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The guanine nucleotide exchange factor (GEF) Ect2 is an oncogene in human cancer

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Rho family GTPases; Rac1; RhoA; Cdc42; Ect2; guanine nucleotide exchange factor (GEF); protein kinase C (PKC); Par6; Mek/Erk; cellular transformation; cellular invasion; lung cancer; oncogene; gene amplification; anchorage-independent growth; tumorigenesis

1. Introduction

Small guanosine triphosphatases (GTPases; EC 3.6.5.2) act as molecular switches in diverse signaling pathways that regulate actin cytoskeleton modeling, cell motility, cell adhesion, cell cycle progression and gene transcription. The discovery of mutations in the *RAS* gene, the prototypical member of the GTPase family, in human tumors prompted a search for mutations in other GTPases, including the well characterized members of the Rho family GTPases, RhoA, Rac1 and Cdc42. To date, mutations in these Rho GTPases have not been shown to occur naturally in human cancers. The Rho GTPases, however, have been implicated in the malignant phenotype of many human cancers as a result of their participation in aberrant signaling in tumor cells and overexpression in human tumors (reviewed in (Gomez del Pulgar et al., 2005). Similar to other GTPases, RhoA, Rac1 and Cdc42 cycle between a GTP bound active state and a GDP bound inactive state. The weak intrinsic GTPase activity of the Rho GTPases is enhanced by association with GTPase activating proteins (GAPs) that promote hydrolysis of GTP and the return of the GTPase to the inactive state. The activity of Rho GTPases is negatively regulated by GTP dissociation inhibitors (GDIs) which bind GTPases and maintain them in the GDP bound state. Guanine nucleotide exchange factors (GEFs) catalyze the exchange of GDP for GTP, thereby activating the Rho GTPases in signal transduction. GEFs for Rho GTPases have also been implicated in cancer, some of which were initially identified as potential oncogenes. One of the Rho-GEFs reported to promote transformation is Epithelial Cell Transforming Sequence 2 (Ect2), a member of the Dbl family of GEFs. This report summarizes the physiologic functions of Ect2, mechanisms of its regulation and its role in cellular transformation. Particular focus will be placed on recent studies demonstrating that Ect2 is an oncogene in human tumors.

2. Ect2 structure and expression

Pebble (Pbl), the fly orthologue of the human *ECT2* gene was the first member of the Ect2 protein family to be identified during a screen for recessive embryonic lethal mutations that result in cuticular pattern defects in *Drosophila melanogaster* (Jügens, 1984). *Pbl* was subsequently found to function as a Rho-GEF required for cytokinesis through activation of Rho1 (a *Drosophila* orthologue of mammalian RhoA) (Lehner, 1992; Prokopenko et al., 1999). Mammalian *ECT2* was later isolated as a proto-oncogene from a murine keratinocyte expression cDNA library using an expression cloning assay (Miki et al., 1993). The human homolog of *ECT2* was subsequently cloned and mapped to 3q26.1→3q26.2. The human

ECT2 and murine *ect2* genes exhibit 95% similarity and 84% identity at the amino acid level. *ECT2* orthologs in *Caenorhabditis elegans* (Let-21) (Dechant and Glotzer, 2003) and in *Xenopus* (*XECT2*) (Tatsumoto et al., 2003) have also been identified and exhibit similarities to the human *ECT2* gene along their entire coding sequence. Thus, *ECT2* is highly evolutionarily conserved.

Human *ECT2* encodes a polypeptide of 883 amino acids, with a predicted molecular weight of approximately 104kDa. The Ect2 protein consists of several structural domains (Figure 1). The N-terminus of Ect2 serves a regulatory function and contains sequences that exhibit high homology to cell cycle control and repair proteins (Saito et al., 2003). An XRCC1 domain resides at the extreme N-terminal region of Ect2, and shows sequence homology to human XRCC1, a protein that repairs defective DNA strand breaks and functions in sister chromatid exchange (Thompson et al., 1990). Adjacent to the XRCC1 domain are sequences that show homology to Clb6, a yeast B-cyclin that promotes transition from the G₁ to S phase of cell cycle. The Clb6 domain is followed by sequences that exhibit homology to yeast Rad4/Cut5, a protein required for entry into S phase and inhibition of M phase entry prior to completion of DNA synthesis (Saka and Yanagida, 1993). Rad4/Cut5 also plays a critical role in replication checkpoint control in yeast (Saka et al., 1994). The Cut5 homology domain contains tandem repeats of the BRCT (Breast Cancer gene 1 Carboxyl-terminal) motif. The BRCT motif is highly conserved in proteins involved in DNA repair and cell cycle checkpoint responses (Bork et al., 1997; Callebaut and Mornon, 1997). The N- and C-terminal domains of Ect2 are separated by a small central (S) domain containing two nuclear localization sequences that appear to be involved in the control of the intracellular localization of Ect2. The catalytic core of Ect2 is found within the C-terminus, and consists of a Dbl-homology (DH) and a pleckstrin-homology (PH) domain which confer guanine nucleotide exchange activity toward Rho-GTPases. The extreme C-terminal (C) region of Ect2 does not exhibit significant homology to any known protein domains or motifs.

