. The fact that correct post-translational modification of Ras is required for its biological activity has made the enzymes involved in this processing very attractive targets for therapeutic intervention (Kloog and Cox, 2004).

Ras family members exhibit a carboxy-terminal CAAX motif, where a cysteine is followed by two aliphatic residues and one random amino acid. This motif is targeted for processing by enzymes which results in the addition of a carboxy-terminal prenyl group. This group, either a farnesyl or a geranylgeranyl moiety, is involved in anchoring the Ras protein to the plasma membrane. The specificity for either farnesylation or geranylgeranylation is mainly determined by the last residue of the CAAX sequence (Michaelson et al., 2005). When X is a serine, methionine, glutamine, alanine or threonine residue the GTPase is farnesylated, whereas a leucine or phenylalanine residue results in gernaylgeranylation. Inhibitors of the enzymes involved in this prenylation process show promise as inhibitors of Ras function by blocking its

localization to the plasma membrane (Martin et al., 2007). The C-termini of both classical Ras proteins and members of the R-Ras family are compared in Figure 2.

	183 C-terminus 208
M-Ras	NQKKKKKTKWRGDRATGTHKLQCVIL
R-Ras	QEQELPPSPPSAPRKKDGGC-PCVLL
R-Ras2 (*Hs*)	QEQECPPSPEPTRKEKDKKGCHCVIF
H-Ras	KLRKLNPPDESGPGCMSC---KCVLS
N-Ras	RLKKLNSSDDGTQGCMGS---PCVLM
K-RasA	RLKKI-SKEEKTPGCVKIK--KCVDM
K-RasB	K-EKM-SKDGKKKKKKSRT--RCTVM

Figure 2. ***C-termini sequences of Ras and Ras-related proteins.*** Comparison of the amino acid sequence of the C-terminus (residues 183-208) of R-Ras, TC21(R-Ras2), M-Ras(R-Ras3), H-Ras, N-Ras, and K-Ras A/B. All sequences are murine with the exception of R-Ras2 (human). Gaps are indicated by a hyphen (-). The CAAX-motifs are boxed. Shaded areas indicate regions of homology between M-Ras (dark gray) and the proline-rich R-Ras box (medium gray) and conserved regions in H-, N-, and K-Ras A (light gray). Poly-lysine motifs in M-Ras and K-RasB are underlined. Cysteine residues that might be palmitoylated are double underlined.

Prenylation alone is insufficient for the functional anchorage of Ras proteins into the plasma membrane, and additional molecular signals are required (Laude and Prior, 2008). The nature of these signals dictates the route by which Ras proteins reach the plasma membrane, and whether they localize to lipid rafts or to the disordered membrane (Apolloni et al., 2000). One such signal is palmitoylation, and the carboxy-terminus of H-Ras includes cysteine residues that undergo further lipid modification by the attachment of palmitoyl moieties which extend far into the plasma membrane (Dudler and Gelb, 1996). Besides subcellular localization, both the intensity and the duration of Ras-signaling profoundly influence downstream signaling networks and the final signal output (Rocks et al., 2006).

The Ras superfamily can be distinguished into six major groupings, namely the Ras, Rho, Ran, Rab, Arf and Kir/Rem/Rad families (Takai et al., 2001). The relations between these groups are represented in Figure 3 as a phylogenetic tree of Ras family members.

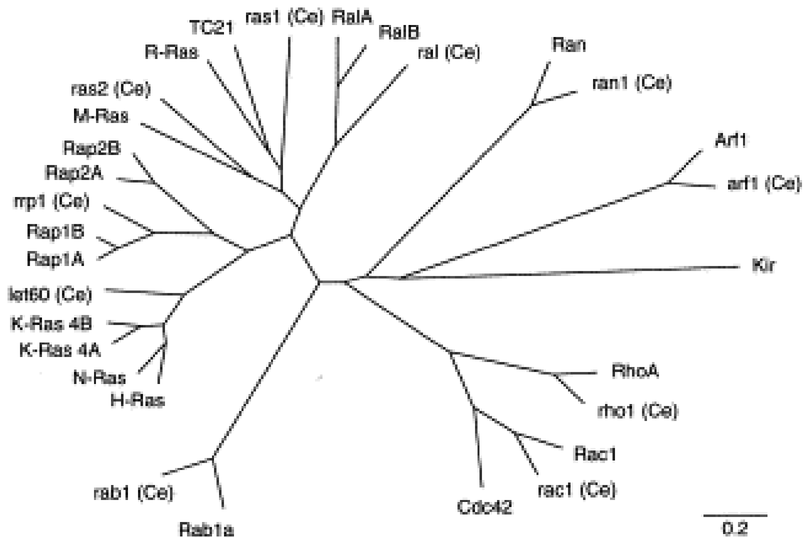


Figure 3. ***The Ras superfamily.***

Of all the Ras family members, the “classical” Ras proteins (H-, K- and N-Ras) are the best understood. These isoforms share 85% amino acid identity and are frequently mutated in human tumors (Moon, 2006). Roughly 20% of human tumors have activating point mutations in Ras, most frequently in K-Ras (about 85% of total), then N-Ras (about 15%), then H-Ras (less than 1%). These mutations are predominantly located near the bound nucleotide, with codons 12, 13, and 61 being the most common targets (Rajalingam et al., 2007). The effect of these mutations is to decrease the intrinsic rate of GTP hydrolysis by Ras, and to make the Ras protein

significantly less sensitive to GAP-stimulated GTP hydrolysis. Thus, the protein is predominantly in a GTP-bound conformation and therefore constitutively active.

Oncogenic variants of the classical Ras proteins are capable of transforming both immortalized rodent fibroblasts and epithelial cells, causing these cells to appear spindle shaped with disorganized actin filaments, to lose contact inhibition, and to gain the ability to proliferate independent of anchorage or in low serum conditions (Moon, 2006). Ras-signaling can also be activated in tumors by the loss of GAPs (Lee and Stephenson, 2007), or where growth-factor-receptor tyrosine kinases such as EGFR or ERBB2 have been overexpressed (Higashiyama et al., 2008). Apart from the classical Ras proteins, only members of the R-Ras subfamily of Ras-related proteins have been reported to induce oncogenic transformation in mammalian cells (Saez et al., 1994) and found to be mutated or aberrantly expressed in human tumors (Clark et al., 1996).

The R-Ras Family

The three members of the R-Ras family, namely R-Ras, TC21(R-Ras2) and M-Ras(R-Ras3), share 50-55% sequence homology with the classical Ras proteins (Ehrhardt et al., 2002). Like Ras, constitutively active mutants of R-Ras proteins have been shown to promote growth and transformation, and to alter cellular differentiation (Graham et al., 1999). Thus, like Ras, R-Ras proteins serve as components of signaling pathways that regulate cell growth and differentiation. R-Ras proteins share functions with Ras proteins at two levels. First, as some Ras GEFs can also serve as GEFs for R-Ras proteins (Ohba et al., 2000), both classical Ras proteins and R-Ras proteins may be activated in a coordinate fashion by the same extracellular stimuli. Second, R-Ras proteins can interact with a number of Ras effectors (Ehrhardt et al., 2002), suggesting they may also activate the same signaling pathways as Ras. Despite possessing strong structural and biochemical similarities, R-Ras proteins are thought to regulate cellular processes distinct from those mediated by classical Ras proteins (Huang et al., 2004).

It has been suggested that M-Ras may reside on a discrete branch of the R-Ras phylogenetic tree (Ohba et al., 2000). This is because in contrast to R-Ras and TC21, M-Ras lacks a C-terminal cysteine residue that is necessary for palmitoylation, as well as a proline-rich motif characteristic of the C-termini in R-Ras and TC21 termed the "R-Ras box". In addition, M-Ras differs from R-Ras and TC21 in its tissue distribution (Zhang et al., 2004), and has been shown to be regulated by a set of GEFs and GAPs which differ from those for R-Ras and TC21 (Ohba et al., 2000). The transforming activity of oncogenic M-Ras is relatively low, which may be due to its weak activation of Ras effector pathways (Ward et al., 2004), and mutations in M-Ras have yet to be identified in human tumors. This research will focus on the role of R-Ras and TC21 in cancer progression.

The R-Ras Oncogene

The founding member of the R-Ras family was originally isolated by low-stringency hybridization using a v-H-*ras* probe (Lowe et al., 1987). The human R-Ras gene was found to encode a 218 amino acid polypeptide that shared 55% sequence homology with prototypic H-Ras. Mutation in R-Ras at position 38 is analogous to the position 12 mutation in oncogenic H-Ras and results in constitutive R-Ras activation (Cox et al., 1994). In addition, mutation in R-Ras at position 87 is analogous to position 61 in H-Ras and also activates the oncogenic potential of R-Ras (Saez et al., 1994). While R-Ras mutations have yet to be identified in human tumors, aberrant expression of R-Ras has been identified in gastric cancers (Nishigaki et al., 2005) and constitutive R-Ras activation was shown to promote the metastasis of cervical cancer epithelial cells (Mora et al., 2007).

The functions of R-Ras are poorly understood at present. R-Ras has been reported to have opposite effects to Ras in that it can promote 32D myeloid apoptosis in response to interleukin-3 withdrawal (Wang et al., 1995) and block the Ras-mediated suppression of integrin function (Sethi et al., 1999). R-Ras is also thought to play roles in the branching morphogenesis of ureteric

bud cells (Pozzi et al., 2006), in the process of exocytosis on endosomes (Takaya et al., 2007), in the regulation of skeletal myogenesis (Suzuki et al., 2000), and in the control of apoptosis through its interaction with Bcl-2 (Cox et al., 1994). R-Ras activity also promotes migration and invasion in breast epithelial carcinoma cells (Keely et al., 1999). One major difference between R-Ras and the classical Ras proteins is the presence of an additional 26 amino acids at the N-terminus of R-Ras, which are thought to mediate Rac activation and the R-Ras-dependent migration of 32D mouse myeloid cells (Holly et al., 2005).

The transforming efficiency of R-Ras is low when compared with other Ras oncogenes (Saez et al., 1994). Activated mutants of R-Ras induce cellular transformation in NIH 3T3 fibroblasts but are unable to transform Rat-1 fibroblasts (Cox et al., 1994). The distinct functions of R-Ras would suggest that its effectors differ from those located downstream of the classical Ras proteins (Huff et al., 1997). Unlike Ras, R-Ras is an inefficient activator of the mitogen activated protein kinases (MAPKs) Raf1, RalGDS, Erk1/2, JNK and p38 MAPK, however this appears to be cell-type dependent (Marte et al., 1997; Mochizuki et al., 2000; Self et al., 2001). One important Ras effector that is shared by R-Ras is phosphatidylinositol-3 kinase (PI3K), which has kinase activity against both lipids and proteins (Cantley, 2002). The catalytic p110 subunit of PI3K interacts directly with GTP-bound R-Ras (Suzuki et al., 1997). By catalyzing the production of 3′ phosphorylated phosphatidylinositols, PI3K targets proteins that contain pleckstrin homology domains to the plasma membrane where they can be activated (i.e. by phosphorylation) and mediate their respective effects. These proteins include the serine/threonine kinases Akt/PKB (Manning and Cantley, 2007) and the mammalian Target of Rapamycin (mTOR) (Hay, 2005).

The activation state of R-Ras is regulated by a set of GAPs and GEFs which differ from those utilized by the classical Ras proteins with a few examples. Negative regulators of R-Ras activity include p120 RasGAP, GAP1m, GAPIII and GAP1(IP4BP) (R-Ras GAP), while R-Ras has been shown to be strongly activated by the GEFs RasGRF1, RasGRP1 (CalDAG-GEF II),

RasGRP3 (CalDAG-GEF III), and weakly by CalDAG-GEF I (Ohba et al., 2000). p120 RasGAP is an example of a GAP that is shared by both H-Ras (Grewal et al., 2005) and R-Ras. The specificity of different GEFs for particular Ras family members may result from differences in the helix 3 region of the Ras protein (residues 91-103 in H-Ras) as differences in this region between H-Ras and R-Ras appear to account for the ability of RasGRF1 but not Sos1 to act on R-Ras (Tian and Feig, 2001).

Another factor affecting GEF specificity may be the nature of the prenyl groups. While both H-Ras and R-Ras undergo palmitoylation, R-Ras possesses a different C-terminal prenylation motif for post-translational modification and undergoes geranylgeranylation rather than farnesylation like H-Ras (Hansen et al., 2002). The importance of this modification on the activation state of the protein was demonstrated in a study where the most carboxy-terminal residue of R-Ras was changed from leucine to serine (as in H-Ras). As a result, R-Ras underwent farnesylation rather than geranylgeranylation and became responsive to the GEF activity of RasGRF2 which is specific for farnesylated GTPases (Gotoh et al., 2001).

The functional difference between virtually identical GTPases can be explained by their differential localization in cells, which is mainly determined by the C-terminal hypervariable region. In R-Ras, a stretch of proline residues at the C-terminus was found to bind the SH3 domain of the adaptor protein Nck, and changing the proline residues at positions 202 and 203 disrupted R-Ras-induced cell-extracellular matrix adhesion (Wang et al., 2000), suggesting that the hypervariable-domain-mediated R-Ras-Nck interaction is important for R-Ras-mediated cell adhesion. The hypervariable domain of R-Ras is also crucial for proper localization of the protein to focal adhesions, since replacement of this domain by that of H-Ras or K-Ras alters its intracellular localization (Furuhjelm and Peranen, 2003). Furthermore, an R-Ras mutant that cannot undergo palmitoylation is no longer present in focal adhesions even though this mutant still has an intact hypervariable domain (Furuhjelm and Peranen, 2003). It might be that palmitoylation regulates the translocation of R-Ras from the Golgi to the plasma membrane,

which is followed by the targeting of R-Ras to focal adhesions through binding to Nck. These findings suggest that both lipid modifications, as well as GTPase-adaptor protein interactions, are necessary for the proper subcellular localization of R-Ras.

The TC21 Oncogene

TC21 was originally identified in a search for Ras-related genes using degenerate oligonucleotide PCR techniques from a human teratocarcinoma (TC) cDNA library (Drivas et al., 1990). The TC21 gene encodes a 203 amino acid polypeptide that shares 55% homology with the classical Ras proteins and 89% amino acid homology with its close relative R-Ras, including complete identity in the effector domain. Mutations in TC21 at positions 23 and 72 are analogous to codons 12 and 61 of H-Ras and result in constitutive activation of the GTPase (Graham et al., 1994). TC21 is the only member of the R-Ras subfamily for which overexpression or mutated versions have been detected in human tumor cells, including cells derived from uterine sarcoma (Huang et al., 1995), ovarian (Chan et al., 1994) and mammary tumors (Barker and Crompton, 1998). Increased expression of TC21 has been found in breast cancer cells (Clark et al., 1996), as well as certain oral (Arora et al., 2005) and esophageal (Sharma et al., 2005) carcinomas. These findings suggest that deregulated TC21 activity may contribute significantly to human oncogenesis.

Like R-Ras, the functions of TC21 are poorly understood. To date, TC21 activity has been shown to mediate cell migration (Huang et al., 2004), transformation (Rong et al., 2002), and survival (McFall et al., 2001). It is the ability of TC21 to induce transformation that is most striking, however, and overexpression of mutant TC21 is highly transforming in a number of cell lines, including NIH 3T3 (Graham et al., 1994), MCF-10A (Clark et al., 1996) and RIE-1 cells (McFall et al., 2001). Mutationally active TC21 can mimic many of the transforming and differentiating activities of oncogenic Ras (Graham et al., 1999), thus it is not surprising that TC21 can interact with a majority of known Ras effectors.

There is conflicting evidence on the ability of TC21 to activate Raf and Erk1/2. Whereas one study found that TC21 fails to interact with Raf kinases (Graham et al., 1996), a second study found that TC21 was indeed an activator of the Raf/Erk effector pathway (Rosario et al., 1999). There is also conflicting data on whether TC21 can activate RalA (Murphy et al., 2002; Rosario et al., 2001). Like R-Ras, TC21 has been shown to interact directly with the p110 catalytic subunit of PI3K, preferentially in its GTP-bound conformation (Murphy et al., 2002). PI3K is a key mediator of TC21-induced transformation, and TC21 is a strong activator of the PI3K target Akt (Rong et al., 2002). TC21 can also activate the JNK and p38 MAPK arms of the MAPK cascade (Graham et al., 1999), as well as NF-κB (Rong et al., 2002) and phospholipase C epsilon (Murphy et al., 2002). In addition, activated TC21 has been shown to strongly interact with RalGDS, RGL, RGL2/Rlf and AF6 (Murphy et al., 2002).

TC21 has been shown to share the same set of regulatory GAPs and GEFs as R-Ras (Ohba et al., 2000). Negative regulators of TC21 activity include p120 RasGAP, GAP1m, GAPIII and GAP1(IP4BP) (R-Ras GAP), while positive regulators include the GEFs RasGRF1, RasGRP1 (CalDAG-GEF II), RasGRP3 (CalDAG-GEF III) and PLCε. Unlike R-Ras, which is geranylgeranylated but not farnesylated, TC21 undergoes post-translational modification by both farnesylation and geranylgeranylation (Hartman et al., 2005). TC21 also differs from R-Ras as it lacks the additional 26 amino acid N-terminal motif found in R-Ras. Beyond the differences mentioned above, it is unclear how R-Ras and TC21 differ in terms of their function and mode of activity within the cell, or whether they exhibit interactive behavior with TGF-β-signaling.

The TGF-β-Signaling Network

TGF-β was originally discovered in the cell culture media of sarcoma virus transformed mouse fibroblasts as an activity that induced the anchorage-independent growth of nonmalignant cells (Moses et al., 1981). In subsequent studies, it was demonstrated that TGF-β can act as a potent tumor suppressor and inhibitor of cell proliferation (Holley et al., 1985). To date, the

TGF-β superfamily of growth factors has grown to more than 35 structurally related secreted polypeptides, including TGF-β's, activins and bone morphogenetic proteins (BMPs) (Bierie and Moses, 2006). These TGF-β family members have diverse roles in the regulation of processes such as proliferation (Huang and Huang, 2005), differentiation (Moses and Serra, 1996), and extracellular matrix deposition (Verrecchia and Mauviel, 2007). Abnormal TGF-β-signaling has been implicated in a number of pathological conditions, including cancer (Neil et al., 2006).

There are three isoforms of TGF-β (TGF-β1, TGF-β2 and TGF-β3) which are ubiquitously expressed in mammalian tissues (Sporn, 2006). These isoforms are secreted as biologically inactive precursors called latent TGF-β's (L-TGF-β). The formation of biologically active TGF-β requires the cleavage of the precursor protein latency-associated peptide from the L-TGF-β. Under physiological conditions, TGF-β's are activated by an acidic cellular microenvironment and by proteolysis from the activity of enzymes such as plasmin and matrix metalloproteinases (MMPs) (Hyytiainen et al., 2004). Once active, TGF-β's are able to bind with their cognate receptors.

There are 3 types of TGF-β receptors: type I, type II and type III (Derynck and Feng, 1997). The type I and type II receptors are very similar transmembrane glycoproteins, each containing a glycosylated extracellular domain, a short transmembrane domain, and an intracellular serine/threonine kinase domain. The type II TGF-β receptors are constitutively autophosphorylated on various serines, while the type I receptors are characterized by a conserved region rich in glycine and serine (GS-region) which precedes the kinase domain (Wieser et al., 1995). To initiate signal transduction, the formation of a heterotetrameric complex between type I and II receptors is required. The TGF-β ligand binds to the extracellular domain of the type II receptor, which subsequently recruits and binds the type I receptor. This leads to cross-phosphorylation of the GS domain in the type I receptor by the type II receptor kinase, resulting in the activation of type I receptor kinase domain and the initiation of downstream signaling events (Wrana et al., 1994).

The growth-inhibitory effects of TGF-β are mediated through the Smad pathway (Massague et al., 2005). The mammalian Smad family consists of 8 members that can be divided into 3 groups according to their function: receptor-activated Smads (R-Smads), common-mediator Smads (Co-Smads) and inhibitory Smads (I-Smads). Smad1, -2, -3, -5 and -8 belong to the group of receptor-activated Smads, Smad4 is a common-mediator Smad, and Smad6 and Smad7 belong to the inhibitory Smad group. Smad1, -5 and -8 mediate signals downstream of BMPs, whereas Smad2/3 mediate signals triggered by TGF-β's and activins. In general, Smads are widely expressed in most adult tissue and cell types (Lange et al., 1999), suggesting the importance of TGF-β-signaling in tissue development and homeostasis.