Ect2 expression is dynamically controlled throughout the cell cycle. During interphase, Ect2 is sequestered within the nucleus. Upon breakdown of the nuclear envelope during mitosis, Ect2 is dispersed throughout the cytoplasm. Ect2 becomes localized to the mitotic spindles during metaphase, the cleavage furrow during telophase, and then the mid-body at the end of cytokinesis (Tatsumoto et al., 1999). Northern blot analysis reveals that Ect2 is expressed in a broad range of adult tissues including kidney, liver, spleen, testis, lung, bladder, ovary and brain (Miki et al., 1993; Saito et al., 2003). *In situ* hybridization of fetal tissues shows Ect2 expression in the liver, thymus, proliferating epithelial cells of the nasal cavity and gut, tooth primordial, costal cartilage, heart, lung and pancreas (Saito et al., 2003).

3. Physiological functions of Ect2

Functional studies in *Drosophila* established a critical role for *Pbl*, the *Drosophila* ortholog of Ect2, in cytokinesis. At the onset of cytokinesis, *Pbl* is associated with the cleavage furrow where it activates Rho1 to initiate assembly of the contractile ring and promote cytokinesis (Prokopenko et al., 1999). Mutations in *Pbl* result in a lack of contractile ring formation and failure of *Drosophila* cells to undergo cytokinesis during embryogenesis. Likewise, Let-21 the *C. elegans* ortholog of Ect2 is required for formation of the cleavage furrow (Dechant and Glotzer, 2003). Interference of mammalian Ect2 function through the expression of a dominant negative *ECT2* allele, microinjection of an Ect2 antibody, or RNAi-mediated knockdown of Ect2 lead to accumulation of multinucleated cells indicative of a failure to undergo cytokinesis (Kim et al., 2005; Liu et al., 2004; Tatsumoto et al., 1999). Ect2 has also been implicated in the control of mitotic spindle assembly in *Xenopus* through activation of Cdc42 (Tatsumoto et al., 2003), and an Ect2→Cdc42→mDia3 signaling pathway has been implicated in

facilitating attachment and stabilization of spindle fibers to kinetochores (Oceguera-Yanez et al., 2005).

Ect2 may also be involved in cell polarity. Cellular polarity is required for asymmetrical cell division, directional cell migration as well as establishment of tissue and organ architecture. The Par complex consisting of Par-6/Par-3 (partition-defective)/atypical protein kinase C (EC 2.7.11.13) and small GTPases, such as Cdc42 or Rac1, is an evolutionary conserved complex that regulates the formation and maintenance of apical-basal polarity in epithelial cells (Joberty et al., 2000). In MDCK cells, small amounts of Ect2 can be detected at cell-cell contacts where it is reported to directly interact with Par6 and PKC ζ and to modulate PKC ζ activity (Liu et al., 2004). Expression of either dominant negative or a constitutively active Ect2 alleles disrupt cell polarity in MDCK cells (Liu et al., 2006). Furthermore, both Ect2 expression and localization to cell-cell contacts is regulated by calcium, a critical regulator of cell-cell adhesion (Liu et al., 2004).