Smads are modular proteins with conserved N-terminal Mad-homology 1 (MH1), intermediate linker and C-terminal MH2 domains (Inman, 2005). The catalytically active TβRI phosphorylates the C-terminal serine residues of the R-Smads (Smad2/3), which then exhibit high affinity for the Co-Smad (Smad4) which is not phosphorylated by receptors but rapidly oligomerizes with phosphorylated Smad2 and Smad3, thus forming functional trimeric protein complexes. The linker domain accepts regulatory phosphorylations by other signaling kinases including JNK (Sekimoto et al., 2007), p38 MAPK and Rho/ROCK (Kamaraju and Roberts, 2005). All monomeric Smad proteins constantly shuttle in and out of the nucleus, but the formation of the receptor-activated R-Smad/Co-Smad complexes favors their nuclear accumulation (Schmierer and Hill, 2005). In the nucleus, the active R-Smad/Co-Smad complexes bind directly to DNA (Smad-binding elements) and associate with a plethora of co-activators or co-repressors that lead to transcriptional induction or repression of a diverse array of genes (roughly 500 in mammalian cells) (Ranganathan et al., 2007). It is through this Smad-mediated transcriptional regulation that TGF-β is able to arrest the cell cycle of epithelial, endothelial and hematopoietic cells at the early G1 phase.

In addition to activation of the Smad pathway, TGF-β increases the activity of several other intracellular pathways (Moustakas and Heldin, 2005). These include elements of the MAPK

cascade such as JNK (Wang et al., 1997) and p38 MAPK (Yu et al., 2002), the TGF-β Associated Kinase 1 (TAK1) (Shibuya et al., 1996), PI3K/Akt (Bakin et al., 2000), p70S6K (Petritsch et al., 2000), mTOR (Lamouille and Derynck, 2007), and some GTPases of the Ras superfamily such as RhoA (Bhowmick et al., 2001). An oversimplified map of various TGF-β-signaling pathways is shown below in Figure 4.

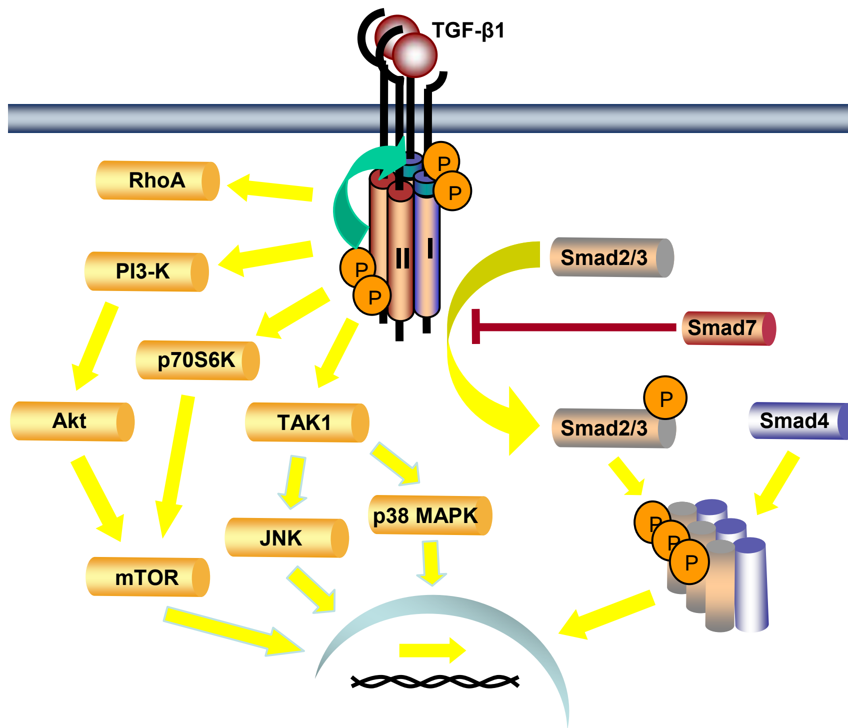


Figure 4. ***TGF-β-signaling pathways (simplified).***

The Smad-independent transducers of TGF-β-signaling also have the potential to regulate the Smad pathway itself (Sekimoto et al., 2007), or to mediate signal transduction by other growth or morphogenetic factors (Shi and Massague, 2003). When specific components of the

TGF-β-signaling pathway are downregulated or overexpressed, as is the case in many human cancers, the growth-inhibitory effects of the pathway are lost (Pardali and Moustakas, 2007). A reduced response of tumor cells to TGF-β-signaling often accompanies an increase in secretion of this ligand. It has been demonstrated in breast cancer patients with poor prognosis that TGF-β1 levels are often elevated in plasma, tumor cells, and associated stroma. Furthermore, the TGF-β1 ligand is known to induce epithelial-to-mesenchymal transition (EMT), resulting in the loss of epithelial polarity, disruption of cellular adhesion, and tumor cell invasion. EMT in response to TGF-β1 is characterized phenotypically by downregulation of epithelial markers such as E-cadherin, specific keratins and ZO-1, and upregulation of mesenchymal markers such as fibronectin, Fsp1, α-smooth muscle actin and vimentin. Interestingly, epithelial cells transformed with oncogenic Ras lose their responsiveness to the growth-inhibitory effects of TGF-β, and respond to exogenous TGF-β1 ligand by undergoing EMT (Oft et al., 1996).

TGF-β and Ras in Cancer

Numerous studies have demonstrated that TGF-β and oncogenic Ras can act in a cooperative fashion to promote tumorigenesis (Janda et al., 2002a; Kim et al., 2005; Kretzschmar et al., 1999), yet the mechanisms that underlie this cooperative behavior are poorly understood. One possibility is that Ras-signaling negatively regulates Smad activity through phosphorylation of the Smad linker region. Oncogenic Ras activates the MAPK Erk1/2, and this kinase can directly phosphorylate specific serine residues in the linker domain of Smad2 and Smad3, which results in cytoplasmic retention of the Smads and blockage of their physiological nuclear function (Kretzschmar et al., 1999). This model of linker phosphorylation has been challenged by a number of independent investigations that have analyzed the modulation of Smad-signaling induced by mitogenic stimuli. In one study, it was demonstrated that linker phosphorylation of Smad 2 and Smad3 by the p38 MAPK and Rho/ROCK pathways was required for the growth-inhibitory effect of TGF-β in human breast cancer cells (Kamaraju and Roberts, 2005). In a

second study, it was found that JNK-dependent phosphorylation of the Smad2/3 linker region correlated with increased invasion and metastasis in sporadic colorectal adenocarcinomas (Yamagata et al., 2005). This observation was supported by a third study which reported that JNK-dependent phosphorylation of the Smad2/3 linker region elicited TGF-β-dependent tumor growth and invasion in rat gastric epithelial cells (Sekimoto et al., 2007). These reports suggest that TGF-β-mediated growth inhibition may require Smad linker phosphorylation, and that oncogenic TGF-β-signaling may result from the functional collaboration of Ras and Smad rather than from Ras-mediated inhibition of the Smad pathway. In addition to these findings, it has been shown that oncogenic H-Ras can block TGF-β-signaling through MAPK-dependent downregulation of Smad4 (Saha et al., 2001). The downregulation of Smad-signaling activity by oncogenic Ras remains an interesting possibility that deserves further attention. Additionally, cell type specificity or quantitative differences in the strength of Ras-signaling between the various systems may explain the observed discrepancies.

A second possible mechanism underlying cooperative behavior between TGF-β and Ras involves the abnormal targeting of TGF-β-mediated cell cycle proteins by oncogenic Ras. For example, it has been reported that oncogenic N-Ras(K61) can disrupt TGF-β-mediated growth inhibition in epithelial cells through mislocalization of the cell cycle inhibitor p27 and cyclin-dependent kinase (CDK) 6 (Liu et al., 2000). Furthermore, it was shown that leukemia cell lines bearing an activating mutation in N-Ras(L61) lack a G_0-G_1 arrest in response to TGF-β due to a lack of p27, which is targeted for degradation by N-Ras through a MAPK-dependent pathway (Schepers et al., 2005). It was also demonstrated that TGF-β-dependent proliferation in U9 colon carcinoma cells requires H-Ras-mediated downregulation of the cell cycle inhibitor p21cip1 (Yan et al., 2002). One key event in the TGF-β antiproliferative program is the inhibition of Myc expression (Orian and Eisenman, 2001), and it was shown that transformation of MCF-10A human mammary epithelial cells with oncogenic H-Ras results in the loss of the growth-inhibitory response to TGF-β, and that this loss correlates with a loss of Myc repression (Chen et

al., 2001). These data suggest that oncogenic Ras may block TGF-β-mediated growth inhibition at the level of the cell cycle, either by sequestering the activity of cell cycle inhibitors such as p27, or by enabling the proliferative activity of cell cycle proteins such as Myc.

A third possible mechanism of interaction between Ras and TGF-β may lie at the level of the TGF-β receptor complex itself. Studies in rat intestinal epithelial (RIE) cells have demonstrated that transformation with oncogenic H-Ras results in a 5- to 10-fold decrease in mRNA and protein levels of the type II TGF-β receptor (TBRII) (Bulus et al., 2000). However, studies also show that signaling through the TBRII is required for H-Ras-induced tumor growth in mammary epithelial cells (Oft et al., 1998). Taken together, these findings would suggest that very few receptors are required to transduce the effects of TGF-β ligand on Ras-transformed cells. In addition to downregulating TBRII expression, oncogenic Ras can also stimulate the increased production of TGF-β1 ligand (Cosgaya and Aranda, 1996). Furthermore, downstream targets of both TGF-β and Ras-signaling such as JNK (Ventura et al., 2004) and p38 MAPK (Gruden et al., 2000) also have the ability to regulate TGF-β1 expression levels.

A fourth possible mechanism to explain the cooperative behavior observed between TGF-β and oncogenic Ras is that they synergistically activate kinases within signaling pathways mediating cell growth and transformation. These kinases include Raf (Janda et al., 2006), Erk1/2 (Janda et al., 2002a) , JNK (Sekimoto et al., 2007), p38 MAPK (Kim et al., 2005), PI3K (Gotzmann et al., 2002), Akt (Chen et al., 1998) and mTOR (Lamouille and Derynck, 2007). Studies have shown that TGF-β and oncogenic H-Ras cooperatively activate Erk1/2 and PI3K-signaling in transforming EpH4 cells (Janda et al., 2002a), and that coordinate activation of Erk1/2 and p38 MAPK is required for TGF-β-mediated migration and invasion in H-Ras-transformed MCF-10A cells (Kim et al., 2005). These findings would suggest that stimulation of these growth promoting pathways by either TGF-β or Ras alone is insufficient to produce a transformed phenotype, while combined stimulation results in cellular transformation. The fact that both R-Ras and TC21 have been reported to activate all of the kinases listed above would

suggest that TGF-β may also show cooperative behavior with these Ras-related GTPases through coordinate activation of growth promoting signaling pathways.

The EpH4 Model System

The EpH4 murine mammary epithelial cell line was originally derived from spontaneously immortalized cells isolated from the fourth mammary glands of mid-pregnant BALB/c mice (Reichmann et al., 1989). These nontumorigenic cells display a stable, polarized epithelial phenotype in 2D culture (Reichmann, 1994), form organotypic, tubular structures in 3D culture (Reichmann et al., 1989), and undergo G_1 cell cycle arrest in response to exogenous TGF-β (Petritsch et al., 2000). Using the EpH4 model, Oft et al. were the first to report that TGF-β and oncogenic H-Ras can collaborate to induce transformation both *in vitro* and *in vivo* (Oft et al., 1996). They also showed that H-Ras-transformed cells (EpRas) produced increased levels of TGF-β1 in an autocrine loop, which was required for their transformation, and that these cells lost their sensitivity to TGF-β-mediated growth arrest. In a subsequent study, it was demonstrated that this process of transformation by oncogenic H-Ras required TGF-β-signaling, and that tumor formation by EpRas cells required functional TBRII (Oft et al., 1998). Additional reports from Janda et al. have shown that TGF-β-dependent transformation of EpRas cells requires activation of the Raf/MAPK and PI3K-signaling pathways (Janda et al., 2002a; Janda et al., 2002b). Thus, the EpH4 model system has been well-characterized for the effects of both TGF-β-signaling and transformation by oncogenic H-Ras.

In the present work, we employed the EpH4 model system to address the transforming properties of R-Ras and TC21, and the question of whether TGF-β-signaling modulates the process of oncogenic transformation by these Ras-related proteins. Using the LZRS-GFP retroviral vector, we generated stable cell populations overexpressing activated mutants of either R-Ras(G38V), TC21(G23V), or H-Ras(G12V). Cells were also transfected with an empty vector construct to act as a control. Cell populations expressing equal amonts of GFP were then isolated

by fluorescence-activated cell sorting (FACS) (Fig. 5A), and levels of both endogenous and mutant Ras expression were confirmed by western blot analysis (Fig. 5B).

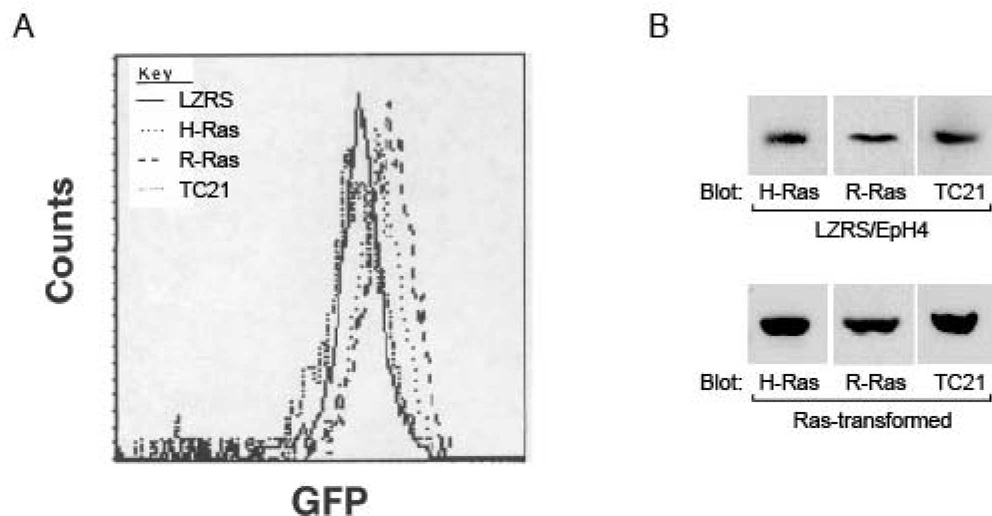


Figure 5. ***Generation of stable cell lines expressing Ras mutants*****. A.** EpH4 cells were infected with retrovirus carrying activated mutants of H-Ras, R-Ras, TC21 or empty vector (LZRS) as described under "Experimental Procedures". Cell populations that expressed equal amounts of GFP were isolated using FACS analysis. **B.** Total cell lysates (20μg) from LZRS/EpH4 or Ras-transformed cells were analyzed by Western blot analysis for levels of endogenous (*upper panel*) or mutant (*lower panel*) Ras expression using H-Ras, R-Ras and TC21 antibodies.

The primary aim of my project was to investigate the transforming properties of R-Ras and TC21 *in vivo* and *in vitro* using the EpH4 model system and to identify the signaling mechanisms mediating transformation by these oncogenes. In chapter 2, I will describe TC21-induced transformation of EpH4 cells and compare it with oncogenic transformation by

prototypic H-Ras. In chapter 3, I will examine transformation induced by oncogenic R-Ras and how it differs from TC21. Chapter 4 will describe how TGF-β-signaling interacts with R-Ras and TC21 in the transformation of EpH4 cells. To conclude, I will discuss the impact of my work on the field of cancer biology and suggest future directions for the research presented here.

CHAPTER II

SIGNALING PATHWAYS MEDIATING TC21-TRANSFORMATION

Introduction

My studies initially focused on TC21, since this GTPase has been demonstrated to hold significant oncogenic potential (Graham et al., 1994) and to be mutated in human tumors (Huang et al., 1995). TC21 has been shown to be highly transforming in both epithelial (Graham et al., 1999) and fibroblast (Graham et al., 1996) cell lines, and to induce rapid tumor formation *in vivo* (Graham et al., 1994). Increased TC21 expression is observed in breast cancer cells (Clark et al., 1996), and TC21 mutations are present in cells derived from uterine sarcoma (Huang et al., 1995), ovarian (Chan et al., 1994) and mammary tumors (Barker and Crompton, 1998). TC21 is also upregulated in oral and esophageal carcinomas (Arora et al., 2005), suggesting a correlation between TC21 expression and the early stages of tumorigenesis.

The signaling pathways activated downstream of TC21 include three members of the mitogen-activated protein kinase (MAPK) family, namely Erk1/2 (Graham et al., 1994), JNK (Graham et al., 1999) and p38 MAPK (Graham et al., 1999), as well as the protein/lipid kinase phosphoinositide 3-kinase (PI3K) (Murphy et al., 2002). Of these, only PI3K, which phosphorylates phosphoinositides to generate the second messenger lipid PIP3, is required for TC21-induced tumorigenesis. The serine/threonine kinase Akt, a key target of PIP3, is activated by TC21 (Rong et al., 2002), resulting in increased cell proliferation, transformation and survival through numerous effectors, including Bad, GSK-3β and mTOR (Manning and Cantley, 2007). Additional targets of PIP3 include PKC, PLCγ and exchange factors for Rac, Rho and Ras GTPases (Kane and Weiss, 2003).

To examine how TC21 induces tumorigenesis, we transformed a non-malignant murine breast line (EpH4) with activated H-Ras (G12V) or TC21 (G23V) mutants. We demonstrate that

G23V TC21 is significantly more oncogenic than G12V H-Ras both *in vivo* and *in vitro* and that TC21-induced proliferation and tumorigenesis was due to activation of p38 MAPK, mTOR and PI3K, but independent of Akt. Thus, expression of oncogenic TC21 is sufficient to highly transform mammary epithelial cells.

Experimental Procedures

Cell culture

Phoenix 293 cells were provided by Dr. Gary Nolan (Stanford University, Stanford, CA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Murine EpH4 cells were obtained from Dr Carlos Arteaga (Vanderbilt University, Nashville, TN) and maintained in DMEM with 10% FBS. PAI/L cells were obtained from Dr. Dan Rifkin (New York University, New York, NY) and maintained in 10% FBS.

Plasmids and cell lines

(G23V)TC21 and (G12V)H-Ras were subcloned into the LZRS-GFP vector modified for bicistronic expression of green fluorescent protein (GFP) and the protein of interest. Vectors were transfected into Phoenix 293 packaging cells using lipofectamine (Invitrogen, Carlsbad, CA), and EpH4 cells were subsequently infected with retrovirus daily for 10 days. Stable populations of cells expressing mutant TC21, H-Ras or empty vector were isolated by GFP using a FACStar Plus cell sorter (BD Biosciences, Franklin Lakes, NJ). The dominant-negative construct pCMV6-AKT-K179M (Franke et al., 1995) was transfected using lipofectamine. Pooled siRNA for Akt was obtained from Ambion (Austin, TX) and transfected using DharmaFECT reagent 2 (Dharmacon, Lafayette, CO). Pooled siRNA for p38 MAPK and mTOR (FRAP), specific siRNA for PI3K(p110β) and control siRNA were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and transfected using the manufacturer's reagents and protocol.

Antibodies and other reagents

Antibodies to TC21, H-Ras and actin were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies to phosphorylated and total Erk1/2, p38 MAPK, Akt, mTOR, p70S6K and Smad2 were from Cell Signaling Technology (Beverly, MA). LY294002, SB203580, U0126, PD98059, Akt-inhibitor II, Akt-inhibitor III and Rapamycin were from Calbiochem (EMD Biosciences, La Jolla, CA).

Tumor formation

5-week old female BALB/c athymic mice were obtained from Harlan Laboratories (Indianapolis, IN) Cells were trypsinized and resuspended in PBS, then injected subcutaneously on either side of the back (1.0×10^6 cells/100 µl PBS per injection). Tumor size was measured after 3 weeks using a dial-caliper and volumes were calculated as (length)x(width)x(height).

Colony formation

1×10^4 cells in suspension (DMEM/1%/FBS/0.3% agar) with or without inhibitors (10µM) were overlaid onto a solidified layer of agar (DMEM/10% FBS/0.7% agar) in 35mm dishes. Cells were incubated at 37ºC for 9 days. Colonies were scored counting multiple fields using an inverted microscope.