Ect2 is reported to interact with several members of the Rho GTPase family including, RhoA, RhoB, RhoC, RhoG, Rac1 and Cdc42 (Miki et al., 1993; Saito et al., 2004; Solski et al., 2004; Tatsumoto et al., 1999; Wennerberg et al., 2002). There are conflicting reports regarding the ability of Ect2 to carry out nucleotide exchange on these Rho GTPases. Ect2 expressed in insect cells has no apparent GEF activity towards Rac1, Cdc42 or RhoA *in vitro* (Miki et al., 1993) whereas full length Ect2 immunoprecipitated from mammalian cells is capable of catalyzing nucleotide exchange on RhoA, Rac1 and Cdc42 *in vitro* (Tatsumoto et al., 1999). This apparent discrepancy may be related to the observation that phosphorylation of mammalian Ect2 can regulate its GEF activity (Tatsumoto et al., 1999). Thus, the ability of Ect2 to catalyze nucleotide exchange on Cdc42 or Rac1 may require specific phosphorylation events that only occur in mammalian cells. It is well established that Ect2 regulates cytokinesis in *Drosophila* and in mammalian cells through RhoA-mediated pathways (Burkard et al., 2007; Kamijo et al., 2006; Kimura et al., 2000; Nishimura and Yonemura, 2006; Yuce et al., 2005). Analysis of *Pbl* indicates that it can catalyze nucleotide exchange on Rho1, but not Rac1 or Cdc42 (Prokopenko et al., 1999). It has been reported that Ect2 may not directly catalyze GTP loading on RhoA, but rather may serve as a signaling platform that is necessary for the recruitment of molecules such as RhoA to the cleavage furrow during cytokinesis (Birkenfeld et al., 2007). Recently, Ect2 and the Rho-GAP, MgcRacGAP, have been implicated in regulating Cdc42 activity and attachment of spindle microtubules to kinetochores during metaphase (Oceguera-Yanez et al., 2005). Thus, whereas Ect2 regulates cytokinesis through activation of RhoA, Ect2 appears to function in mitosis through activation of Cdc42. Collectively, these data indicate that Ect2 can act as a GEF for multiple Rho family GTPases, including RhoA, Rac1 and Cdc42. An important area for further study is to determine the role of phosphorylation, localization and protein-protein interactions in regulating the specificity of Ect2 GEF activity.

4. Regulation of Ect2 function

Ect2 function is regulated through a number of mechanisms including phosphorylation, intracellular localization and intra- and inter-molecular interactions. Ect2 becomes phosphorylated during the G₂ phase of the cell cycle, remains phosphorylated during mitosis and becomes dephosphorylated at the time of cytokinesis. Phosphatase treatment of immunoprecipitated Ect2 significantly decreases its GEF activity, suggesting that Ect2 exchange activity is regulated by phosphorylation. At least 2 sites on Ect2, T341 and T412, have been reported to be phosphorylated during G₂/M phase most likely by cyclin-dependent kinase 1 (Cdk1; EC 2.7.11.22) (Hara et al., 2006; Niiya et al., 2006). Phosphorylation at T412 is required for subsequent binding and phosphorylation of Ect2 by polo-like kinase 1 (Plk1; EC 2.7.11.21), however the sites of Plk1-mediated phosphorylation have not been determined.

Phosphorylation at T341 and T412 both appear to be important for Ect2-mediated activation of transcription by RhoA (Hara et al., 2006; Niiya et al., 2006). Phosphorylation of T341 is thought to induce a conformational change that may release Ect2 from an auto-inhibitory state, generating an active conformation that is able to associate with putative binding partners (Hara et al., 2006).

A second level of Ect2 regulation is mediated through control of its intracellular localization. Ect2 is localized almost exclusive to the nucleus of non-transformed cells during interphase where it exists in a hypo-phosphorylated state. During mitosis Ect2 is dispersed in the cytoplasm where it becomes phosphorylated and presumably activated. Nuclear localization of Ect2 is likely mediated by two functional nuclear localization sequences (NLS) in the S domain of Ect2. Truncation mutants of Ect2 that remove the S domain, or Ect2 mutants in which the NLS sites are disrupted, localize to the cytoplasm throughout the cell cycle (Saito et al., 2004).

Like other members of the Dbl family of GEFs, Ect2 becomes constitutively active upon removal of its N-terminal sequences, indicating that the domains found in the N-terminus function as negative regulators of Ect2 GEF activity. Kim et al identified an autoinhibitory interaction between the BRCT and DH/PH domains of Ect2 (Kim et al., 2005). Pull down assays demonstrate that the N-terminus of Ect2, spanning the amino acids containing the BRCT domains (Ect2-N), is able to bind to both full length Ect2 and a Ect2-DH/PH mutant, but does not dimerize with itself. Mutation of W304, a conserved amino acid in the BRCT domain, eliminated the interaction of the BRCT domain with full length Ect2 or the Ect2-DH/PH mutant, and results in a mutant Ect2 with increased GEF activity (Kim et al., 2005). Thus, disruption of the intramolecular interaction between the DH/PH and BRCT domains appears to release Ect2 from autoinhibition by inducing a conformation that exposes the catalytic domain of Ect2 leading to increased GEF activity.

Another mechanism by which Ect2 GEF activity is regulated involves specific interactions of Ect2 with other proteins. During cytokinesis, Ect2 associates with MgcRacGAP, a GTPase activating protein for Rac1 (Kamijo et al., 2006; Somers and Saint, 2003; Yuce et al., 2005). This interaction may prevent Ect2-mediated activation of Rac1 during cytokinesis. MKlp1 (EC 3.6.4.4), a mitotic kinesin, and MgcRacGAP co-precipitate with Ect2 from HeLa cells arrested in mitosis. MKlp1 forms a stable complex with MgcRacGAP known as centralspindlin that is found at the central spindle of cells during mitosis (Mishima et al., 2002). Thus, the centralspindlin complex may be directly involved in targeting Ect2 to the central spindle of mitotic cells and regulating its GEF activity there. Ect2 may also play a role in cell polarity as a result of its interaction with the Par polarity complex at areas of cell-cell contact (Liu et al., 2004; Liu et al., 2006). Finally, Ect2 has also been reported to interact with a kelch-related protein, KLEIP (KLHL20), an interaction that appears to be involved in VEGF-induced activation of Rho GTPases (Hara et al., 2004).

5. Ect2 and Cancer

The *ECT2* gene was initially identified as a proto-oncogene capable of transforming NIH/3T3 fibroblasts (Miki et al., 1993). Subsequent analysis revealed that the originally characterized oncogenic Ect2 clone actually consisted of a carboxyl-terminal truncation of the full-length *ECT2* gene. This truncated clone encoded a protein consisting of the DH-PH-C domains of Ect2 similar to that described above. This mutant localized to the cytoplasm, possessed constitutive GEF activity and could transform fibroblasts *in vitro* (Saito et al., 2004). In contrast, full-length Ect2 localized almost exclusively to the nucleus and was not capable of transforming fibroblasts (Saito et al., 2004), consistent with the observation that sequences within the N-terminal region of Ect2 serve to regulate Ect2 localization and function.

Whereas these results indicated that Ect2 is theoretically capable of causing cellular transformation, the relevance of these initial observations to human cancer remained obscure until recently. Expression analysis revealed that established human cancer cell lines express only full-length Ect2, indicating that the transforming C-terminal Ect2 fragment originally cloned is not directly relevant to the cancer biology (Saito et al., 2003). Paradoxically, full-length Ect2 is overexpressed in several human tumor types, suggesting a role for elevated Ect2 expression in these tumors (Hirata et al., 2009; Salhia et al., 2008; Sano et al., 2006; Zhang et al., 2008). Furthermore, transient knockdown of Ect2 expression by small interfering RNA leads to a transitory effect on cell cycle progression in lung cancer cells *in vitro*, suggesting that Ect2 may be involved in tumor cell growth (Hirata et al., 2009), though the specific role of Ect2 in cancer cell growth was not determined. More recent studies have firmly established that Ect2 is a human oncogene in lung and possibly other human tumor types.

5.1 Ect2 is overexpressed in human tumors as a result of tumor-specific ECT2 gene amplification

Ect2 is highly expressed in a variety of human tumors including brain (Salhia et al., 2008; Sano et al., 2006) lung (Hirata et al., 2009; Justilien and Fields, 2009), bladder (Saito et al., 2004), esophageal (Hirata et al., 2009), pancreatic (Zhang et al., 2008) and ovarian tumors (Saito et al., 2004). Ect2 is overexpressed at the mRNA and protein levels in established NSCLC cell lines as well as primary NSCLC tumors (Justilien and Fields, 2009). Ect2 overexpression was found in 10 of 15 lung cancer cell lines examined (Hirata et al., 2009) and in 82% of primary NSCLC tumors (Justilien and Fields, 2009). Immunohistochemical analysis revealed that Ect2 is largely restricted to the nucleus of normal lung epithelial cells (Justilien and Fields, 2009), but that primary NSCLC tumors display increased Ect2 staining in both the nucleus and cytoplasm with little or no staining in the tumor-associated stroma (Figure 2) (Hirata et al., 2009; Justilien and Fields, 2009). Analysis of 242 NSCLC cases found that 46% exhibited strong Ect2 staining, 38% stained weakly, and 16% were negative for Ect2 (Hirata et al., 2009). Consistent with this finding, we independently observed elevated cytoplasmic Ect2 staining in ~84% of primary NSCLC tumors analyzed (Justilien and Fields, 2009). Thus, Ect2 is frequently overexpressed and mislocalized in primary NSCLC tumors. Likewise, Ect2 mRNA and protein is overexpressed in glioblastoma multiforme (GBM), compared to normal brain tissue and low-grade astrocytomas (LGA) (Salhia et al., 2008). Interestingly, whereas LGAs show predominantly nuclear Ect2 staining, GBMs display prominent staining of Ect2 in both the cytoplasm and nucleus, consistent with findings in NSCLC (Hirata et al., 2009; Justilien and Fields, 2009). Similar increases in Ect2 mRNA levels have been reported in pancreatic cell lines and primary pancreatic tumors (Zhang et al., 2008), however it remains to be determined whether overexpression of Ect2 in pancreatic cancer cells plays a role in their transformation.