Cell proliferation

3×10^3cells were plated per well in 24-well plates and maintained in DMEM (2% FBS) for 70h, then pulsed for 2h with 4µCi/well [3H]thymidine (Perkin Elmer Life Sciences, Boston, MA). Cells were washed with 10% trichloroacetic acid, solubilized with 0.2N NaOH, and radioactivity was measured using a scintillation counter. Cell counting assays were performed by plating 2.5×10^2 cells (subconfluent) or 3×10^3 cells (confluent) per 35mm dish and counting cell number over 5 days using a hemocytometer.

Immunoblotting

Cells were serum starved overnight and stimulated with 10% FBS for the times indicated. Cells were lysed in RIPA buffer (50mM TrisHCl pH8, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 5mM EDTA) supplemented with protease and phosphatase inhibitors. Total cell lysates were run onto 10% SDS gels, then transferred to nitrocellulose membranes and blocked with 5% milk in Tris buffered saline with Tween-20 (TBS-T; 150mM NaCl, 100mM Tris pH 7.5, 0.1% Tween-20). Immunoblotting was performed with primary (1:1000) and secondary (1:5000) antibodies in TBS-T with 5% milk, and visualized using the ECL Western blotting detection system (Perkin-Elmer Biosystems, Foster City, CA).

Statistical analysis

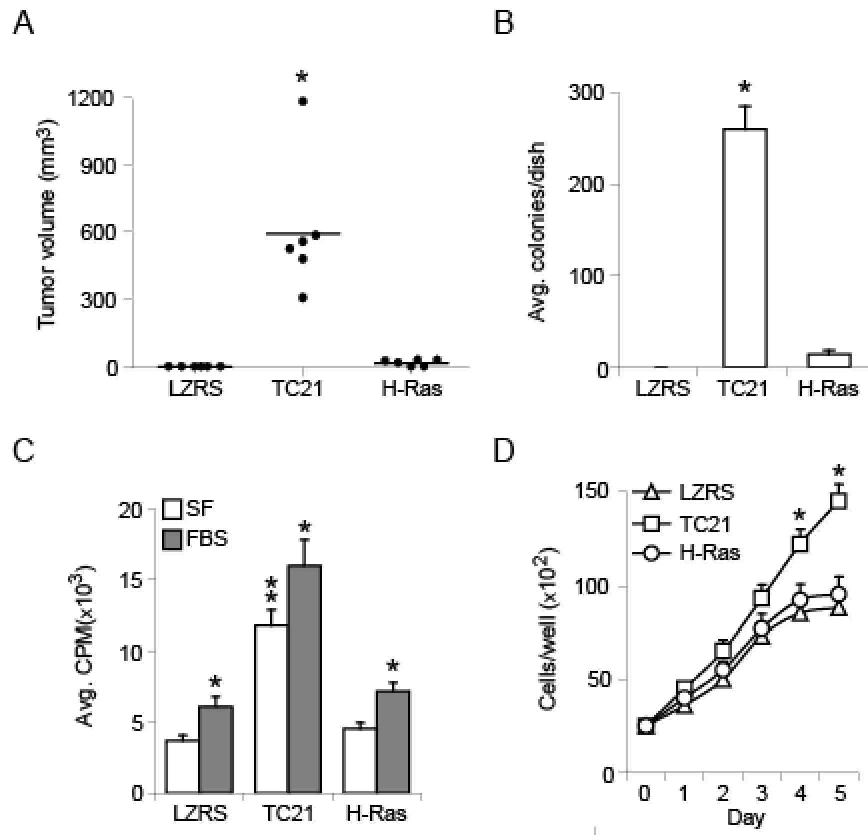
The Student's t test was used to compare two groups. Values with $p \leq 0.05$ were considered significant. Results from colony formation and proliferation assays are representative of three independent experiments.

Results

(G23V) TC21-transformed EpH4 cells are highly tumorigenic

EpH4 cells expressing active (G23V) TC21 (TC21/EpH4) or active (G12V) H-Ras (H-Ras/EpH4) or empty LZRS-GFP retroviral vector (LZRS/EpH4) were sorted by fluorescence-activated cell sorting (FACS) (Fig. 5A) and mutant Ras expression was verified by Western blot analysis (Fig. 5B). To test tumorigenicity *in vivo*, cells were injected subcutaneously into nude

Figure 6. ***TC21/EpH4 cells induce transformation* in vitro *and* in vivo. A.** EpH4 cells were infected with retrovirus carrying activated mutants of H-Ras, TC21 or empty vector (LZRS) as described in *Experimental Procedures.* Tumorigenicity *in vivo* was determined by injecting BALB/c athymic mice subcutaneously on either side of the back with 1 x 10^6 cells expressing TC21(G23V), H-Ras(G12V) or LZRS vector. After 3 weeks tumor volumes were measured using a dial-caliper. The open circles represent individual tumors (n = 6) and the bars the mean. Tumor volumes were significantly higher in TC21/EpH4 cells (*p< 0.01). **B.** Soft agar colony formation assays were performed as described in *Experimental Procedures*, and colonies were scored after 9 days. Colony number was significantly higher in TC21/EpH4 (*p< 0.01) compared to H-Ras/EpH4 and LZRS/EpH4 cells. **C.** Cell proliferation was measured by performing [3H]-thymidine incorporation assays as described in *Experimental Procedures*. Cells were grown on plastic in serum free media (SF) or 2% FBS for 72 hours. TC21/EpH4 cells proliferated significantly faster than H-Ras/EpH4 or LZRS/EpH4 cells (**p<0.01). Proliferation of TC21/EpH4, H-Ras/EpH4 and LZRS/EpH4 cells was significantly increased when grown in 2% FBS (*p<0.05). **D.** Cell proliferation in confluent conditions was determined by plating 3 x 10^3 cells/well and sequential cell counting. TC21/EpH4 cells proliferated significantly faster than H-Ras/EpH4 or LZRS/EpH4 cells (*p<0.01). Values from transformation and proliferation assays are means ± standard deviation (SD) from triplicate wells of a single representative experiment.


A
B
C
D
Tumor volume (mm3)
1200
900
600
300
0
LZRS
TC21
H-Ras
Avg. colonies/dish
300
200
100
0
Avg. CPM(x10^3)
20
15
10
5
SF
FBS
Cells/well (x10^2)
150
100
50
LZRS
TC21
H-Ras
0
1
2
3
4
5
Day

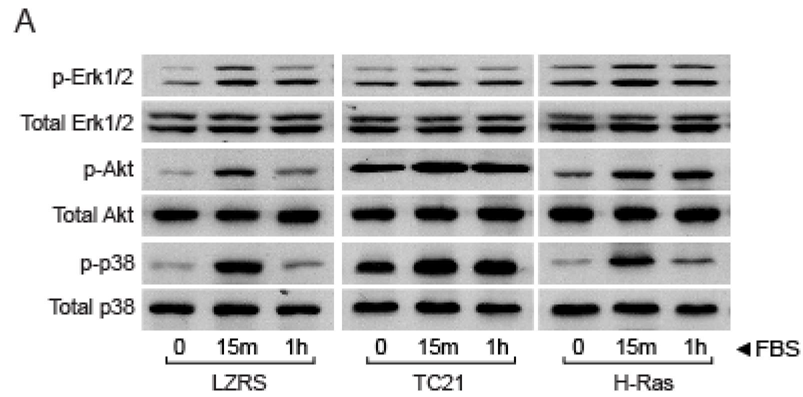
mice. TC21/EpH4 cells formed large tumors within 14 days post injection; H-Ras/EpH4 cells formed very small tumors, while LZRS/EpH4 cells were non-tumorigenic (Fig. 6A).

The differences in tumorigenicity between cell populations *in vivo* were correlated *in vitro* by evaluating soft agar colony formation. Within 9 days in culture, TC21/EpH4 cells formed numerous large-sized colonies, while H-Ras/EpH4 cells formed very few colonies and LZRS/EpH4 cells failed to grow (Fig. 6B). Relative cell proliferation rates using [^{3}H]-thymidine incorporation and cell-counting assays were performed on the different cell populations. TC21/EpH4 cells proliferated approximately three times faster than H-Ras/EpH4 or LZRS/EpH4 cells (Fig. 6C). Furthermore, when plated at high density, H-Ras/EpH4 and LZRS/EpH4 cells were contact-inhibited, while TC21/EpH4 cells continued to grow (Fig. 6D). Thus, TC21/EpH4 cells are significantly more tumorigenic than H-Ras/EpH4 cells both *in vivo* and *in vitro*.

TC21-induced tumorigenicity requires p38 MAPK and PI3K but not Akt

Since TC21 is known to activate Erk1/2 (Graham et al., 1994), p38 MAPK (Graham et al., 1999) and PI3K (Murphy et al., 2002), we investigated the roles of these pathways in TC21-induced transformation of EpH4 cells. Serum starved TC21/EpH4 cells showed markedly elevated basal levels of phosphorylated Akt and p38 MAPK, but not Erk1/2 (Fig. 7A). In contrast, H-Ras/EpH4 cells showed a slight increase in Akt activity relative to LZRS/EpH4 cells (Fig. 7A). Serum stimulation induced a similar transient increase in p38 MAPK and Erk1/2 activity in LZRS and H-Ras/EpH4 cells, while Akt activation was slightly increased in H-Ras/EpH4expressing cells (Fig. 7A). In contrast, marked and sustained activation of Akt and p38 MAPK was evident in TC21/EpH4 cells. To determine whether these pathways were required for TC21-induced growth and tumorigenicity, soft agar and cell proliferation assays were performed in the presence of specific inhibitors for these pathways as well as following gene silencing with siRNA. As shown in Figure 7B, significant decreases in PI3K(110β), Akt or p38 MAPK expression was obtained following gene silencing. Inhibition of the Erk1/2 pathway

Figure 7. ***TC21/EpH4 cells show increased p38 MAPK and PI3K activation.*** **A.** Cell populations were analyzed for Erk1/2, PI3K and p38 MAPK activation by western blot analysis as described in *Experimental Procedures.* Serum starved cells were stimulated with 10% FBS for the times indicated, and immunoblots were performed on 20μg of total cell lysate using the antibodies indicated. A representative of three experiments performed is shown. **B.** Gene silencing was performed as described in *Experimental Procedures*. Cell populations were transfected with target siRNAs for PI3K(p110β), Akt, p38 MAPK or a scrambled control. Immunoblots were performed on 20μg of total cell lysate from transfected cells to determine levels of target gene expression using the antibodies indicated. Immunoblotting for actin was performed as a loading control. **C.** Colony formation of TC21/EpH4 or H-Ras/EpH4 cells in soft agar treated with 10μM LY294002, 10μM SB203580, 10μM U0126, or transfected with siRNA for PI3K, Akt, p38 MAPK or a scrambled control. Colonies were scored after 9 days. Differences in colony formation of cells treated with inhibitors or transfected with siRNA were significant (*$p < 0.05$). **D.** 72 hour cell proliferation was evaluated by [3H]-thymidine incorporation assay in cells transfected with siRNA for PI3K, p38 MAPK, Akt or a scrambled control, and in cells treated with or without 10μM LY294002, 10μM SB203580 or 10μM U0126. Differences in proliferation of TC21/EpH4, H-Ras/EpH4 and LZRS/EpH4 cells treated with inhibitors or treated with siRNA (*$p<0.01$). Values from transformation and proliferation assays are means ± SD from triplicate wells of a representative experiment.


A
p-Erk1/2
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p-Akt
Total Akt
p-p38
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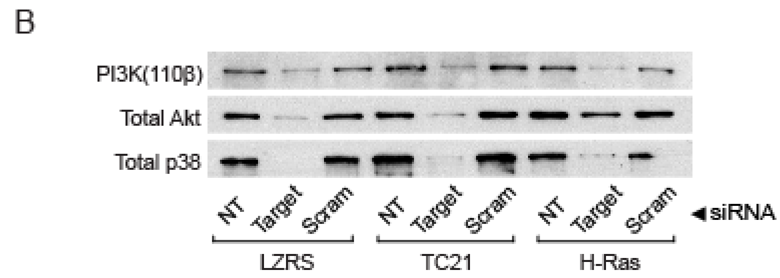
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Total Akt
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siRNA
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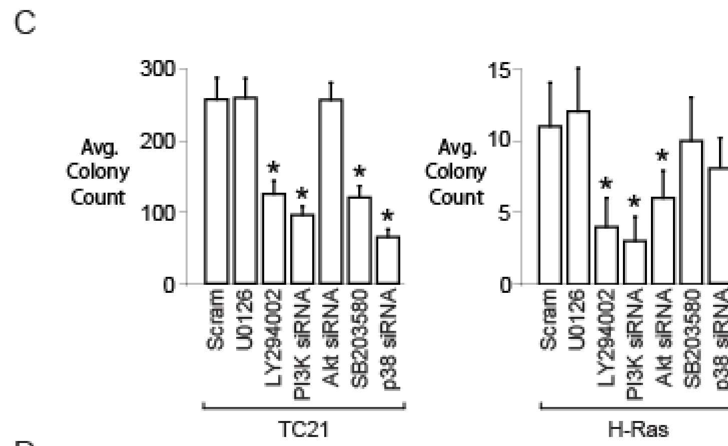
TC21
H-Ras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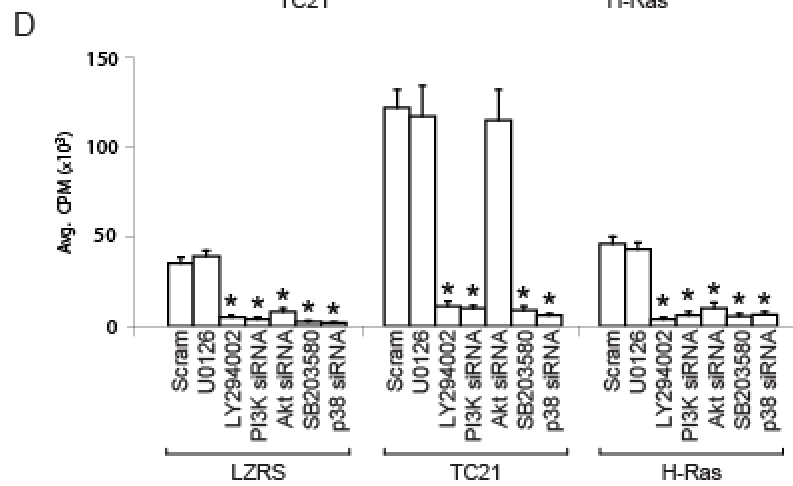
TC21
H-Ras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Akt siRNA
SB203580
p38 siRNA
LZRS
TC21
H-Ras

with the MEK inhibitors U0126 (Fig. 7C) or PD98059 (data not shown) had no effect on colony formation of either the TC21 or H-Ras/EpH4 cells. In contrast, TC21/EpH4 colony formation was decreased 50-60% with PI3K-inhibition (LY294002, PI3K siRNA) or p38 MAPK-inhibition (SB203580, p38 MAPK siRNA) (Fig. 7C). Combined inhibition of PI3K and p38 MAPK had no additional effect (data not shown). Surprisingly, inhibition of Akt activity by siRNA (Fig. 7C), specific inhibitors (Akt inhibitor II, Akt inhibitor III) (data not shown) or transfection of a dominant negative Akt construct (pCMV6-AKT-K179M) (data not shown) did not affect TC21/EpH4 colony formation. In H-Ras/EpH4 cells, PI3K-inhibition blocked approximately 80% of colony formation and Akt-inhibition reduced colony formation by 50% (Fig. 7C), while inhibiting p38 MAPK had little effect.

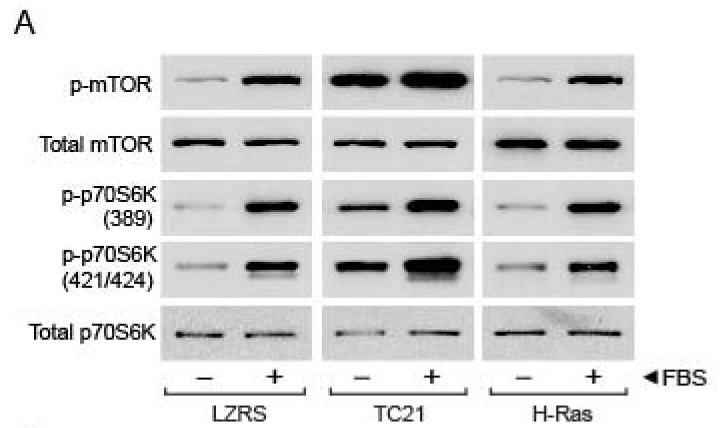
The same strategies described above were utilized to determine which pathways played a role in cell proliferation as determined by [^{3}H]-thymidine incorporation assays. As shown in Figure 7D, inhibition of PI3K or p38 MAPK reduced TC21/EpH4 cell proliferation by roughly 90%, while blocking Akt activity had no effect. The proliferation rates of both H-Ras/EpH4 and LZRS/EpH4 cells were decreased by approximately 80% with inhibition of PI3K, Akt or p38 MAPK activity (Fig. 7D). Inhibition of Erk1/2 activity did not affect growth of any of the cell populations. Thus, transformation and increased growth of TC21/EpH4 cells is mediated by p38 MAPK and PI3K, but not Akt.

TC21 activates mTOR-signaling

As TC21-mediated transformation of EpH4 cells was PI3K-dependent but Akt-independent, we investigated whether mTOR played a role in this process, as mTOR is known to act downstream of PI3K/Akt (Hay, 2005) and mediate Ras-transformation (Shaw and Cantley, 2006). TC21/EpH4 cells demonstrated a marked increase in phosphorylation of mTOR and its effector p70S6K, both basally and following serum stimulation compared to H-Ras/EpH4 and LZRS/EpH4 cells (Fig. 8A). To determine whether mTOR activation was required for

Figure 8. ***TC21/EpH4 cell tumorigenesis is induced by mTOR*****. A.** Activation of mTOR and p70S6K was determined in cell populations stimulated with 10% FBS for 10min. Total cell lysates (20μg/lane) were analyzed by western blot with the antibodies indicated. Results were similar in three independent experiments. **B.** Cell populations were transfected with siRNA for mTOR or a scrambled control. Immunoblots were performed on 20μg of total cell lysate to determine levels of total mTOR expression. Soft agar colony formation assays were performed using knockdown TC21/EpH4 and H-Ras/EpH4 cells, or treating TC21/EpH4 and H-Ras/EpH4 cells with the mTOR-inhibitor rapamycin (2ng/ml). Colony formation was scored after 9 days. Knockdown of mTOR expression or treatment with rapamycin significantly reduced colony formation (*$p<0.01$). **C.** 72 hour cell proliferation assays were performed using cells transfected with mTOR siRNA or treated with rapamycin (2ng/ml). Inhibition of mTOR activity significantly reduced basal TC21/EpH4 cell proliferation (*$p<0.01$). Values from transformation and proliferation assays are means ± SD from triplicate wells of a representative experiment.

A



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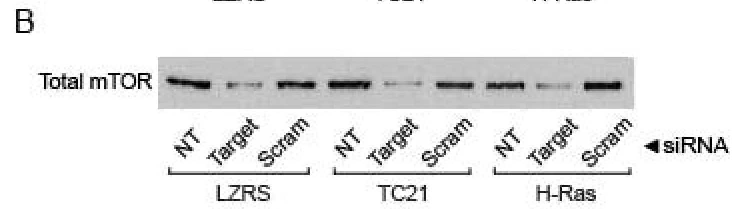

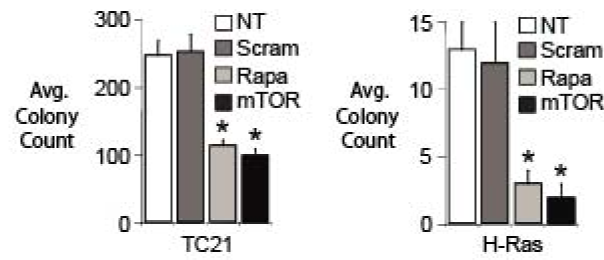

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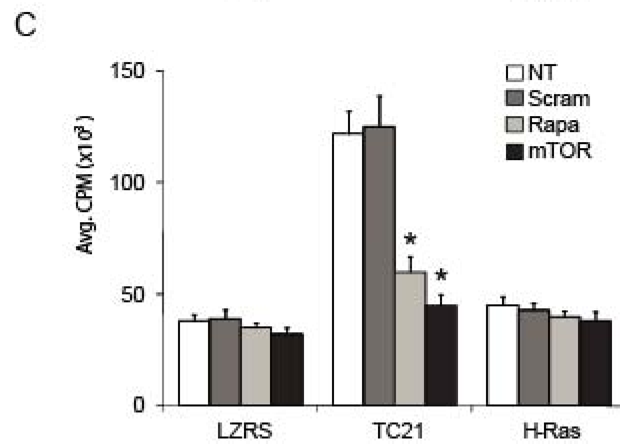


transformation and growth, colony formation and proliferation assays were performed using the mTOR-inhibitor rapamycin as well as siRNA directed against mTOR. Expression levels of mTOR were significantly decreased in all the cell populations following gene silencing with siRNA (Fig. 8B). Inhibition of mTOR activity decreased TC21/EpH4 and H-Ras/EpH4 colony formation by 60% and 80% respectively (Fig. 8C). Interestingly, the basal growth of H-Ras/EpH4 and LZRS/EpH4 cells on plastic was not significantly reduced by rapamycin or siRNA directed against mTOR, while TC21/EpH4 cell proliferation was decreased by 50-60% (Fig. 8D). These observations suggest a role for mTOR in TC21-induced tumorigenesis.