The Ect2 gene (*ECT2*) resides on chromosome 3q26, a region frequently targeted for chromosomal alterations in human tumors (Eder et al., 2005; Han et al., 2002; Lin et al., 2006; Zhang et al., 2006). *ECT2* amplification frequently occurs in lung squamous cell carcinomas (LSCC) (~70%), but rarely in lung adenocarcinoma (LAC) (Justilien and Fields, 2009). These findings are consistent with the prevalence of chromosome 3q26 amplification in LSCCs and the rare occurrence of 3q26 amplification in LACs (Balsara et al., 1997; Brass et al., 1996). Interestingly, *ECT2* gene copy number correlates with copy number of the PKC α gene (*PRKCI*), a relevant oncogene previously shown to be amplified as part of the 3q26 amplicon in human tumors (Regala et al., 2005b; Zhang et al., 2006). In addition, Ect2 mRNA expression in LSCCs correlates with gains in *ECT2* gene copy number, demonstrating that tumor-specific *ECT2* amplification is an important mechanism driving Ect2 overexpression in LSCCs (Justilien and Fields, 2009). 3q26 amplification is also commonly present in esophageal squamous cell carcinomas (ESCCs), and ~40% of primary ESCC tumors contain *ECT2*

amplification (Yang et al., 2008; Yen et al., 2005). Although Ect2 is found to be overexpressed in ESCC tumors, a direct correlation was not seen between *ECT2* amplification and expression in these tumors. The exposure of refinery workers to nickel compounds correlates with an increased risk for the development of tumors of the respiratory tract (Shen and Zhang, 1994). Interestingly, C3H/10T1/2 mouse embryo fibroblast cells transformed with insoluble carcinogenic nickel compounds show increased expression of Ect2 mRNA and protein compared to non-transformed 10T1/2 cells as result of *Ect2* amplification (Clemens et al., 2005). It remains to be determined whether *ECT2* is also amplified and overexpressed in tumors of the respiratory tract that develop in nickel refinery workers. In addition to LSCC and ESCC tumors, ovarian tumors also show tumor-specific amplification at 3q26 (Eder et al., 2005; Zhang et al., 2006). Ect2 is overexpressed in ovarian tumors harboring *ECT2* gene amplification compared to whole normal ovary (Haverty et al., 2009) indicating that tumor-specific *ECT2* amplification also drives Ect2 expression in ovarian tumors. Thus, a major mechanism driving Ect2 expression is tumor-specific amplification of *ECT2* as part of the 3q26 amplicon. 3q26 amplification is also prevalent in SCC of the cervix (Heselmeyer et al., 1997; Heselmeyer et al., 1996) and head and neck (Singh et al., 2002), suggesting that *ECT2* may also be a relevant target of amplification in these tumors.

ECT2 amplification is likely not the only mechanism by which Ect2 is overexpressed in human tumors. Many tumors, including lung adenocarcinomas (LACs), which do not harbor *ECT2* amplification, also overexpress Ect2. Indeed, elevated Ect2 mRNA and protein expression is equally prevalent in LAC and LSCC tumors despite the fact that *ECT2* amplification is confined to LSCC tumors (Justilien and Fields, 2009). Though relatively little is known about the transcriptional mechanisms controlling Ect2 gene expression, a recent report demonstrated that *ECT2* contains a p53 response element that binds p53 in response to DNA damage or Nutlin-3. Upon p53 binding, Ect2 expression is suppressed (Scoumanne and Chen, 2006). *ECT2* promoter activity may also be inhibited by p21 (Scoumanne and Chen, 2006). Whether these mechanisms occur in the context of human tumors remains to be resolved. It is reasonable to hypothesize however that in tumors with mutant p53, Ect2 promoter activity may be increased. Other potential mechanisms for oncogenic activation of Ect2 such as post-translational modifications and/or somatic mutations also warrant further investigation.

Ect2 overexpression is associated with poor prognosis in patients with NSCLC and ESCC (Hirata et al., 2009). Specifically, NSCLC and ESCC patients whose tumors exhibit strong Ect2 staining had a poorer prognosis than patients whose tumors showed weak or no Ect2 staining. In ESCC patients, high Ect2 expression also positively correlated with tumor size and tumor metastasis to lymph nodes (Hirata et al., 2009), suggesting that Ect2 expression is important for these clinicopathological factors of tumor progression. Ect2 expression may also serve as a prognostic indicator in glioblastoma patients, where high Ect2 expression was associated with a shorter survival time (Salhia et al., 2008; Sano et al., 2006). Further investigation is required to determine if Ect2 expression is useful as a prognosis marker in other human cancer types.

5.2 Function of Ect2 domains in transformation

As mentioned above, mammalian Ect2 was initially isolated as a complementary DNA (cDNA) from murine epithelial cells whose ectopic expression was capable of transforming NIH 3T3 fibroblasts (Miki et al., 1993). The original Ect2 cDNA was found to encode a truncation mutant of full length Ect2 consisting of the DH and PH domains that catalyze nucleotide exchange for Rho GTPases. Similar to other members of the Dbl family, the N-terminal sequences of Ect2 serve as negative regulators of its GEF activity. The Ect2 BRCT domains form an intramolecular association with the DH domain, inducing a conformation in which Ect2 GEF activity may be blocked. Consistent with this observation, removal of the N-terminus of Ect2

leads to constitutive activation and gain of transforming activity (Saito et al., 2004). Deletion of the S domain also results in an Ect2 mutant that exhibits transforming activity, albeit weaker than the N-terminal truncation mutant. The DH, PH and C domains have all been reported to be required for the transforming activity of Ect2. Point mutations of key residues in the DH domain abolish Ect2 transforming activity (Saito et al., 2004). Likewise, deletions in Ect2 encompassing the PH domain also inhibit Ect2 transforming activity (Saito et al., 2004). In a conflicting study, mutation of tryptophan 752, a key conserved residue in the PH domain of most Dbl GEFs, does not impair Ect2 transforming activity (Solski et al., 2004). Deletion of sequences in the C domain also significantly reduces Ect2 transforming activity (Saito et al., 2004; Solski et al., 2004). An Ect2 mutant consisting of the isolated DH/PH domains (Ect2-DH/PH) is able to activate only RhoA *in vitro* and *in vivo* (Solski et al., 2004). In contrast, an Ect2 mutant consisting of the DH, PH and C domains (Ect2-DH/PH/C) activates RhoA, Rac1, and Cdc42 *in vivo*. Interestingly, expression of the Ect2-DH/PH mutant enhances actin stress-fiber formation indicative of RhoA activation, whereas expression of the Ect2-DH/PH/C mutant caused lamellipodia formation characteristic of Rac1 activation (Solski et al., 2004). Thus, sequences in the C terminal domain of Ect2, coupled with cytoplasmic mislocalization, may influence the specificity of its GEF activity in transformation. However, since Ect2 truncation mutants have not been identified in human cancers, and ectopic expression of full length Ect2 in non-transformed cells is not sufficient in and of itself to induce transformation (Saito et al., 2004), it appears that other mechanisms regulate Ect2 function in transformed cells.

5.3 Ect2 is important for cell proliferation, migration, invasion and tumorigenicity of human cancer cells

Ect2 plays a promotive role in transformation in various tumor model systems. Transient knockdown of Ect2 by short interfering RNAs (siRNA) decreases cell growth of NSCLC cells, suggesting a role for Ect2 in cellular proliferation (Hirata et al., 2009). Consistent with this finding, stable knockdown (KD) of Ect2 expression by short hairpin RNAs (shRNAs) significantly impairs anchorage-independent growth and cellular invasion of multiple NSCLC cell lines (Justilien and Fields, 2009). Expression of an shRNA-resistant, wild-type Ect2 allele in Ect2 KD cells restored anchorage-independent growth and invasion to Ect2 KD cells, confirming that inhibition of these cellular phenotypes was due to loss of Ect2. Ect2 KD in A549 NSCLC cells induces impaired tumor growth when injected into the flanks of athymic nude mice, demonstrating that Ect2 also plays a role in NSCLC tumorigenicity *in vivo* (Justilien and Fields, 2009). Ect2 KD inhibits NSCLC cell proliferation *in vivo* with no apparent effects on tumor cell apoptosis or tumor associated vasculature, indicating that the primary function of Ect2 in NSCLC tumorigenicity is to promote tumor cell proliferation (Justilien and Fields, 2009).

Ect2 is also important for proliferation, migration and invasion of glioma cells. RNAi-mediated KD of Ect2 caused a significant decrease in glioma cell proliferation (Salhia et al., 2008; Sano et al., 2006). Glioma cells depleted of Ect2 showed increased multinucleation (Salhia et al., 2008), a hallmark characteristic of a cytokinesis defect, suggesting that the decrease in proliferation observed in Ect2 KD glioma cells was due to an inability of Ect2-deficient cells to undergo cell division. Using a two-dimensional radial cell migration assay, Salhia et al showed that Ect2 KD in glioblastoma cell lines significantly inhibited the rate of migration of these cells (Salhia et al., 2008). Depletion of Ect2 in glioma cells also decreased invasion in an ex vivo rat brain slice assay, and in *in vitro* Matrigel invasion assays (Sano et al., 2006), consistent with results observed in NSCLC cells (Justilien and Fields, 2009). Ect2 is also important for proliferation of ESCC cells *in vitro* (Hirata et al., 2009), though it has not yet been established whether Ect2 expression is essential for transformation in ESCC cells.