Signaling through PI3K and mTOR in TC21/EpH4 cells

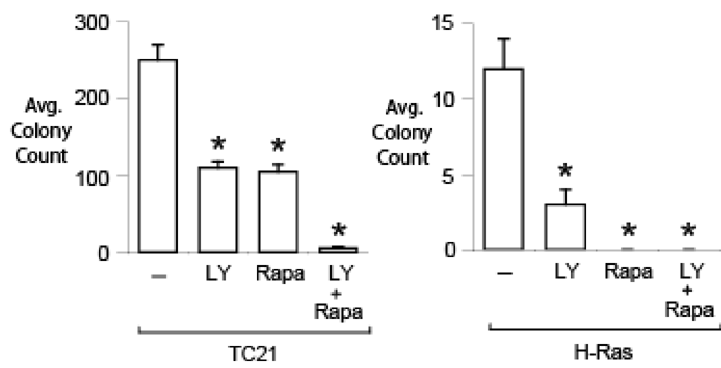
Based on the finding that transformation of EpH4 cells depended on PI3K and mTOR, but not Akt, we investigated whether PI3K and mTOR were signaling through a common pathway. As seen in Figure 9A, inhibition of either PI3K or mTOR reduced TC21/EpH4 colony formation by approximately 50%, while inhibition of both PI3K and mTOR abolished colony formation completely. Combined inhibition of PI3K and mTOR also had an additive effect on TC21/EpH4 cell proliferation (Fig. 9B). As rapamycin virtually abolished all H-Ras/EpH4 colony formation and proliferation, it was not possible to determine an additive effect of these inhibitors. To determine if mTOR activation was dependent on PI3K, we tested whether the inhibitors indicated above could block activation of Akt or mTOR by serum stimulation (Fig. 9C). Akt phosphorylation was unaffected by rapamycin treatment in all cell lines, suggesting PI3K is not dependent or downstream of mTOR activity. Phosphorylation of mTOR was slightly decreased by LY294002 in H-Ras/EpH4 and LZRS/EpH4 cells, but not in TC21/EpH4 cells, suggesting that PI3K-independent pathways are predominantly responsible for mTOR activation.

mTOR activation is downstream of p38 MAPK

As p38 MAPK mediates TC21-induced transformation independent of PI3K activity

Figure 9. PI3K *and mTOR signal by discrete pathways in TC21/EpH4 cells.* A. TC21/EpH4 (top) or H-Ras/EpH4 cells (bottom) were seeded in soft agar with or without LY294002 (10µM) or rapamycin (2ng/ml) and colony formation was scored after 9 days. Treatment with rapamycin and/or LY294002 significantly reduced basal TC21/EpH4 and H-Ras/EpH4 colony formation (*$p<0.01$). **B.** 72 hour cell proliferation assays was performed in the presence or absence of LY294002 (10µM) and rapamycin (2ng/ml). Treatment with rapamycin and/or LY294002 significantly reduced basal TC21/EpH4 cell proliferation, while LY294002 alone or in combination with rapamycin significantly decreased basal H-Ras/EpH4 and LZRS/EpH4 cell proliferation (*$p<0.01$). **C.** PI3K and mTOR-signaling was investigated by stimulating serum-starved cells with 10% FBS for 10min in the presence or absence of LY294002 (10µM) and rapamycin (2ng/ml). Total cell lysates were analyzed (20µg/lane) by western blot for levels of activated as well as total Akt and mTOR. Results were similar in three independent experiments. Values from transformation and proliferation assays are means ± SD from triplicate wells of a representative experiment.

A



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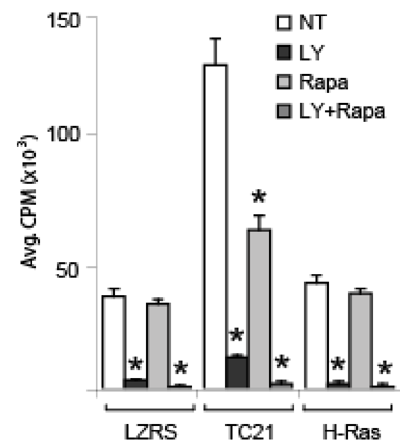

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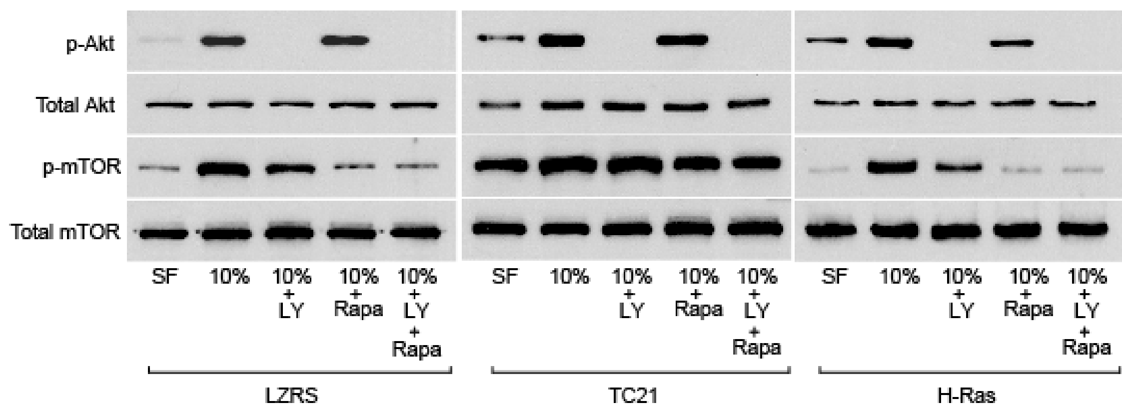


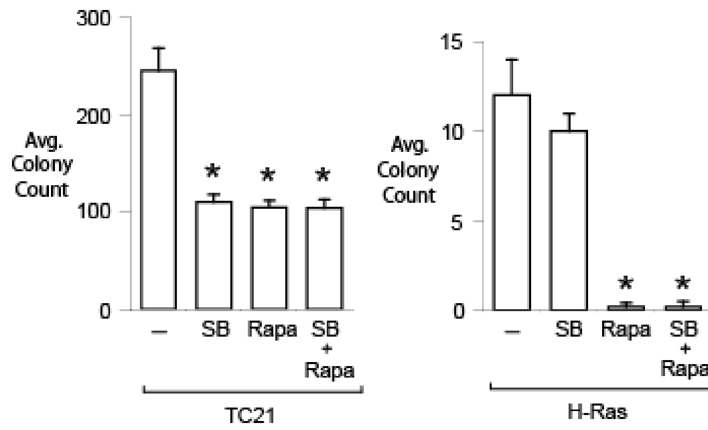
(data not shown), we investigated whether p38 and mTOR were signaling through a common pathway. As shown in Figures 10A and B, combined inhibition of p38 MAPK and mTOR was no more effective than inhibiting either one alone in reducing TC21/EpH4 colony formation or cell proliferation. The combinatorial effect on H-Ras/EpH4 colony formation could not be tested as rapamycin alone completely blocked growth in soft agar, however combined inhibition did not decrease H-Ras/EpH4 or LZRS/EpH4 cell proliferation any further. We then examined the effect of these inhibitors on the activation of p38 MAPK or mTOR following serum stimulation (Fig. 10C). The serum induced phosphorylation of mTOR in TC21/EpH4 cells was blocked by p38 MAPK inhibition, while treatment with rapamycin blocked serum induced mTOR phosphorylation and reduced mTOR activation below basal levels. Combined inhibition of p38 MAPK and mTOR had no additive effect of mTOR phosphorylation, and rapamycin treatment alone did not affect the activation of p38 MAPK. Activation of mTOR was also partially blocked by p38 MAPK inhibition in H-Ras/EpH4 and LZRS/EpH4 cells, but rapamycin alone did not block activation of p38 MAPK. These data suggest that TC21-induced transformation and proliferation is mediated in part by a p38/mTOR-dependent pathway.

Discussion

TC21 is known to be a powerful oncogene, yet it is not clear how constitutive TC21 activity induces cell proliferation and transformation. We demonstrate here that TC21/EpH4 cells are significantly more oncogenic than H-Ras/EpH4 cells both *in vivo* and *in vitro*, and that TC21-induced proliferation and transformation requires PI3K, p38 MAPK and mTOR activity.

TC21/EpH4 cells were highly tumorigenic both *in vitro* and *in vivo* compared to H-Ras/EpH4 cells. These results contrast with earlier observations where TC21-transformed NIH3T3 fibroblasts formed the same number of soft agar colonies as H-Ras-transformed cells despite forming more aggressive tumors in nude mice (Chan et al., 1994), and where TC21-transformed MCF10A human epithelial cells formed significantly more colonies than H-Ras

Figure 10. ***p38 MAPK and mTOR signal via the same pathway in TC21/EpH4 cells.*** **A.** TC21/EpH4 (top) or H-Ras/EpH4 cells (bottom) were seeded in soft agar with SB203580 (10μM) or rapamycin (2ng/ml) and colony formation was scored after 9 days. Rapamycin and/or SB203580 significantly reduced basal TC21/EpH4 colony formation (*$p<0.01$). Rapamycin treatment alone or with SB203580 significantly blocked basal H-Ras/EpH4 colony formation (*$p<0.01$). **B.** 72 hour cell proliferation was performed in the presence or absence of SB203580 (10μM) and rapamycin (2ng/ml). Treatment with rapamycin and/or SB203580 significantly reduced basal TC21/EpH4 cell proliferation, while SB203580 alone or in combination with rapamycin significantly decreased basal H-Ras/EpH4 and LZRS/EpH4 cell proliferation (*$p<0.01$). **C.** p38 MAPK and mTOR-signaling was investigated by stimulating serum-starved cells with 10% FBS for 10min in the presence or absence of SB203580 (10μM) and rapamycin (2ng/ml). Total cell lysates were analyzed (20μg/lane) for levels of activated as well as total p38 MAPK and mTOR. Results were similar in three independent experiments. Values from transformation and proliferation assays are means ± SD from triplicate wells of a representative experiment.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300
200
100
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Avg. Colony Count
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SB
Rapa
SB + Rapa
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10
5
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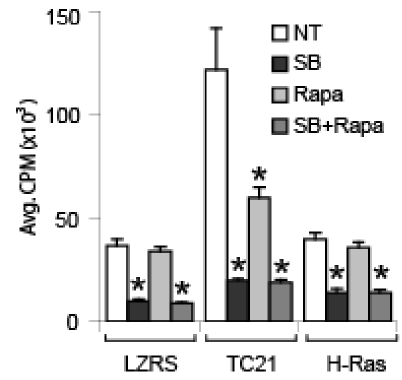
H-Ras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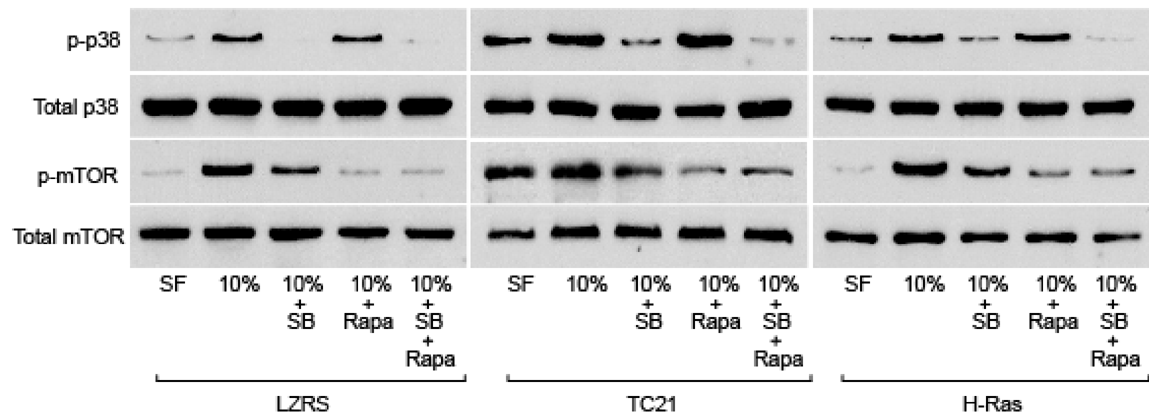
C
p-p38
Total p38
p-mTOR
Total mTOR
SF
10%
10% + SB
10% + Rapa
10% + SB + Rapa
LZRS
TC21
H-Ras

expressing cells but neither formed tumors *in vivo* (Clark et al., 1996). The low level of tumorigenicity in H-Ras/EpH4 cells was surprising as others report EpH4 cells transformed with H-Ras (EpRas) form tumors by 5-7 days in BALB/c mice (Oft et al., 1996) and 4 weeks in nude mice (Janda et al., 2002a).

The pathways that mediate TC21-induced tumorigenesis are not well established. Our data show TC21-mediated transformation of EpH4 cells is independent of Erk1/2, which contrasts with data that TC21 overexpression in NIH3T3 cells increases Erk1/2 (Rong et al., 2002) and Raf activity, which is required for transformation (Rosario et al., 1999). These data in turn contrast with other studies that suggest TC21 does not activate Erk1/2 directly (Graham et al., 1999) and TC21 can transform NIH3T3 cells independent of Raf (Graham et al., 1994). A marked increase in basal levels of phosphorylated p38 MAPK was noted in TC21/EpH4 cells, which was important in promoting colony formation and cell proliferation. These data are consistent with findings that TC21 can activate p38 MAPK in Cos7 cells (Graham et al., 1999) and p38 MAPK activation is important for TC21-induced ureteric bud cell proliferation (Pozzi et al., 2006). The requirement of p38 MAPK but not Erk1/2 for EpH4 cell transformation, once again demonstrates the heterogeneity by which TC21 induces its effects in different cell types.

Like others we demonstrate that TC21 activates Akt and that TC21-transformation is PI3K-dependent (Rong et al., 2002), however transformation of EpH4 cells was independent of Akt. While this finding was surprising, TC21-induced migration of murine Schwann cells is dependent on Erk1/2 and PI3K, but not Akt activation (Huang et al., 2004). Treatment of TC21/EpH4 cells with rapamycin reduced cell proliferation and transformation by 50%, suggesting a role for mTOR in these processes. Although mTOR has not previously been associated with TC21 transformation, it does mediate K-Ras-induced alveolar epithelial neoplasia in mice (Wislez et al., 2005). Our finding that mTOR mediates TC21 transformation downstream

of p38 MAPK and not PI3K/Akt was unexpected. Though current models suggest that mTOR signals both downstream and in parallel with PI3K to converge on common downstream targets (Fingar and Blenis, 2004), it is not known whether mTOR is directly activated by p38 MAPK.

In summary, these studies show that activated TC21 causes marked transformation of non-tumorigenic mammary cells, and that the transforming potential is significantly greater than that of oncogenic H-Ras in the EpH4 model. We show that TC21-induced transformation was dependent on increased activity through the PI3K and p38 MAPK/mTOR-signaling pathways, but was independent of Akt. By identifying the effectors and pathways activated by this Ras superfamily member, we have furthered our understanding of the signaling mechanisms that underlie malignant transformation.

CHAPTER III

R-RAS AND TC21 HAVE DISTINCT TRANSFORMING PROPERTIES

Introduction

R-Ras is the closest relative to TC21 in the Ras superfamily (Ehrhardt et al., 2002). These highly homologous proteins share 89% amino acid sequence identity, including identical Switch I and II domains that form critical interactions with regulatory proteins and downstream effectors (Self et al., 2001). Though their functions are still poorly understood, both have been implicated to play roles in cell adhesion, migration and invasion (Huang et al., 2004; Jeong et al., 2005; Keely et al., 1999). R-Ras and TC21 undergo regulation by the same set of guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) and show similar subcellular localization predominantly at the plasma membrane (Ohba et al., 2000). While TC21 mutations have been identified in human tumors (Huang et al., 1995), mutations in R-Ras have yet to be reported. However, there is evidence that increased R-Ras activity correlates with carcinogenesis (Nishigaki et al., 2005) and the promotion of metastasis (Mora et al., 2007).

Despite their similarities, R-Ras and TC21 exhibit differential transforming properties in a variety of cell lines. When expressed in NIH 3T3 fibroblasts, TC21 is more efficient than R-Ras at inducing focus formation, colony formation in soft agar and tumor formation in nude mice (Cox et al., 1994; Graham et al., 1994). TC21 also potently transforms Rat-1 fibroblasts (Graham et al., 1994) and a number of epithelial cell lines including MCF-10A (Clark et al., 1996) and RIE-1 cells (McFall et al., 2001). By comparison, R-Ras is unable to transform Rat-1 fibroblasts (Lowe et al., 1987) but does promote tumor growth in cervical epithelial cells (Rincon-Arano et al., 2003). Studies have identified the predominant effector of R-Ras and TC21 activity to be phosphoinositide 3-kinase (PI3K) (Marte et al., 1997; Rong et al., 2002). Additionally, they both activate the Raf1, Ral-GDS, Erk1/2, JNK and p38 MAPK pathways in a cell-type specific manner

(Graham et al., 1999; Rosario et al., 1999; Self et al., 2001). Though they share numerous regulators and effectors in common, R-Ras and TC21 have distinct roles within the cell.

In the previous chapter we demonstrated that oncogenic TC21 was highly transforming in EpH4 cells. Here we show that expression of oncogenic R-Ras also induces transformation of EpH4 cells, albeit far less efficiently than TC21. Furthermore, this difference in oncogenic potential between R-Ras and TC21 correlates with the ability of these proteins to activate signaling through the JNK/p38 MAPK, PI3K/Akt and mTOR-signaling pathways.

Experimental Procedures

Cell culture

Phoenix 293 cells were provided by Dr. Gary Nolan (Stanford University, Stanford, CA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Murine EpH4 cells were obtained from Dr Carlos Arteaga (Vanderbilt University, Nashville, TN) and maintained in DMEM with 10% FBS. PAI/L cells were obtained from Dr. Dan Rifkin (New York University, New York, NY) and maintained in 10% FBS.

Plasmids and cell lines

R-Ras(G38V) and TC21(G23V) were subcloned into the LZRS-GFP vector modified for bicistronic expression of green fluorescent protein (GFP) and the protein of interest. Vectors were transfected into Phoenix 293 packaging cells using lipofectamine (Invitrogen, Carlsbad, CA), and EpH4 cells were subsequently infected daily with retrovirus for 10 days. Stable populations of cells expressing mutant R-Ras, TC21 or empty vector were isolated by GFP using a FACStar Plus cell sorter (BD Biosciences, Franklin Lakes, NJ). Pooled siRNA for Akt was obtained from Ambion (Austin, TX) and transfected using DharmaFECT reagent 2 (Dharmacon, Lafayette, CO). Pooled siRNA for p38 MAPK, JNK and mTOR (FRAP), specific siRNA for PI3K(p110β)

and control siRNA were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and transfected using the manufacturer's reagents and protocol.

Antibodies and other reagents

Antibodies to R-Ras, TC21, PI3Kβ and Actin were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies to phosphorylated and total Erk1/2, JNK, p38 MAPK, Akt and mTOR were from Cell Signaling Technology (Beverly, MA). LY294002, SB203580, and Rapamycin were from Calbiochem (EMD Biosciences, La Jolla, CA). SP600125 was purchased from Biomol (Plymouth Meeting, PA).