5.4 Cellular mechanisms in Ect2 mediated transformation

5.4.1 Ect2 function in NSCLC transformation is distinct from its functions in cytokinesis

A major physiologic function of Ect2 is the regulation of cytokinesis (Hara et al., 2006; Niiya et al., 2006; Tatsumoto et al., 1999). Ect2 KD in non-transformed cells leads to a cytokinesis defect characterized by decreased cell growth and accumulation of multinucleated cells (Tatsumoto et al., 1999). Strikingly however, NSCLC cells stably transduced with Ect2 shRNAs do not show significant changes in population doubling time (PDT) or accumulation of multinucleated cells (Justilien and Fields, 2009). Likewise, ectopic NSCLC Ect2 KD tumors exhibit a dramatic decrease in cellular proliferation but no accumulation of multinucleated cells *in vivo* (Justilien and Fields, 2009). In contrast, Ect2 KD in non-transformed MDCK cells results in a profound increase in PDT and accumulation of large, multinucleated cells indicative of a cytokinesis defect (Justilien and Fields, 2009; Tatsumoto et al., 1999). These data indicate that the role of Ect2 in transformed growth and invasion of NSCLC cells *in vitro*, and tumorigenicity *in vivo*, is distinct from its role in cytokinesis. In contrast, transient Ect2 KD in glioma and A549 NSCLC cells leads to transitory cell cycle arrest (Hirata et al., 2009; Salhia et al., 2008). This discrepancy may result from the use of transient Ect2 KD by siRNA as opposed to stable Ect2 KD by lentiviral shRNA. It is possible that NSCLC cells with stable Ect2 KD retain sufficient Ect2 that is above a critical threshold required for cytokinesis. Interestingly, Ect2 KD in fibrosarcoma cells does not induce a cytokinesis defect due to the fact these cells have acquired a novel, Ect2-independent cytokinesis mechanism (Kanada et al., 2008). These results raise the possibility that a similar Ect2-independent cytokinesis mechanism may be operative in NSCLC and other tumor cells. In either case, these results indicate that Ect2 plays a prominent role in transformed growth that is distinct from its physiologic role in cytokinesis.

5.4.2 Rac1 is a critical downstream effector of Ect2 in NSCLC—Since Ect2 is a GEF that can regulate the activity of the Rho GTPases Rac1, Cdc42 and RhoA *in vitro* (Miki et al., 1993), we determined whether Ect2 mediates transformation in NSCLC cells through activation of Rho GTPases. Ect2 KD in NSCLC cells leads to a significant decrease in Rac1 activity but no apparent changes in Cdc42 or RhoA activity (Justilien and Fields, 2009). Furthermore, expression of a constitutively active Rac1 allele (RacV12) restores anchorage-independent growth and invasion in Ect2 KD cells, indicating that Rac1 is an important effector of Ect2 transformation (Justilien and Fields, 2009). Rac1 mediates transformation by activating a Pak-Mek1,2-Erk1,2 proliferative signaling pathway (Regala et al., 2005a; Mek EC 2.7.12.2; Erk EC 2.7.11.24). Consistent with a role for Ect2 in activating this pathway, Ect2 KD tumors exhibit decreased phospho-298 Mek1,2 and phospho-Erk1,2 levels (Justilien and Fields, 2009). Furthermore, Ect2 KD did not induce PARP (EC 2.4.2.30) cleavage, a biochemical marker of cellular apoptosis, or changes in tumor vascularization (Justilien and Fields, 2009). Thus, Ect2 KD inhibits the Rac1-Pak-Mek1,2-Erk1,2 proliferative signaling pathway *in vivo* while having no apparent effect on cell survival.