Tumor formation

5-week old female BALB/c athymic mice were obtained from Harlan Laboratories (Indianapolis, IN). Cells were trypsinized and resuspended in PBS, then injected subcutaneously on either side of the back ($1.0x10^6$ cells/100 μl PBS per injection). Tumor size was measured after 3 weeks using a dial-caliper and volumes were calculated as (length)x(width)x(height).

Colony formation

$1x10^4$ cells in suspension (DMEM/10% FBS/0.3% agar) with or without inhibitors (10μM) were overlaid onto a solidified layer of agar (DMEM/10% FBS/0.7% agar) in 35mm dishes. Cells were incubated at 37ºC for 9 days. Colonies were scored counting multiple fields using an inverted microscope.

Cell Proliferation

3×10^3 cells were plated per well in 24-well plates and maintained in DMEM (2% FBS) for 70h, then pulsed for 2h with 4μCi/well [3H]thymidine (Perkin Elmer Life Sciences, Boston, MA). Cells were washed with 10% trichloroacetic acid, solubilized with 0.2N NaOH, and

radioactivity was measured using a scintillation counter. Cell counting assays were performed by plating 2.5 x 10^2 cells (subconfluent) or 3 x 10^3 cells (confluent) per 35mm dish and counting cell number over 5 days using a hemocytometer.

Immunoblotting

Cells were serum starved overnight and stimulated with 10% FBS for the times indicated. Cells were lysed in RIPA buffer (50mM TrisHCl pH8, 150mM NaCl, 1% NP-40, 0.5% sodium Deoxycholate, 0.1% SDS, 5mM EDTA) supplemented with protease and phosphatase inhibitors. Total cell lysates were run onto 10% SDS gels, then transferred to nitrocellulose membranes and blocked with 5% milk in Tris buffered saline with Tween-20 (TBST; 150mM NaCl, 100mM Tris pH 7.5, 0.1% Tween-20). Immunoblotting was performed with primary (1:1000) and secondary (1:5000) antibodies in TBS-T with 5% milk, and visualized using the ECL Western blotting detection system (Perkin-Elmer Biosystems, Foster City, CA).

Statistical analysis

The Student's t test was used to compare two groups. Values with $p \leq 0.05$ were considered significant. Results from colony formation and proliferation assays are representative of three independent experiments.

Results

R-Ras and TC21 induce tumor growth *in vivo* and colony formation *in vitro*

EpH4 cells were used in this study as they have been well characterized for the effects of Ras-transformation (Janda et al., 2002; Oft et al., 1996; Sekimoto et al., 2007). Tumorigenicity *in vivo* was determined by injecting EpH4 cells expressing activated mutants of R-Ras (G38V) or TC21 (G23V) subcutaneously into nude mice. As shown in Figure 11A, R-Ras/EpH4 cells form

Figure 11. ***R-Ras and TC21 differentially induce soft agar growth* in vitro *and tumor formation* in vivo. A.** EpH4 cells were infected with retrovirus carrying activated mutants of R-Ras, TC21 or empty vector (LZRS) as described in *Experimental Procedures*. Tumor formation *in vivo* was determined by subcutaneously injecting athymic BALB/c mice on either side of the back with 1 x 10^6 cells expressing R-Ras, TC21 or empty LZRS vector. Tumor volumes were measured 3 weeks later using a dial caliper. The open circles represent individual tumors ($n = 6$) and the bars the mean. Tumor volumes were significantly higher in TC21/EpH4 cells than R-Ras/EpH4 cells. **B.** Soft agar colony formation assays were performed as described in *Experimental Procedures*, and colonies were scored after 9 days. Colony number was significantly higher in TC21/EpH4 cells compared to R-Ras/EpH4 cells (*$p<0.01$). **C.** Cell proliferation was measured by performing 72-hour [3H]-thymidine incorporation assays as described in *Experimental Procedures*. R-Ras/EpH4 and TC21/EpH4 cells proliferated significantly faster than control LZRS/EpH4 cells (*$p<0.01$). **D.** Cell proliferation in confluent conditions was determined by plating 3 x 10^3 cells/well and sequential cell counting. R-Ras/EpH4 and TC21/EpH4 cells proliferated significantly faster than LZRS/EpH4 cells (*$p<0.01$). Values from transformation and proliferation assays are the mean ± standard deviation (SD) from triplicate wells of a single representative experiment.

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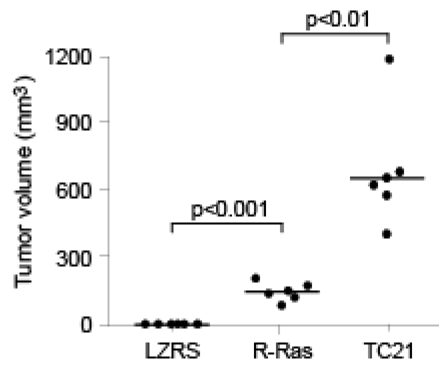

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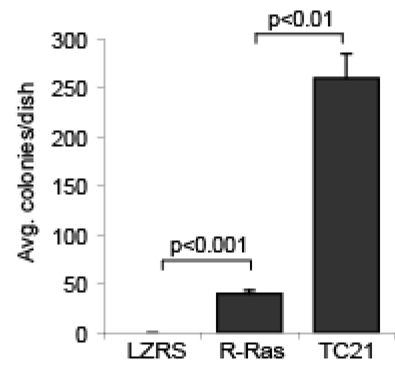

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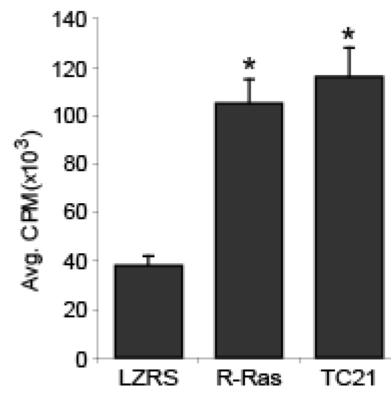

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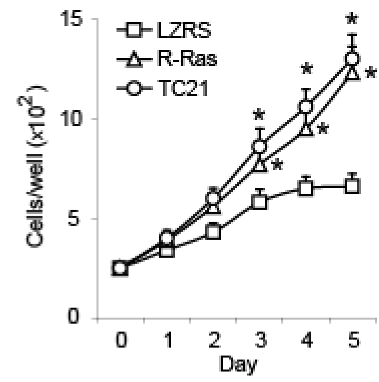

progressive tumors *in vivo* that were significantly smaller than tumors formed by TC21/EpH4 cells at 21 days post-injection, while control LZRS/EpH4 cells were nontumorigenic.

As an *in vitro* correlate of tumorigenicity, we used the soft agar assay for anchorage-independent growth and measured colony formation after 9 days. Similar to the results *in vivo*, R-Ras/EpH4 cells formed significantly fewer colonies in soft agar than TC21/EpH4 cells (Fig. 11B). To determine the relative proliferation rates of R-Ras and TC21-expressing cells, we performed [^{3}H]thymidine incorporation and sequential cell counting assays. Both R-Ras/EpH4 and TC21/EpH4 cells proliferated 2-3 times faster than LZRS/EpH4 cells (Fig. 11C). When initially plated at a higher concentration, R-Ras/EpH4 and TC21/EpH4 cells continued to proliferate after reaching confluence while control cells were contact-inhibited (Fig. 11D). Thus, while both R-Ras and TC21-expression increased EpH4 cell proliferation 2-3 fold, TC21 was significantly more transforming than R-Ras *in vitro* and *in vivo*.

JNK/p38 MAPK-signaling in R-Ras/EpH4 and TC21/EpH4 cells

We investigated the role of JNK and p38 MAPK, two important kinases that are activated by both R-Ras and TC21 (Graham et al., 1999; Mochizuki et al., 2000). R-Ras/EpH4 and TC21/EpH4 cells both showed increased basal JNK and p38 MAPK activation, as determined by Western blot analysis (Fig. 12A). TC21/EpH4 cells showed higher basal levels of phosphorylated JNK and p38 MAPK. To test whether these MAPKs were mediating cell transformation, we knocked down JNK and p38 MAPK expression using siRNA (Fig. 12B) and performed colony formation and proliferation assays. Loss of p38 MAPK expression reduced R-Ras/EpH4 colony formation by 50%, while silencing JNK expression reduced colony formation by more than 90% (Fig. 12C). In comparison, TC21/EpH4 cells were less dependent on JNK for transformation, showing a 30% reduction in colony formation following knockdown of JNK expression, while p38 MAPK knockdown reduced TC21/EpH4 colony formation by 40%. Silencing JNK or p38 MAPK expression also reduced the basal proliferation rate of R-Ras/EpH4 cells by 80-90%,

Figure 12. ***Signaling through JNK and p38 MAPK in R-Ras and TC21-transformed cells.*** **A.** Cell populations were analyzed for levels of activated and total JNK and p38 MAPK by western blot analysis. Serum starved cells were stimulated with 10% FBS for the times indicated, and immunoblots were performed on 20μg of total cell lysate using the antibodies indicated. A representative of three experiments performed is shown. **B.** Gene silencing was performed on cell populations using siRNA targeting JNK or p38 MAPK, or using a scrambled control. Immunoblots were performed on 20μg of total cell lysate from transfected cells to determine levels of target gene expression using the antibodies indicated. **C.** Soft agar colony formation assays were performed using knockdown R-Ras/EpH4 and TC21/EpH4 cells, and colony formation was scored after 9 days. Knockdown of JNK or p38 MAPK expression significantly reduced colony formation in R-Ras/EpH4 (**$p<0.001$) and TC21/EpH4 (*$p<0.01$) cells. **D.** 72-hour cell proliferation assays were performed using knockdown cells. Silencing JNK or p38 MAPK expression significantly reduced R-Ras/EpH4 (**$p<0.01$) and TC21/EpH4 (*$p<0.05$) cell proliferation, while p38 MAPK knock down significantly reduced proliferation in control cells (*$p<0.05$). Values from transformation and proliferation assays are means ± SD from triplicate wells of a representative experiment.

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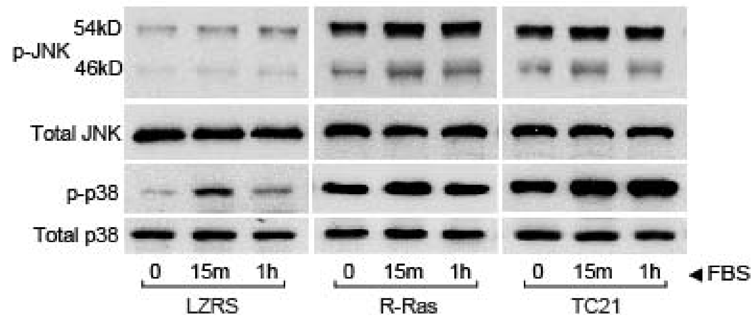

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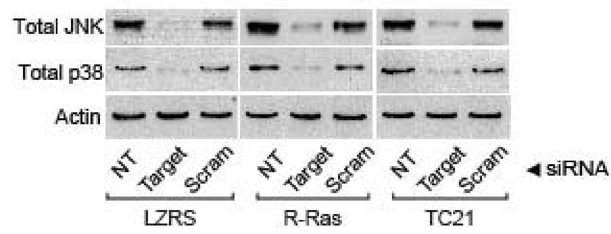

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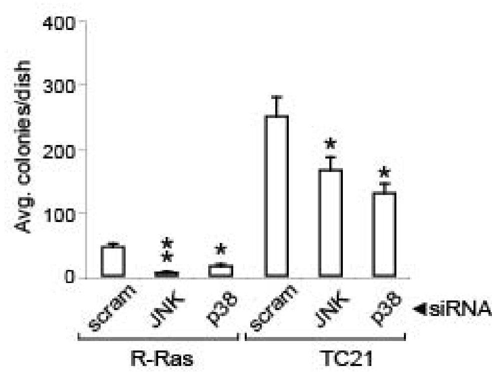

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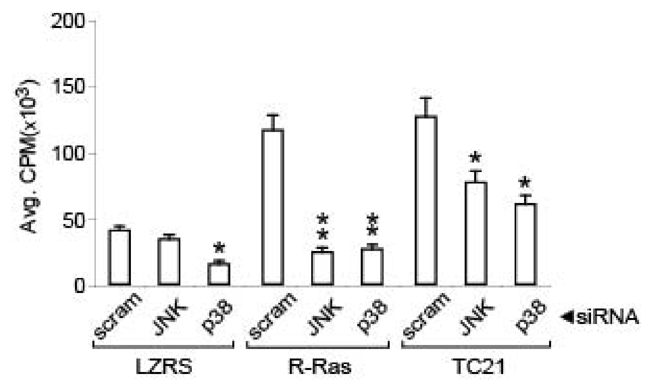

while blocking JNK or p38 MAPK reduced basal proliferation of TC21/EpH4 cells by roughly 50% (Fig. 12D). We observed similar effects on colony formation and cell proliferation using specific inhibitors of JNK (SP600125) or p38 MAPK (SB203580) (data not shown). These results suggest that JNK and p38 MAPK play a greater role in R-Ras-transformation than TC21-transformation.

PI3K/Akt and mTOR-signaling in R-Ras and TC21-induced transformation

To further dissect the pathways mediating transformation in R-Ras/EpH4 and TC21/EpH4 cells, we investigated signaling events downstream of PI3K. Both R-Ras and TC21-expressing cells showed increased basal activation of Akt and mTOR in comparison to control cells, as determined by Western blot analysis (Fig. 13A). While basal Akt and mTOR activity was highest in TC21/EpH4 cells, R-Ras/EpH4 cells showed a relatively greater increase in Akt and mTOR phosphorylation following serum stimulation. To determine the role of PI3K/Akt and mTOR activity in cell transformation, we used specific siRNA to silence either PI3K, Akt or mTOR expression (Fig. 13B) and performed colony formation and proliferation assays. As shown in Figure 13C, knocking down PI3K, Akt or mTOR expression markedly reduced basal R-Ras/EpH4 colony formation. In contrast, silencing either PI3K or mTOR expression in TC21/EpH4 cells reduced colony formation by 30-40% basally and with serum stimulation, while blocking Akt expression had no effect. Moreover, the proliferation rate of R-Ras/EpH4 cells following knockdown of PI3K, Akt or mTOR was reduced by 60-70% basally as determined by [^{3}H]thymidine incorporation (Fig. 13D). While blocking PI3K or mTOR expression reduced the proliferation of TC21/EpH4 cells by 50-60% basally, silencing Akt expression did not affect proliferation in TC21-expressing cells. We observed similar effects on colony formation and cell proliferation using specific inhibitors of PI3K (LY294002), Akt (Akt inhibitor II, Akt inhibitor III) or mTOR (rapamycin) (data not shown). These results suggest that R-Ras/EpH4 cells are more dependent on PI3K/Akt and mTOR-signaling for transformation than TC21/EpH4 cells.

Figure 13. ***PI3K/Akt and mTOR-signaling in R-Ras and TC21-transformed cells.*** **A.** Cell populations were analyzed for levels of activated and total Akt and mTOR by western blot analysis. Serum starved cells were stimulated with 10% FBS for the times indicated, and immunoblots were performed on 20μg of total cell lysate using the antibodies indicated. A representative of three experiments performed is shown. **B.** Gene silencing was performed on cell populations using siRNA targeting PI3Kβ, Akt or mTOR, or with a scrambled control. Immunoblots were performed on 20μg of total cell lysate from transfected cells to determine levels of target gene expression using the antibodies indicated. **C.** Soft agar colony formation assays were performed using knockdown R-Ras/EpH4 and TC21/EpH4 cells, and colony formation was scored after 9 days. Knockdown of PI3K, Akt or mTOR expression significantly reduced colony formation in R-Ras/EpH4 and TC21/EpH4 cells (**$p<0.001$) (*$p<0.01$). **D.** 72-hour cell proliferation assays were performed using knockdown cells. Silencing PI3K, Akt or mTOR significantly reduced R-Ras/EpH4 cell proliferation (*$p<0.01$). Knockdown of PI3K or mTOR significantly reduced TC21/EpH4 cell proliferation while silencing PI3K or Akt significantly reduced proliferation in control cells (*$p<0.01$). Values from transformation and proliferation assays are means ± SD from triplicate wells of a representative experiment.

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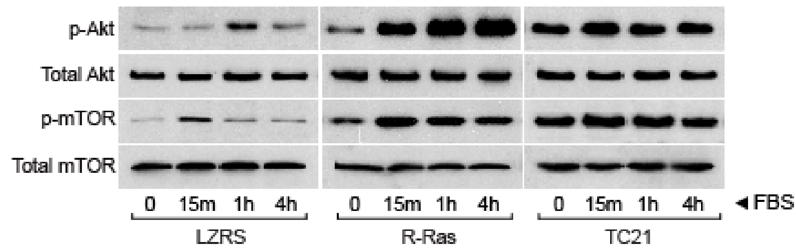

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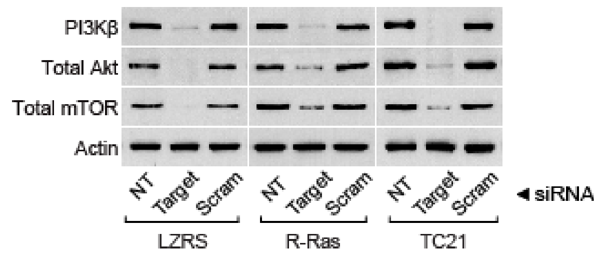

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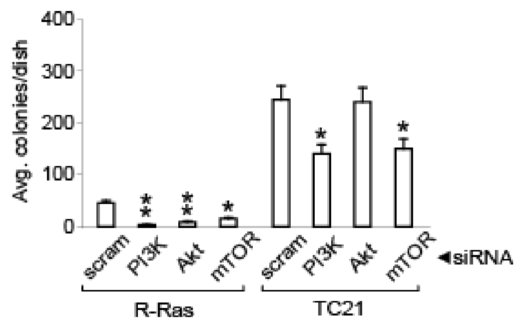

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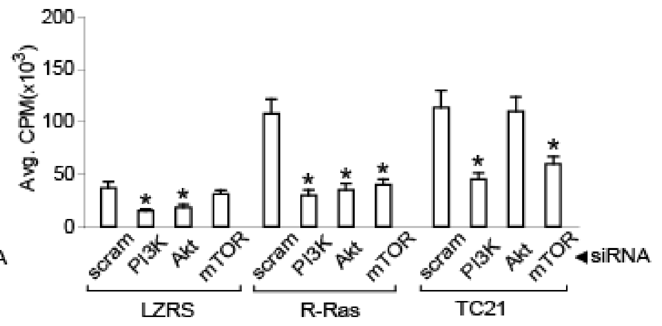


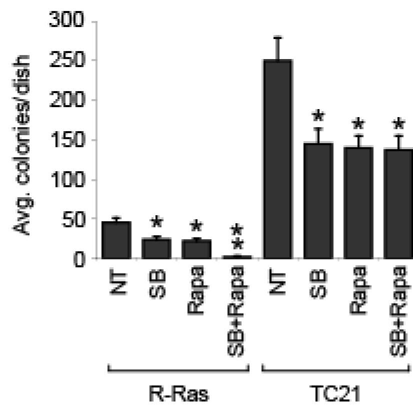
Differential signaling via p38 MAPK and mTOR in R-Ras/EpH4 and TC21/EpH4 cells

In the previous chapter we demonstrated that p38 MAPK acts upstream of mTOR in promoting the transformation of TC21/EpH4 cells. To determine whether cells expressing R-Ras were using a similar mechanism, we performed colony formation and proliferation assays with specific inhibitors of p38 MAPK (SB203580) and mTOR (Rapamycin) activity. As shown in Figure 14A, treatment of R-Ras/EpH4 cells with either SB203580 or rapamycin decreased colony formation by approximately 50%, while combined inhibition of p38 MAPK and mTOR blocked colony formation completely. In contrast, inhibiting either p38 MAPK or mTOR blocked TC21/EpH4 colony formation by 40-50%, yet combined inhibition had no additive effect on colony formation. Similarly, combined inhibition of p38 MAPK and mTOR decreased the proliferation rate of R-Ras/EpH4 cells further than treatment with either inhibitor alone, while proliferation in TC21/EpH4 and control cells showed no additive effect with combined inhibition (Fig. 14B). These results would suggest that p38 MAPK and mTOR are acting in parallel in R-Ras/EpH4 cells, yet signaling through a common pathway in TC21/EpH4 cells. To further investigate the role of p38 MAPK and mTOR, we examined the effect of specific pathway inhibition on p38 MAPK and mTOR activation in R-Ras and TC21-expressing cells using western blot analysis (Fig. 14C). While treatment with rapamycin did not affect p38 MAPK activation in any cell populations, treatment with SB203580 blocked activation of mTOR in TC21/EpH4 cells but not in R-Ras/EpH4 cells, suggesting that p38 MAPK acts upstream of mTOR in TC21/EpH4 cells but not R-Ras/EpH4 cells. Thus, R-Ras and TC21 induce transformation in part through differential p38 MAPK and mTOR-signaling.

Discussion

In this study we used EpH4 mammary epithelial cells to compare the transforming properties of oncogenic R-Ras and TC21. We demonstrate here that TC21(G23V) transforms EpH4 cells more efficiently than R-Ras(G38V) due to higher basal activation of PI3K, mTOR,

Figure 14. ***Differential signaling through p38 MAPK and mTOR in R-Ras and TC21-transformation*. A.** Cell populations were seeded in soft agar in the presence or absence of 10μM SB203580 (SB) or 2ng/ml rapamycin (Rapa), and colony formation was scored after 9 days. Differences in colony formation of cells treated with inhibitors compared to basal levels were significant (*$p<0.01$) (**$p<0.001$). **B.** 72-hour cell proliferation was evaluated in cells grown on plastic treated in the presence or absence of 10μM SB203580 (SB) and 2ng/ml rapamycin (Rapa). Differences in proliferation of R-Ras/EpH4, TC21/EpH4 and LZRS/EpH4 cells treated with inhibitors were significant (*$p<0.01$). Values from transformation and proliferation assays are means ± SD from triplicate wells of a representative experiment. **C.** p38 MAPK and mTOR-signaling was investigated by stimulating serum starved cells with 10% FBS for 15min in the presence or absence of SB203580 (10μM) and rapamycin (2ng/ml). Total cell lysates were analyzed (20μg/lane) for levels of activated as well as total p38 MAPK and mTOR. Results were similar in three independent experiments.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Avg. colonies/dish
300
250
200
150
100
50
0
NT
SB
Rapa
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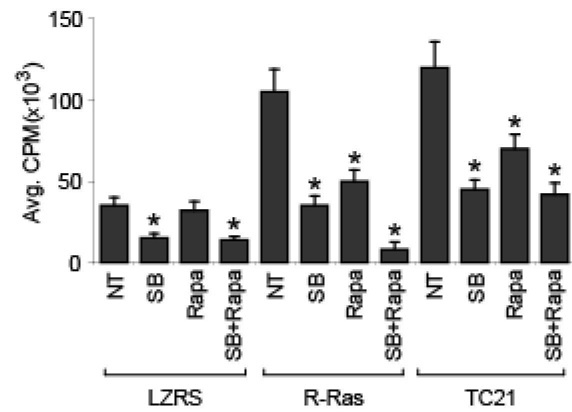
R-Ras
TC21


B
Avg. CPM(x10^3)
150
100
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NT
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Rapa
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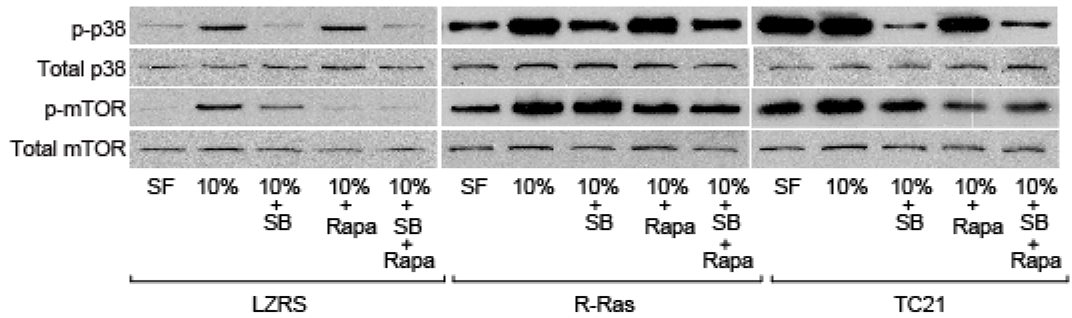
TC21


C
p-p38
Total p38
p-mTOR
Total mTOR
SF
10%
10% + SB
10% + Rapa
10% + SB + Rapa
LZRS
R-Ras
TC21

JNK and p38 MAPK. Furthermore, we show that R-Ras and TC21 require differential activation of JNK/p38 MAPK and PI3K/Akt/mTOR-signaling for transformation to occur.

Previously we showed that TC21 is significantly more transforming than H-Ras in EpH4 cells. Here we show that R-Ras expression also induces transformation of EpH4 cells, though less efficiently than TC21. This is consistent with earlier studies showing TC21 to be more efficient than R-Ras at transforming either NIH 3T3 or Rat-1 cells (Cox et al., 1994; Graham et al., 1994). While R-Ras is less transforming than TC21 in EpH4 cells, R-Ras/EpH4 cells induced greater tumor formation *in vivo* and colony formation *in vitro* than we observed in H-Ras/EpH4 cells. Thus, constitutively active mutants of H-Ras, R-Ras and TC21 are progressively more efficient at transforming EpH4 cells.

While the basis for this trend has not been established, one possibility is the divergence in amino acid sequence within the hypervariable carboxy terminal of these GTPases, a region which determines their subcellular localization (Hancock et al., 1989; Hancock et al., 1990). Another possibility for the observed differences is that the unique N-terminal 26 amino acid extension of R-Ras confers specificity of R-Ras function, as these amino acids have been demonstrated to play a role in Rac activation, cell spreading and cell migration (Holly et al., 2005). Finally, the hypervariable C-termini of these proteins may undergo cooperative interactions with their respective effector loops to create signaling specificity (Oertli et al., 2000).

In this study we established novel roles for JNK and p38 MAPK as mediators of R-Ras- and TC21-induced growth and transformation, which is not surprising as these kinases are key regulators of both tumor promotion and suppression (Dolado et al., 2007; Heasley and Han, 2006). This is also the first report demonstrating the requirement of Akt and mTOR-signaling for R-Ras-induced transformation. These observations are different from TC21, which induces EpH4 cell transformation by a PI3K-dependent and Akt-independent pathway, but are consistent with studies demonstrating that constitutive Akt activity is required for R-Ras to induce estrogen-independent proliferation in MCF-7 breast cancer cells (Yu and Feig, 2002), and that PI3K/Akt

and mTOR activity is critical for the increased proliferation and migration of cervical epithelial cells transfected with mutant R-Ras (Mora et al., 2007). Our observation that p38 MAPK acts upstream of mTOR in TC21/EpH4 cells, but not in R-Ras/EpH4 cells, is further evidence that R-Ras and TC21 use distinct mechanisms to induce cell transformation.

Taken together, these findings demonstrate that the highly homologous proteins R-Ras and TC21 utilize distinct mechanisms to cause cellular transformation in mammary epithelial cells. These results underscore the heterogeneity with which these GTPases act in different cell types.

CHAPTER IV

THE ROLE OF TGF-β IN R-RAS AND TC21-TRANSFORMED CELLS

Introduction

The multifunctional cytokine transforming growth factor-beta (TGF-β) modulates cell transformation through activation of the Smad, MAPK and PI3K-signaling pathways (Bierie and Moses, 2006; Moustakas and Heldin, 2005). Upon binding the TGF-β ligand, the type II TGF-β receptor (TBRII) recruits the type I receptor (TBRI) into a heterotetrameric signaling complex and transactivates it by phosphorylation. TBRI then phosphorylates Smad2/3, which translocate along with Smad4 to the nucleus and initiate gene transcription resulting in G_1/S cell cycle arrest (Massague et al., 2005). TGF-β can also initiates Smad-independent signaling through PI3K/Akt (Horowitz et al., 2004), the mammalian target of rapamycin (mTOR) (Das et al., 2007), and the JNK/p38 MAPK arms of the MAPK cascade via TGF-β-associated kinase 1 (TAK1) (Delaney and Mlodzik, 2006; Kim et al., 2007).

TGF-β is known to cooperate with oncogenic members of the Ras superfamily in promoting cellular transformation and tumor progression (Kim et al., 2005; Oft et al., 1996), however, it is unknown whether similar behavior occurs between TGF-β and the Ras-related proteins R-Ras and TC21.

In this study we show that R-Ras-transformation of EpH4 cells is dependent on TGF-β-signaling and requires autocrine TGF-β production, while TC21-transformation is largely independent of TGF-β. Furthermore, we demonstrate that R-Ras/EpH4 cells are far more susceptible to the transforming ability of exogenous TGF-β than TC21/EpH4 cells. Consistent with these findings, TGF-β stimulation activates TAK1, JNK, p38 MAPK, Akt and mTOR-signaling to a greater degree in R-Ras/EpH4 cells than in TC21/EpH4 cells. Finally, we demonstrate that TAK1 is required for TGF-β-dependent R-Ras/EpH4 cell transformation, but

not for TC21/EpH4 cell transformation, suggesting a critical role for TAK1 in the TGF-β-dependent transformation of mammary epithelial cells by oncogenic R-Ras.

Experimental Procedures

Cell culture

Phoenix 293 cells were provided by Dr. Gary Nolan (Stanford University, Stanford, CA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Murine EpH4 cells were obtained from Dr. Carlos Arteaga (Vanderbilt University, Nashville, TN) and maintained in DMEM with 10% FBS. PAI/L cells were obtained from Dr. Dan Rifkin (New York University, New York, NY) and maintained in 10% FBS.

Antibodies and other reagents

TGF-β1 was obtained from R&D Systems (Minneapolis, MN). The 2G7 TGF-β-neutralizing antibody was kindly provided by Dr. Carlos Arteaga (Vanderbilt University, Nashville, TN). Antibodies to R-Ras, PI3Kβ, TGF-β type II receptor and Actin were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies to phosphorylated and total Erk1/2, JNK, p38 MAPK, Akt, mTOR and Smad2 were from Cell Signaling Technology (Beverly, MA), as were antibodies to phosphorylated MKK3/6 and total TAK1. Total Smad3 antibodies were from Zymed Laboratories (San Francisco, CA) and antibodies to phosphorylated Smad3 were kindly provided by Dr. Ed Leof (Mayo Clinic, Rochester, MN). LY294002, U0126, PD98059, SB203580, Akt inhibitor II, Akt inhibitor III and Rapamycin were from Calbiochem (EMD Biosciences, La Jolla, CA). SP600125 was purchased from Biomol (Plymouth Meeting, PA). Recombinant MKK6 was purchased from Sigma-Aldrich (St. Louis, MO). Pooled siRNA for p38 MAPK, JNK and mTOR (FRAP), specific siRNA for TBRII, TAK1 and PI3K(p110β), and

control siRNA were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and transfected using the manufacturers reagents and protocol.

Plasmids and cell lines

R-Ras(G38V) and TC21(G23V) were subcloned into the LZRS-GFP vector modified for bicistronic expression of green fluorescent protein (GFP) and the protein of interest. Vectors were transfected into Phoenix 293 packaging cells using lipofectamine (Invitrogen, Carlsbad, CA), and EpH4 cells were subsequently infected daily with retrovirus for 10 days. Stable populations of cells expressing mutant R-Ras, TC21, or empty vector were isolated by GFP using a FACStar Plus cell sorter (BD Biosciences, Franklin Lakes, NJ). Pooled siRNA for Akt was obtained from Ambion (Austin, TX) and transfected using DharmaFECT reagent 2 (Dharmacon, Lafayette, CO).

Tumor formation

5-week old female BALB/c athymic mice were obtained from Harlan Laboratories (Indianapolis, IN). Cells were trypsinized and resuspended in PBS, then injected subcutaneously on either side of the back (1.0×10^6 cells/100 µl PBS per injection). Tumor size was measured after 3 weeks using a dial-caliper and volumes were calculated as (length)x(width)x(height).

Colony formation

1×10^4 cells in suspension (DMEM/10% FBS/0.3% agar) with TGF-β (5ng/ml) or inhibitors (10µM) were overlaid onto a solidified layer of agar (DMEM/10% FBS/0.7% agar) in 35mm dishes. Cells were incubated at 37ºC for 9 days. Colonies were scored counting multiple fields using an inverted microscope.

Cell Proliferation

3×10^3 cells were plated per well in 24-well plates and maintained in DMEM (2% FBS) for 70h with or without TGF-β (1ng/ml), then pulsed for 2h with 4μCi/well [3H]thymidine (Perkin Elmer Life Sciences, Boston, MA). Cells were washed with 10% trichloroacetic acid, solubilized with 0.2N NaOH, and radioactivity was measured using a scintillation counter. Cell counting assays were performed by plating 3×10^3 cells per 35mm dish and counting cell number over 5 days using a hemocytometer.

Immunoblotting

Cells were serum starved overnight and stimulated with TGF-β (5ng/ml) for the times indicated. Cells were lysed in RIPA buffer (50mM TrisHCl pH8, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 5mM EDTA) supplemented with protease and phosphatase inhibitors. Total cell lysates were run onto 10% SDS gels, then transferred to nitrocellulose membranes and blocked with 5% milk in Tris buffered saline with Tween-20 (TBST; 150mM NaCl, 100mM Tris pH 7.5, 0.1% Tween-20). Immunoblotting was performed with primary (1:1000) and secondary (1:5000) antibodies in TBS-T with 5% milk, and visualized using the ECL Western blotting detection system (Perkin-Elmer Biosystems, Foster City, CA).

Reporter Assays

Cells were transiently transfected using lipofectamine with the 3TP-Lux luciferase or CAGA reporter construct in conjunction with a cytomegalovirus-driven renela luciferase plasmid. Subsequently cells were treated with TGF-β (5ng/ml) for 24 hours, then lysed and dual-luciferase assays were performed as indicated by the manufacturer (Promega, Madison, WI) and measured on a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). Ratios of firefly and renela luciferase were calculated in normalizing data to relative luminescent units (RLU). The PAI/L assay was performed as previously described. Briefly, PAI/L cells were

incubated for 24h in serum free media with or without TGF-β (5ng/ml), or in conditioned serum free media collected from R-Ras/EpH4 or LZRS/EpH4 cells. Cells were then lysed and luciferase assays were performed as described above.

In Vitro Kinase Assay

300 μg of cell lysates were immunoprecipitated using anti-TAK1 antibodies. Immunoprecipitates were incubated with 1 μg of bacterially expressed MKK6 in 10 μl of kinase buffer containing 10 mM HEPES pH 7.4, 1 mM dithiothreitol, 5 mM $MgCl_2$ and 5 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol) at 25 °C for 2 minutes. The reactions were terminated by adding SDS sample buffer and boiling for 5 minutes. Samples were then fractionated by 10% SDS-PAGE followed by western blotting with antibodies to phosphorylated MKK3/6 or TAK1.

Statistical analysis

The Student's *t* test was used to compare two groups. Values with $p \leq 0.05$ were considered significant. Results from colony formation, proliferation, reporter and kinase assays are representative of three independent experiments.

Results

TGF-β-signaling is required for transformation by R-Ras but not TC21

Previous reports show that TGF-β-signaling through the type II TGF-β receptor (TBRII) is required for tumor formation by EpH4 cells expressing mutant H-Ras (Oft et al., 1998), and that these cells produce increased levels of TGF-β (Oft et al., 1996). To test whether TGF-β modulates the growth of R-Ras and TC21-expressing cells, we performed colony formation and [^{3}H]thymidine incorporation assays. As shown in Figure 15A, TGF-β stimulation dramatically increased R-Ras/EpH4 colony formation in soft agar, yet had minimal effect on TC21/EpH4

Figure 15. ***TGF-β-signaling is required for R-Ras, but not TC21-induced transformation.*** **A.** Colony formation of R-Ras/EpH4 or TC21/EpH4 cells in soft agar treated with TGF-β (5ng/ml). Colonies were scored after 9 days and differences in colony formation of cells treated with TGF-β were significant (*$p< 0.05$) (**$p<0.01$). **B.** 72-hour cell proliferation assays was performed in the presence or absence of TGF-β (5ng/ml). Treatment with TGF-β significantly increased R-Ras/EpH4 and TC21/EpH4 cell proliferation, while the proliferation of LZRS/EpH4 cells was significantly decreased (*$p<0.05$). **C.** Gene silencing was performed as described in *Experimental Procedures*. Cell populations were transfected with target siRNA for TBRII or a scrambled control. Immunoblotting was performed on 20μg of total cell lysate from transfected cells to determine levels of target gene expression using the antibodies indicated. Blotting for Actin was performed as a loading control. **D.** Soft agar colony formation assays were performed using TBRII knockdown R-Ras/EpH4 and TC21/EpH4 cells, or treating R-Ras/EpH4 and TC21/EpH4 cells with the TGF-β-neutralizing antibody 2G7. Colony formation was scored after 9 days. Knockdown of TBRII expression or treatment with 2G7 significantly reduced colony formation in R-Ras/EpH4 cells (*$p<0.01$). **E.** 72-hour proliferation assays were performed using cells transfected with TBRII siRNA or treated with 2G7. TGF-β inhibition significantly reduced R-Ras/EpH4 cell proliferation (*$p<0.01$). Values from transformation and proliferation assays are means ± SD from triplicate wells of a representative experiment. **F.** Production of autocrine TGF-β was determined using the PAI/L assay as described in *Experimental Procedures*. PAI/L cells were incubated for 24h in serum free media with or without TGF-β (5ng/ml), or in conditioned serum free media collected from R-Ras/EpH4, TC21/EpH4 or LZRS/EpH4 cells. PAI/L cells were then harversted and assayed for luciferase activity. Differences in relative luciferase activity were significant (*$p<0.01$). Values are means ± SD from triplicate wells of a representative experiment.

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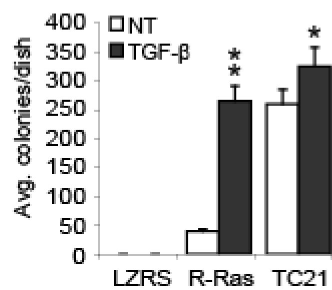

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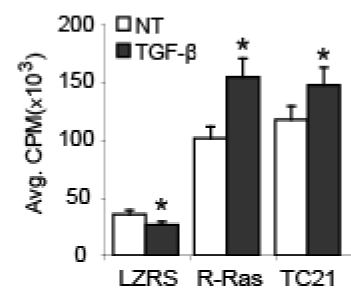

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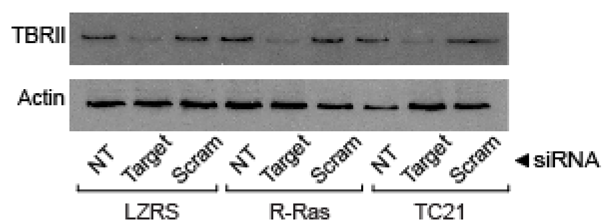

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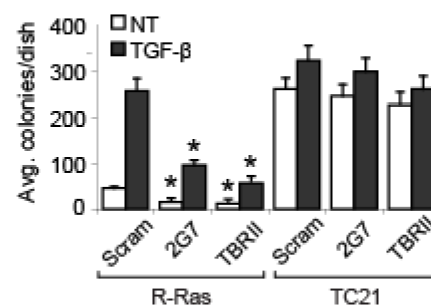

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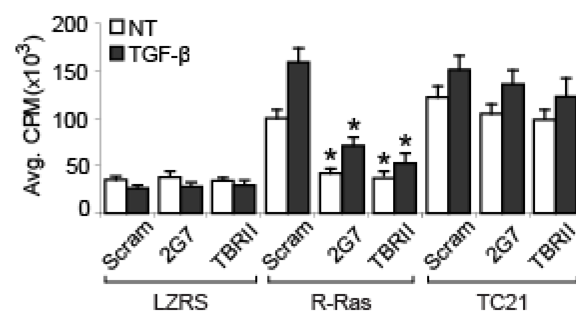

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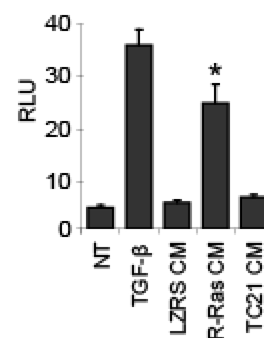

cells. While control cells were approximately 30% growth-inhibited by TGF-β, the proliferation rate of both R-Ras/EpH4 and TC21/EpH4 cells was increased by 30-40% following TGF-β stimulation (Fig. 15B). To test whether TGF-β-signaling was required for transformation by R-Ras or TC21, we blocked TBRII expression using siRNA (Fig. 15C) and performed colony formation and [^{3}H]thymidine incorporation assays. As shown in Figure 15D, using TBRII siRNA or a TGF-β-neutralizing antibody (2G7) significantly reduced colony formation in R-Ras/EpH4 cells, while TC21/EpH4 cells were unaffected. Similarly, blocking TGF-β-signaling decreased the rate of proliferation in R-Ras/EpH4 cells by 50%, while TC21/EpH4 and control cells were not affected (Fig. 15E). Neither R-Ras nor TC21-expressing cells showed any change in TBRII expression (Fig. 15C). As the basal levels of TBRII were similar in both cell populations, we hypothesized that R-Ras/EpH4 cells might be producing more activated TGF-β than TC21/EpH4 or control cells. To test this possibility, PAI/L reporter cells were incubated with conditioned media from each cell population and luciferase activity was measured. As shown in Figure 15F, R-Ras/EpH4 cells produced significantly higher levels of TGF-β than either TC21/EpH4 or LZRS/EpH4 cells. These data demonstrate that R-Ras/EpH4 cells are markedly transformed by exogenous TGF-β and require autocrine TGF-β production for basal transformation, in contrast to TC21/EpH4 cells which undergo transformation independent of TGF-β.