5.4.3 Ect2 mislocalization in NSCLC cells is mediated through binding to the oncogenic PKC α /Par6 α complex—Like Ect2, the PKC α -Par6 α complex regulates Rac1 activity in NSCLC cells and is required for NSCLC transformation (Frederick et al., 2008; Regala et al., 2005a), suggesting that Ect2 and the oncogenic PKC α -Par6 α complex converge on Rac1. Co-immunoprecipitation experiments show that Ect2 associates with the PKC α -Par6 α complex in NSCLC cells (Justilien and Fields, 2009). Interestingly, Ect2 preferentially binds to the PKC α -Par6 α complex rather than PKC α or Par6 α alone since Ect2 does not bind to mutants of PKC α and Par6 α that can not associate with each other (Justilien and Fields, 2009). Earlier studies using Ect2 truncation mutants demonstrated that Ect2 transforming activity requires both Ect2 GEF activity and mis-localization of Ect2 to the cytoplasm (Saito et al., 2004; Soltski et al., 2004). Consistent with these results, we observed that Ect2 is largely

mis-localized to the cytoplasm of cultured NSCLC cells (Figure 3), but not in non-transformed MDCK cells, consistent with our findings in primary NSCLC tumors (Justilien and Fields, 2009). Interestingly, when PKC ϵ or Par6 α expression is inhibited in NSCLC cells by RNAi-mediated KD, a significant redistribution of Ect2 from the cytoplasm to the nucleus is observed, indicating that binding of Ect2 to the PKC ϵ -Par6 α complex is important in regulating cytoplasmic Ect2 localization (Figure 3) (Justilien and Fields, 2009). Taken together, these results demonstrate an interesting paradigm in which two oncogenes, Ect2 and PKC ϵ , are genetically linked in human tumors through coordinate amplification as part of the 3q26 amplicon, and biochemically linked through their physical association in a multi-protein complex that functions to drive transformation. It seems likely that such a dual genetic and biochemical link exists between PKC ϵ and Ect2 in other tumors, such as ESCC, glioma and ovarian tumors, which harbor *PRKCI* and *ECT2* amplification.

Summary

Ect2 is an oncogene in multiple human cancers. Ect2 is aberrantly overexpressed and mis-localized in multiple human tumor types, often as a result of targeted amplification of the *ECT2* gene as part of the 3q26 amplicon. Ect2 is important for proliferation, migration and invasion of various types of cancer cells *in vitro*, and for NSCLC tumorigenicity *in vivo*. The role of Ect2 in cellular transformation is distinct from its physiologic role in cytokinesis, and many tumor cells appear to have evolved Ect2-independent cytokinesis mechanisms. In NSCLC cells, the ability of Ect2 to support transformation is linked to its mislocalization to the cytoplasm and activation of a Rac1-Pak-Mek1,2-Erk1,2 signaling axis that is regulated through its binding to the oncogenic PKC ϵ /Par6 α complex (Figure 4). Therefore, Ect2 and PKC ϵ are genetically linked due to their frequent co-amplification as part of the 3q26 amplicon, and functionally and biochemically linked through formation of an oncogenic PKC ϵ -Par6-Ect2 complex that drives transformation. Further experiments will be required to determine if Ect2 and PKC ϵ are similarly linked in other tumors, particularly those harboring 3q26 amplification. In addition, further work is needed to elucidate the molecular mechanisms that regulate the formation, dynamics and activity of the oncogenic PKC ϵ -Par6 α -Ect2 complex. These studies hold the promise of identifying novel therapeutic approaches to cancer treatment based on inhibiting Ect2 function in cancer cells.

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Figure 1. Schematic diagram of the domain structure of the Ect2 protein

N, Amino-terminal region; **XRCC1**, X-ray repair complementing defective repair in Chinese hamster cells 1 domain; **Cyclin B6**, cyclin B6-like domain; **BRCT**, BRAC1 C-terminal domain; **S**, small central region; **NLS**, nuclear localization sequence; **DH**, Dbl homology domain; **PH**, pleckstrin homology domain; **C**, Carboxyl-terminal region.

Normal Lung Epithelium

Lung Adenocarcinoma

Figure 2. Ect2 is mislocalized to the cytoplasm of primary non-small cell lung cancer tumor cells Immunohistochemical staining of Ect2 in normal human lung epithelium (**left panel**) and a primary lung adenocarcinoma (**right panel**). Ect2 localizes predominantly to the nucleus of normal lung epithelial cells, but is localized to both the nucleus and cytoplasm of lung cancer cells.

Figure 3. PKC ι and Par6 α regulate the cytoplasmic localization of Ect2 in NSCLC cells
Immunofluorescence staining of H1703 NSCLC cells expressing Non-target (NT) RNAi, PKC ι RNAi or Par6 α RNAi for Ect2 (**red**) and DAPI (**blue**). Ect2 is localized to both the nucleus and cytoplasm (arrows) of NT cells, but redistributes to the nucleus of PKC ι RNAi and Par6 α RNAi cells.

Figure 4. Schematic diagram of the PKC1-Par6α-Ect2 oncogenic signaling axis
Cytoplasmic Ect2 binds to the PKC1-Par6α complex and activates a Rac1/Pak1/Mek 1,2/Erk 1,2 signaling axis that drives transformed growth and invasion of NSCLC cells.