Transformation by R-Ras or TC21 does not disrupt Smad-signaling

Based on our finding that R-Ras and TC21-expressing cells were insensitive to TGF-β-mediated growth inhibition, we wanted to test whether TGF-β-signaling via the Smad pathway was reaching the nucleus. To do this, we performed 3TP-Lux and CAGA reporter assays. As shown in Figures 16A and B, there were no differences in TGF-β-induced transcriptional activation between control and transformed cells, suggesting that transformation by R-Ras or TC21 does not inhibit Smad-signaling. Additionally, we performed Western blot analysis to measure relative levels of activating Smad2/3 phosphorylation. R-Ras/EpH4, TC21/EpH4 and

Figure 16. ***Smad-signaling is unaffected by transformation with R-Ras or TC21.*** **A.** Reporter assays for transcriptional activation of TGF-β-induced genes were performed as described in *Experimental Procedures*. Cells were co-transfected with 3TP-Lux and Renilla constructs, treated with TGF-β (5ng/ml) for 24h and then harvested to measure luciferase activity. Values are means ± SD from triplicate wells after being normalized to Renilla activity. **B.** Smad-induced transcriptional activation was determined by co-transfecting cells with CAGA-luciferase and Renilla constructs, then treating cells with TGF-β (5ng/ml) for 24h and performing dual-luciferase assays. Values are means ± SD from triplicate wells after being normalized to Renilla activity. **C.** Cell populations were analyzed for levels of activated and total Smad2 and Smad3 by western blot analysis as described in *Experimental Procedures.* Serum starved cells were stimulated with TGF-β (5ng/ml) for the times indicated, and immunoblots were performed on 20μg of total cell lysate using the antibodies indicated. A representative of three experiments performed is shown.

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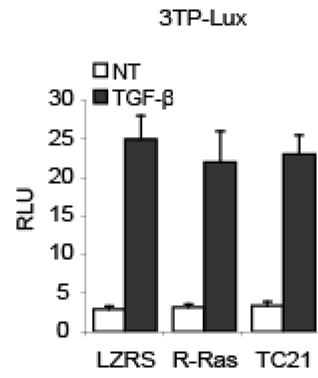

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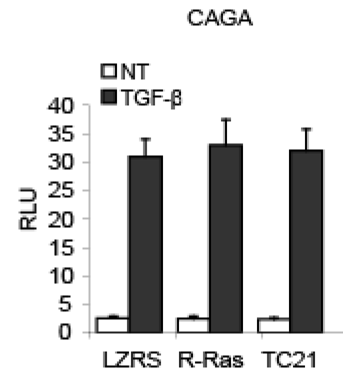

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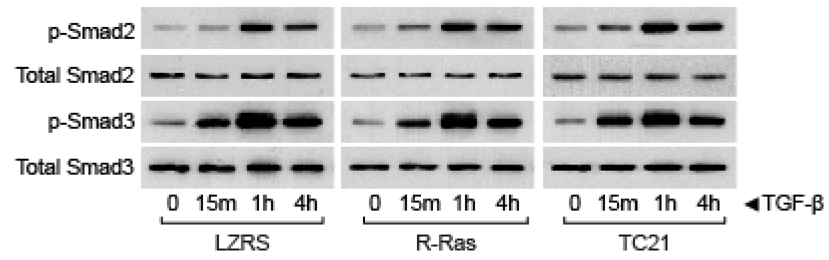

LZRS/EpH4 control cells all showed similar levels of Smad2/3 phosphorylation both basally and in response to TGF-β stimulation (Fig. 16C). These data suggest the effects of TGF-β on R-Ras and TC21-expressing cells are mediated through Smad-independent signaling pathways.

Signaling pathways mediating R-Ras-transformation and the effects of TGF-β on R-Ras/EpH4 cells

Since R-Ras/EpH4 cells were dependent on TGF-β-signaling for transformation, and the Smad pathway remained intact in R-Ras-EpH4 cells, we investigated other well-described TGF-β effectors. We previously demonstrated that R-Ras-induced transformation and proliferation in EpH4 cells was not dependent on the activation of Erk1/2. When we evaluated the JNK and p38 MAPK pathways, increased basal activation of both kinases was evident in R-Ras/EpH4 and TC21/EpH4 cells when compared to LZRS/EpH4 cells (Fig. 17A). Upon treatment with TGF-β, R-Ras/EpH4 cells showed a dramatic increase in JNK and p38 MAPK activity, while control cells showed a minimal increase in activation of p38 MAPK but not JNK following TGF-β stimulation. To test whether these MAPKs were required for TGF-β-dependent transformation in R-Ras/EpH4 cells, expression of JNK or p38 MAPK was silenced using siRNA (Fig. 17B) and colony formation and proliferation assays were performed. Downregulating either JNK or p38 MAPK completely blocked TGF-β-induced colony formation in R-Ras/EpH4 cells (Fig. 17C). However, R-Ras/EpH4 knockdown cells treated with TGF-β still showed greater colony formation and proliferation than untreated knockdown cells, suggesting that additional pathways are involved in R-Ras-induced transformation. The proliferation rate of R-Ras/EpH4 cells was also significantly reduced by decreasing either JNK or p38 MAPK expression, in both the presence and absence of TGF-β (Fig. 17D). Similar results for transformation and cell proliferation were obtained using specific inhibitors of JNK or p38 MAPK (data not shown). Thus, JNK and p38 MAPK are critical in mediating the transforming effects of TGF-β on R-Ras/EpH4 cells, but play a less significant role in TC21/EpH4 cell transformation.

Figure 17. ***TGF-β-dependent signaling through JNK and p38 MAPK in R-Ras-transformed cells*****.** **A.** Cell populations were analyzed for levels of activated and total JNK and p38 MAPK by western blot analysis. Serum starved cells were stimulated with TGF-β (5ng/ml) for the times indicated, and immunoblots were performed on 20μg of total cell lysate using the antibodies indicated. A representative of three experiments performed is shown. **B.** Gene silencing was performed on cell populations using siRNA targeting JNK or p38 MAPK, or using a scrambled control. Immunoblots were performed on 20μg of total cell lysate from transfected cells to determine levels of target gene expression using the antibodies indicated. **C.** Soft agar colony formation assays were performed using knockdown R-Ras/EpH4 cells, and colony formation was scored after 9 days. Knockdown of JNK or p38 MAPK expression significantly reduced colony formation in R-Ras/EpH4 cells (*p<0.01). **D.** 72-hour cell proliferation assays were performed using knockdown cells. Silencing JNK or p38 MAPK expression significantly reduced R-Ras/EpH4 (*p<0.01) cell proliferation, while p38 MAPK knock down significantly reduced proliferation in LZRS/EpH4 (**p<0.05). Values from transformation and proliferation assays are means ± SD from triplicate wells of a representative experiment.

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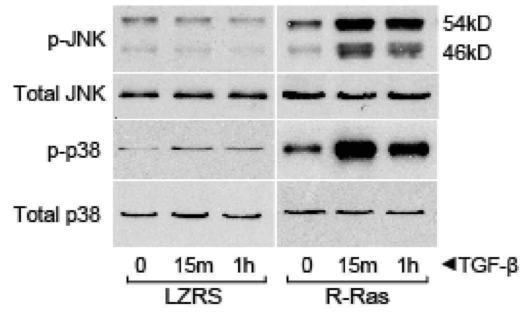

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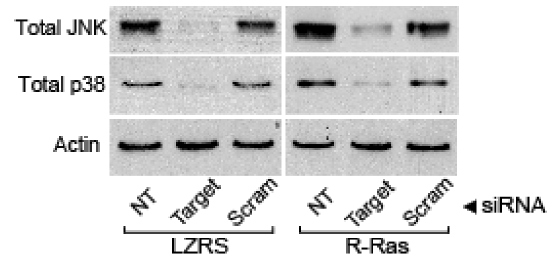

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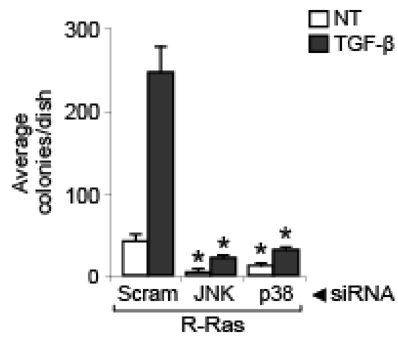

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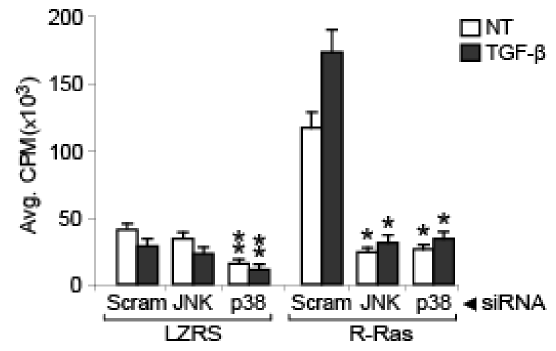

From the above data it is clear that pathways apart from JNK and p38 MAPK were required for the TGF-β-dependent transformation of R-Ras/EpH4 cells. As we previously demonstrated an important role for PI3K and mTOR-signaling in the transformation of TC21/EpH4 cells, we investigated the role of these pathways in the TGF-β-dependent transformation of R-Ras/EpH4 cells. R-Ras/EpH4 cells expressed elevated basal levels of phosphorylated Akt and mTOR relative to LZRS/EpH4 cells as determined by Western blot analysis and stimulation with TGF-β induced significantly more activation of these pathways in R-Ras/EpH4 cells (Fig. 18A). The roles of PI3K/Akt and mTOR in TGF-β-dependent transformation of R-Ras/EpH4 cells was determined by downregulating these proteins using specific siRNAs (Fig. 18B) and performing colony formation and proliferation assays. As shown in Figure 18C, silencing PI3K, Akt or mTOR expression markedly reduced basal R-Ras/EpH4 colony formation and completely blocked the TGF-β-induced increase in R-Ras/EpH4 colony formation. Furthermore, the proliferation rate of R-Ras/EpH4 cells following knockdown of PI3K, Akt or mTOR was reduced by 60-70% both basally and with TGF-β treatment (Fig. 18D). Blocking PI3K or Akt expression also reduced the proliferation rate in LZRS/EpH4 cells by 50%, while blocking mTOR expression had no effect. Similar effects on colony formation and cell proliferation were observed using specific inhibitors of PI3K, Akt or mTOR (data not shown). Thus, the PI3K/Akt and mTOR signaling pathways are also required for the transforming effects of TGF-β on R-Ras/EpH4 cells.

R-Ras-induced transformation is TAK1-dependent

From our observations that R-Ras/EpH4 cells required signaling through TBRII, JNK and p38 MAPK for transformation, we examined the role of the TGF-β effector TAK1 which acts upstream of both JNK and p38 MAPK (Choo et al., 2006; Delaney and Mlodzik, 2006). Using *in vitro* kinase assays we determined that R-Ras/EpH4 cells have significantly higher levels of

Figure 18. ***TGF-β-dependent PI3K/Akt and mTOR-signaling in R-Ras-transformed cells.*** **A.** Cell populations were analyzed for levels of activated and total Akt and mTOR by western blot analysis. Serum starved cells were stimulated with TGF-β (5ng/ml) for the times indicated, and immunoblots were performed on 20μg of total cell lysate using the antibodies indicated. A representative of three experiments performed is shown. **B.** Gene silencing was performed on cell populations using siRNA targeting PI3Kβ, Akt or mTOR, or with a scrambled control. Immunoblots were performed on 20μg of total cell lysate from transfected cells to determine levels of target gene expression using the antibodies indicated. **C.** Soft agar colony formation assays were performed using knockdown R-Ras/EpH4 cells, and colony formation was scored after 9 days. Knockdown of PI3K, Akt or mTOR expression significantly reduced colony formation in R-Ras/EpH4 and TC21/EpH4 cells (** $p<0.001$) (* $p<0.01$). **D.** 72-hour cell proliferation assays were performed using knockdown cells. Silencing PI3K, Akt or mTOR significantly reduced R-Ras/EpH4 cell proliferation (* $p<0.01$). Knockdown of PI3K or mTOR significantly reduced TC21/EpH4 cell proliferation while silencing PI3K or Akt significantly reduced proliferation in control cells (* $p<0.01$). Values from transformation and proliferation assays are means ± SD from triplicate wells of a representative experiment.

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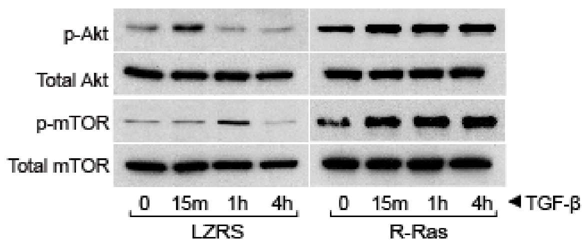

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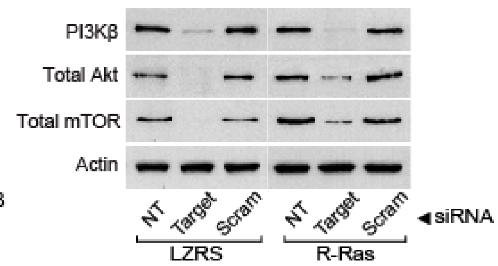

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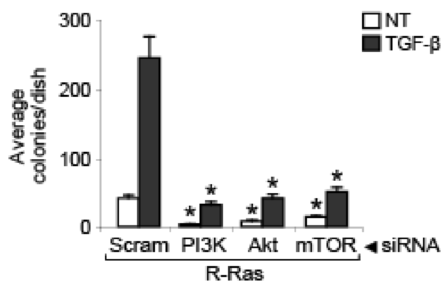

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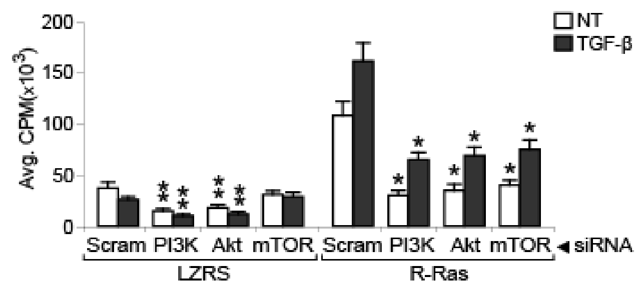

R-Ras-induced transformation is TAK1-dependent

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Discussion

TGF-β cooperates with oncogenic H-Ras in promoting transformation of EpH4 cells (Oft et al., 1996), however its role in transforming R-Ras/EpH4 or TC21/EpH4 cells is not known. In this study we demonstrate that oncogenic R-Ras collaborates with TGF-β to highly transform EpH4 cells, while TC21/EpH4 cells are relatively unaffected by the transforming effects of TGF-β. Furthermore, R-Ras/EpH4 cells produce significantly increased levels of autocrine TGF-β and require TGF-β-signaling to undergo transformation. The TGF-β-dependent effects on R-Ras

Figure 19. ***TAK1 is required for transformation by R-Ras, but not TC21.*** **A.** TAK1 activation was determined by *in vitro* kinase assay as described in *Experimental Procedures.* Immunoprecipitates were incubated with 1 µg of bacterially expressed MKK6 in 10 µl of kinase buffer containing [γ-^{32}P]ATP at 25 °C for 2 min. The samples were then fractionated by 10% SDS-PAGE and immunoblotted using antibodies to phosphorylated MKK3/6. Blotting with total TAK1 was performed as a control. **B.** Gene silencing was performed on cell populations using siRNA targeting TAK1 or a scrambled control. Immunoblots were performed on 20µg of total cell lysate from transfected cells to determine levels of target gene expression using the antibodies indicated. **C.** Soft agar colony formation assays were performed using TAK1 knockdown R-Ras/EpH4 and TC21/EpH4 cells, and colony formation was scored after 9 days. Knockdown of TAK1 expression significantly reduced colony formation in R-Ras/EpH4 cells (*p<0.01) **D.** 72-hour proliferation assays were performed using TAK1 knockdown cells. Silencing TAK1 significantly reduced R-Ras/EpH4 cell proliferation (*p<0.01). Values from transformation and proliferation assays are means ± SD from triplicate wells of a representative experiment.

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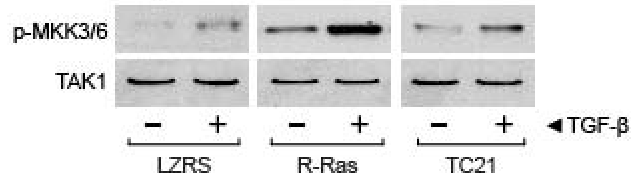

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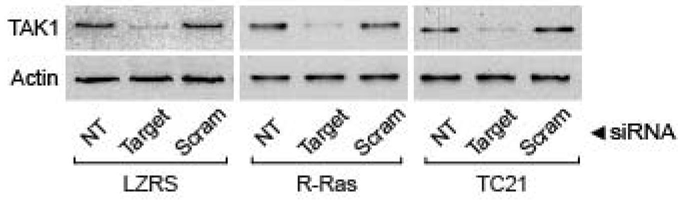

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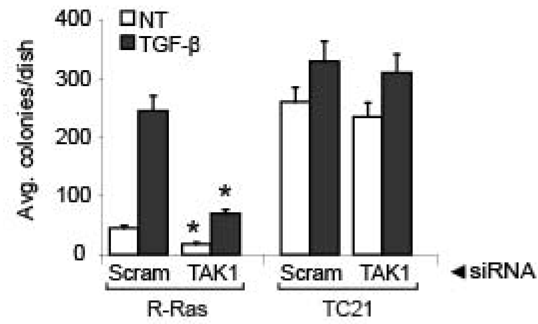

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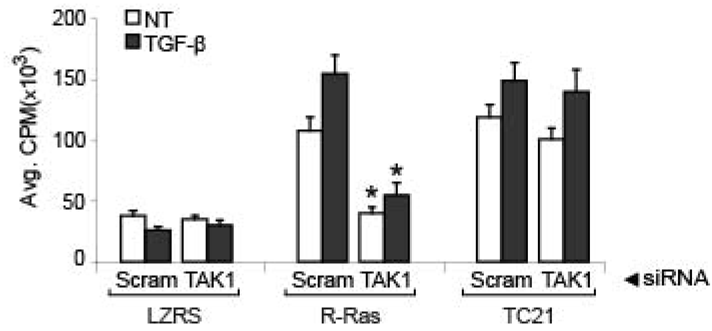

transformed EpH4 cells are mediated through activation of the TAK1/JNK/p38 MAPK, PI3K/Akt and mTOR-pathways. Thus, R-Ras and TGF-β cooperatively transform mammary epithelial cells through coordinate activation of Smad-independent signaling pathways.

Mutant R-Ras and H-Ras behave in a similar manner with respect to their cooperation with TGF-β in cell transformation. EpH4 cells expressing oncogenic H-Ras undergo an epithelial-fibroblastoid conversion following TGF-β stimulation, which is maintained through autocrine TGF-β production (Oft et al., 1996). Furthermore, these cells require TGF-β-signaling to induce tumor formation *in vivo* (Oft et al., 1998). We demonstrate here that R-Ras-transformed EpH4 cells also depend on TGF-β-signaling and show increased TGF-β production, while TC21/EpH4 cells are independent of TGF-β. The transformation of EpH4 cells with activated mutants of H-Ras, R-Ras or TC21 does not affect TGF-β-signaling through the Smad pathway, and this pathway is not required for transformation of R-Ras/EpH4 or TC21/EpH4 cells. This contrasts with reports that oncogenic transformation by H-Ras alters the TGF-β-dependent phosphorylation of Smad2/3 in RGM1 cells derived from the rat gastric epithelium (Sekimoto et al., 2007), and in mammary and lung epithelial cells (Kretzschmar et al., 1999). Thus, interactions between Ras proteins and the Smad-signaling network are highly dependent on the cell type.

Previously we established novel roles for JNK and p38 MAPK as mediators of R-Ras- and TC21-induced growth and transformation. Unlike studies where JNK and p38 MAPK were shown to mediate TGF-β-dependent phosphorylation of Smad2 and Smad3 (Hayes et al., 2003; Sekimoto et al., 2007), we did not observe any changes in Smad-signaling following transformation with R-Ras. The observation that R-Ras/EpH4 cells are reliant on JNK and p38 MAPK-signaling for cell transformation may be due to the ability of these kinases to regulate TGF-β expression levels (Gruden et al., 2000; Ventura et al., 2004). It is also known that TGF-β regulation of cell size, migration, invasion (Lamouille and Derynck, 2007) and protein synthesis (Das et al., 2007) requires activation of both Akt and mTOR, and we demonstrate here that signaling through PI3K/Akt and mTOR is also required for the transforming effects of TGF-β on

R-Ras/EpH4 cells. Although it has been reported that Akt and mTOR can inhibit TGF-β-dependent Smad-signaling by interacting with Smad3 and preventing its phosphorylation (Song et al., 2006), this behavior was not observed in R-Ras/EpH4 cells.

In examining Smad-independent signaling pathways, we identified TAK1 as a novel effector of R-Ras activity with a critical role in cell transformation. We demonstrate that TAK1 is activated by both R-Ras and TGF-β in EpH4 cells, and that TAK1 activation is required by TGF-β to mediate the transformation of R-Ras/EpH4 cells. Like TGF-β, TAK1 can act as either a tumor suppressor or tumor promoter under varying conditions. For example, TAK1 enhances the *in vitro* migration and lung metastasis of colon CT26 cancer cells (Choo et al., 2006) and is required for the TGF-β-dependent invasion and metastasis of MDA-MB-231 breast cancer cells (Safina et al., 2007). However, TAK1 also mediates TGF-β-induced apoptosis via p38 MAPK in PC-3U prostate cancer cells (Edlund et al., 2003) and targets the SnoN oncoprotein for degradation, thereby relieving its inhibition of TGF-β-dependent Smad-signaling (Kajino et al., 2007). Although it is currently unknown how R-Ras alters TAK1-signaling, it is possible that R-Ras directly interacts with TAK1 as TAK1 can form a tertiary complex with H-Ras and PI3K at the plasma membrane (Chen et al., 2007). Thus, TAK1 may provide a exciting new link through which signals from TGF-β and R-Ras converge to promote increased cell proliferation and transformation.

In conclusion, we have demonstrated that noncanonical TGF-β-signaling plays a significant role in the promotion of transformation by oncogenic R-Ras, but not TC21, in mammary epithelial cells. Furthermore, we demonstrate a critical role for TAK1 in this process, suggesting a novel role for this kinase in TGF-β-mediated oncogenic transformation. Together, our results suggest that differences in signaling through the TGF-β/TAK1, MAPK and PI3K pathways account for the differential transforming potential of R-Ras and TC21 in EpH4 cells. Understanding how these oncogenes differ in their mechanism of transformation may reveal novel strategies for targeting cancers that harbor mutations in R-Ras proteins.

CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

The primary focus of my research has been to investigate the transforming properties of the Ras-related proteins R-Ras and TC21 in an epithelial model of tumorigenesis, and to determine the role of TGF-β-signaling in R-Ras and TC21-induced transformation. I believe the work presented here adds important information to the field of cancer biology, and offers future opportunities to understand the normal and abnormal regulation of these processes. Below, I will discuss the key findings of my research and their implications, while interspersing interesting future experiments which they suggest.

R-Ras and TC21 in Cancer

In this work we demonstrate that the closely-related GTPases R-Ras and TC21(R-Ras2) induce oncogenic transformation in mammary epithelial cells via differential activation of the TGF-β/TAK1, JNK/p38 MAPK, PI3K/Akt and mTOR-signaling pathways. We show that transformation with either R-Ras or TC21 resulted in a loss of cellular responsiveness to the growth-inhibitory effects of TGF-β. Rather, with exogenous TGF-β stimulation TC21/EpH4 cells showed a modest increase in transformation while R-Ras/EpH4 cells became highly transformed. Furthermore, R-Ras/EpH4 cells showed significantly increased levels of TGF-β production, and this was required for their transformation. These findings establish a number of novel links between the R-Ras family of Ras-related proteins and TGF-β-signaling.

The prevalence of R-Ras and TC21 mutations in human cancers is unknown at present. The frequency of Ras mutations in human cancers varies according to tumor type, with rates as high as 90, 50, and 30% for pancreatic, colorectal and lung carcinoma respectively (Moon, 2006). Thus, aberrant Ras function is believed to contribute to the development of a significant portion

of these neoplasms. However, activating mutations in H-, K- and N-Ras are rarely found in neoplasms developed in the breast, ovaries or cervix (Rajalingam et al., 2007). While mutations in TC21 have been found in ovarian carcinoma and leiomyosarcoma cell lines (Chan et al., 1994; Huang et al., 1995), studies have shown mutations in TC21 to be infrequent in both primary and metastatic breast tumors examined (Barker and Crompton, 1998). Interestingly, increased expression of TC21 has been shown to be correlated with the advanced stages of esophageal cancer, and TC21 was found to be increasingly localized within the nucleus in these cells (Sharma et al., 2005). The function of TC21 in the nucleus is unknown, and in general, the function of Ras GTPases in the nucleus is unknown. It is possible that this action may simply sequester these proteins from their normal regulatory enzymes at the plasma membrane, or perhaps Ras GTPases can interact with transcription factors within the nucleus and influence the upregulation of growth-promoting genes. Our finding that TC21 is more transforming than H-Ras in a mammary epithelial model of tumorigenesis is significant and illustrates the heterogeneity with which these oncogenes can operate in different cell types.

Although mutations in R-Ras have not been reported in human tumors, this is likely because the prevalence of R-Ras mutations in human cancers has not been widely investigated. R-Ras has been found to be altered in some types of tumors. For example, elevated levels of R-Ras are reported to be sufficient for inducing estrogen-independent proliferation of breast cells and the progression of breast cancer cells to tamoxifen resistance (Yu and Feig, 2002). Also, upregulation of R-Ras was found in transformed colorectal crypt cells and in gastric cancers (Nishigaki et al., 2005). In addition, functional blocking of R-Ras in these cells resulted in the disappearance of adhered cells, confirming the role of R-Ras in gastric tumorigenesis. Moreover, R-Ras expression and phosphorylation correlated with increasing grade of gliomas in human brain tumor specimens (Nakada et al., 2005). These reports show that R-Ras has a relevant role in tumor progression of various types of cancer. Additional work is necessary to develop a better

understanding of the roles played by R-Ras and TC21 in normal cells and in the process of mammalian tumorigenesis.

Anti-Ras Drugs

The prevalence of *ras* mutations alone does not provide complete validation of the importance of aberrant Ras function in cancer development. However, the considerable body of experimental studies in cell culture and animal model systems argues strongly that aberrant Ras function contributes significantly to malignancy (Graham and Olson, 2007), and that therapeutic approaches to correct defects in Ras function may have an important impact on tumor progression (Alvarado and Giles, 2007; Smith et al., 2006). Moreover, since *ras* mutations are especially frequent in human cancers that are ineffectively cured by current therapeutic approaches (Smith et al., 2006), the development of anti-Ras drugs as novel anti-cancer agents has been a very active endeavour by the pharmaceutical and biotechnology industries.

Two main approaches have been considered and pursued for the development of anti-Ras drugs. First, much attention has been focused on the development of inhibitors of FTase, the enzyme that catalyzes Ras processing and attachment to the plasma membrane (Basso et al., 2006). A key advantage of FTase inhibitors (FTIs) as anti-Ras drugs is the fact that Ras biological function is dependent absolutely on farnesylation-mediated membrane association. However, since farnesylation is also required for the function of normal Ras proteins, as well as other proteins, FTIs will not specifically block the function of mutated Ras proteins (Pan and Yeung, 2005). Despite this concern from selectivity, FTIs have shown remarkable anti-tumor activity in cell-based and animal model studies, with surprisingly limited toxicity to normal cells (Gotlib, 2005; Zhu et al., 2003).

A number of FTIs are now under evaluation in Phase II clinical trials for anti-tumor activity in cancer patients (eg. R115777, SCH66336) (Appels et al., 2005; Perabo and Muller, 2007). The action of one specific inhibitor against farnesyltransferases, FTI-277, was tested in a

pancreatic carcinoma cell line that responds to TGF-β (Adnane et al., 2000). Interestingly, FTI-277 led to enhanced expression of TβRII mRNA, protein and cell surface ligand-binding activity, thus increasing the responsiveness of the tumor cells to TGF-β with respect to downstream transcriptional and cytostatic responses. This study established that drugs targeting oncogenic proteins like Ras have additional beneficiary effects as they simultaneously enhance the cytostatic functions of TGF-β against tumor cell growth. However, if we consider the pro-tumorigenic actions of TGF-β, then such drugs, if used systemically, may also have detrimental effects by promoting tumor invasiveness and suppression of cytolytic T cell activities. Interestingly, one unexpected complexity in the development of FTIs as anti-Ras drugs is the widely held belief that the anti-tumor activity of FTIs may not be due to inhibition of Ras function at all. Instead, while FTase is still the likely target for these inhibitors, whether a farnesylated Ras-related protein such as TC21 or another FTase substrate is the real target is another area that is currently being investigated (Karp and Lancet, 2007).

A second approach for the development of anti-Ras drugs has been to target the downstream signaling pathways that are activated by Ras. In particular, pharmacologic inhibitors of MEK, for example PD98059 and U0126, have been developed that prevent Ras activation of the Erk cascade (Friday and Adjei, 2008). Both cell culture and animal studies have documented the potent anti-tumor activity of these kinase inhibitors (Brown et al., 2007; Cole et al., 2006). However, in light of the fact that Ras must activate a multitude of effector signaling pathways for oncogenesis, the effectiveness of blocking only the Raf/MEK/Erk pathway as a means of abrogating Ras-mediated tumor proliferation is not clear. In addition to the MEK pathway, other pathways that are being targeting for therapeutic intervention include PI3K/Akt (Chee et al., 2007), JNK (Mehrotra et al., 2004), p38 MAPK (Wada et al., 2005), and mTOR (Awada et al., 2008). With the involvement of Ras proteins in a multitude of signaling pathways that regulate normal cell physiology, it is not surprising that aberrant activation of Ras can contribute significantly to the aberrant growth properties of cancer cells.

Whether anti-Ras drugs will become effective anti-cancer drugs is presently uncertain. Nevertheless, the rational design of pharmacologic inhibitors of Ras and other specific signal transduction molecules is widely believed to be the direction of drug development in the future. It is generally acknowledged that the current arsenal of cytotoxic compounds that are in use today in the clinic have reached their limits in efficacy for cancer treatment (Colombo et al., 2004). Therefore, it is hoped that anti-Ras and other target-based inhibitors will make a significant impact in drug development for the cure or palliative treatment of cancer.

Mechanisms of R-Ras and TC21 Activity

Ras family GTPases, when in their active GTP-bound state, interact with a wide array of downstream effectors to regulate many biological functions in different cell types (Takai et al., 2001). How signal specificity among the closely related family members is achieved is still poorly understood. There is both promiscuity and specificity in the ability of these proteins to interact with and regulate the various effector families, as well as isoforms within those families (Rodriguez-Viciana et al., 2004). Ras proteins seem to have individual blueprints of effector interactions, and specificity should be considered in the context of the full spectrum of effectors they regulate. The sequencing of the genome has identified a remarkably diverse number of proteins with domains homologous to the Ras-binding domain (RBD) of known Ras effectors and, thus, with the potential to interact with Ras and/or other Ras family GTPases (Kiel et al., 2007). In addition, other proteins without known RBD types are known to behave as Ras effectors (Linnemann et al., 2002), suggesting even more complexity in the number of effector interactions. Determining which of these many candidates are "true" effectors and characterizing their specificity is a critical step to understanding the specific signaling properties and biological functions of the various Ras family GTPases.

The "gold standard" for identifying a new protein as a direct Ras effector is to show that the purified proteins can interact *in vitro* in a GTP-dependent manner (Rodriguez-Viciana and

McCormick, 2005). Knowing that some Ras proteins have a remarkable degree of overlap in their effector interactions, it is also important to address the issue of specificity and compare the ability of the new effector to interact with other Ras family members. While it should be acknowledged that the overexpression of (exogenous) proteins could result in a loss of specificity and the detection of interactions that would not take place at physiological levels of expression, however, it should also be noted that even under these conditions of overexpression, remarkable specificity is still maintained, such that within the same cell type and under the same conditions, there are differential patterns of interactions among Ras proteins and their various effectors, with some Ras proteins, but not all, binding to particular effectors but not others, and doing so with distinct relative affinities (Rodriguez-Viciana 2004). It is important to note that our findings are only reflective of R-Ras and TC21 activity in one murine epithelial cell line. It will be necessary to explore the functions of these oncogenes in additional human and rodent model systems if we are to develop a more complete understanding of R-Ras and TC21-induced tumorigenesis.

One exciting prospect for future investigation is to study the structural domains within R-Ras and TC21 that mediate the transforming effects of these closely-related oncogenes. R-Ras and TC21 share 89% overall amino acid identity, including complete identity in their Switch I and Switch II domains, yet show remarkably different activity and transforming properties in a variety of cell systems (Cox et al., 1994; Graham et al., 1994). One method to determine the specific domains or residues responsible for the observed effects would be to generate a panel of R-Ras/TC21 chimeras and measure their potential to induce transformation in EpH4 or alternative cell systems. Areas of amino acid homology are shown in red in Figure 20.

Since R-Ras has an additional 26 amino acids at its N-terminus which are absent from TC21, one interesting chimera to generate would incorporate full-length TC21 fused with an R-Ras N-terminal domain. While the function of this domain is unclear, it has been shown by others to mediate R-Ras-dependent Rac activation and cell migration (Holly et al., 2005). As numerous reports have demonstrated the C-terminal hypervariable region to be critical for proper

Figure 20. ***Sequence homology between TC21, R-Ras and H-Ras.***

localization and thus normal functioning of Ras proteins (Gotoh et al., 2001; Konstantinopoulos et al., 2007; Michaelson et al., 2005), it would also be of interest to generate R-Ras/TC21 chimeras where varying lengths of the respective C-terminal regions were exchanged, and to test whether these chimeras showed differential transforming properties, subcellular localization, or activation of previously identified R-Ras and TC21 effectors. By identifying the specific domains mediating transforming activity by these oncogenes, we will further our understanding of the molecular basis of Ras-induced tumor formation and may even discover novel targets for pharmacological intervention.

The most notable finding from our studies with R-Ras and TC21 is that these highly homologous proteins induce transformation via distinct signaling mechanisms in EpH4 cells. In particular, R-Ras-transformation was dependent on TGF-β-signaling and activation of the TGF-β effector TAK1, while TC21-transformation was independent of TGF-β/TAK1. These findings suggest that TAK1 may play an important role as a mediator of crosstalk between the TGF-β and Ras-signaling pathways. Since others have demonstrated that TAK1 can form a tertiary complex at the plasma membrane with H-Ras and PI3K (Chen et al., 2007), it would be interesting to determine firstly whether R-Ras interacts directly with TAK1, and secondly whether a similar tertiary complex forms between PI3K, TAK1, and either R-Ras or TC21, both of which have been shown previously to interact directly with PI3K (Marte et al., 1997; Murphy et al., 2002). Studies have also reported that TGF-β can bind and activate PI3K (Yi et al., 2005), though it

remains unclear whether this is a direct interaction. Thus, one possible explanation for the observed differences in responsiveness between R-Ras/EpH4 and TC21/EpH4 cells may involve the ability of R-Ras and TC21 to induce the formation of a signaling-complex with TAK1 and TGF-β at the plasma membrane. A model for this possibility is illustrated below in Figure 21.

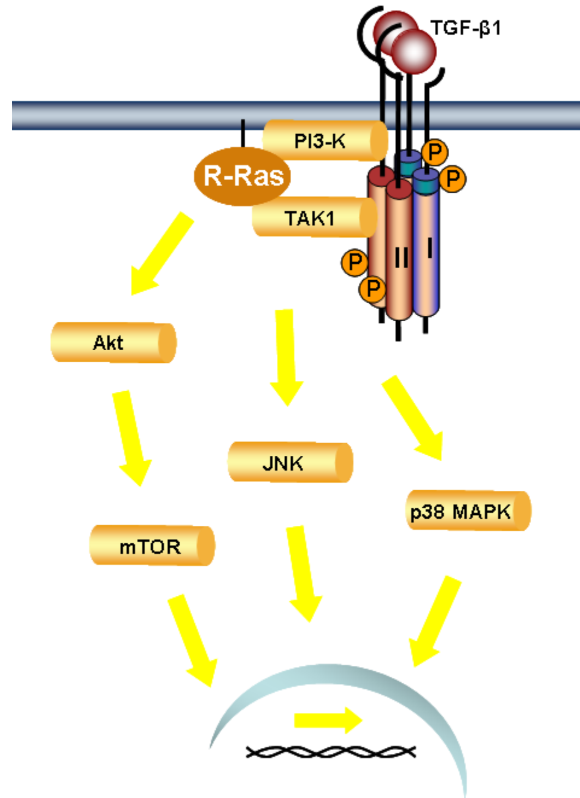

Figure 21. ***Model for interaction between R-Ras and TGF-β-signaling.***

All Roads Lead to TGF-β

Through our studies we have established a novel role for TGF-β in the regulation of proliferation and transformation induced by oncogenic R-Ras. As a global regulator of cell growth and proliferation (Bierie and Moses, 2006), it is not surprising to find a role for TGF-β in the process of oncogenic transformation. What was surprising, however, is that R-Ras/EpH4 and

TC21/EpH4 cells differed so dramatically in their response to exogenous TGF-β and in their requirement for TGF-β/TAK1-signaling to induce transformation, despite the high degree of homology and numerous similarities between R-Ras and TC21. It would be interesting to map out the specific residues or domains within R-Ras that are required for cooperative behavior with TGF-β to take place, and this could be accomplished through the generation of various R-Ras/TC21 chimeras. Now that we have established an *in vitro* relationship between TGF-β and R-Ras, it will be necessary to determine whether similar cooperative behavior is present in a more physiologically relevant context using *in vivo* models of tumorigenesis.

The ability of TGF-β to synergize with transforming oncogenes further complicates the task of developing targeting therapies for specific types of cancer. The data presented in this work have several implications for the bigger picture of designing rational treatments for individual patients: first, oncogenes such as R-Ras may engage and require TGF-β for tumor progression; second, tumors with activating mutations in R-Ras are attractive candidates for the testing of TGF-β inhibitors; and third, combinations of anti-Ras agents, pathway-specific inhibitors, and inhibitors of TGF-β may reduce tumor burden more effectively than either of these treatment options alone.

Concluding Remarks

The research presented here demonstrates that R-Ras and TC21 induce oncogenic transformation of mammary epithelial cells through distinct signaling mechanisms. While we do not yet know the prevalence of R-Ras and TC21 mutations in human cancers, this work has contributed to understanding the molecular mechanisms through which these oncogenes operate to promote malignant transformation. Most notably, we have identified TAK1 as a novel link that may function to coordinate signals between TGF-β and Ras proteins. Through these studies, we have generated a powerful new model with which to study the transforming properties of R-Ras proteins.

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