ECT2 and RASAL2 Mediate Mesenchymal-Amoeboid Transition In Human Astrocytoma Cells

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Malignant astrocytomas are highly invasive brain tumors. The Rho family of cytoskeletal GTPases are key regulators of astrocytoma migration and invasion; expression of the guanine nucleotide exchange factor ECT2 is elevated in primary astrocytomas and predicts both survival and malignancy. Mice bearing orthotopically implanted astrocytoma cells with diminished ECT2 levels following ECT2 knockdown exhibit longer survival. Although ECT2 is normally expressed in the nucleus, we show that ECT2 is aberrantly localized to the cytoplasm in both astrocytoma cell lines and primary human astrocytomas, and colocalizes with RAC1 and CDC42 at the leading edge of migrating astrocytoma cells. Inhibition of ECT2 expression by RNA interference resulted in decreased RAC1 and CDC42 activity, but no change in RHO activity, suggesting that ECT2 is capable of activating these pro-migratory Rho family members. ECT2 overexpression in astrocytoma cells resulted in a transition to an amoeboid phenotype that was abolished with the ROCK inhibitor, Y-27632. Cytoplasmic fractionation of astrocytoma cells followed by ECT2 immunoprecipitation and mass spectrometry were used to identify protein-binding partners that modulate the activity of ECT2 toward RHO and ROCK. We identified RASAL2 as an ECT2-interacting protein that regulates RHO activity in astrocytoma cells. RASAL2 knockdown leads to a conversion to an amoeboid phenotype. Our studies reveal that ECT2 has a novel role in mesenchymal-amoeboid transition in human astrocytoma cells. (Am J Pathol 2012, 181:662–674; http://dx.doi.org/10.1016/j.ajpath.2012.04.011)
and the downstream effector Rho-associated protein ki-
nase ROCK, promoting formation of lamellipodia and ac-
tivating invasion.\(^2\) Recent studies have shown that some
cancers, such as melanoma, are capable of undergoing a
switch in migratory phenotype between a mesenchymal
phenotype dominated by RAC1 activity and an amoeboid
phenotype dominated by RHO/ROCK.\(^{9,10}\) Mesenchy-
mal–amoeboid transition (MAT) of cancer cells is medi-
atized by the antagonistic relationship between RAC1 and
RHO/ROCK whereby RAC1 activity suppresses RHO/
ROCK activity and promotes mesenchymal migration.\(^{11}\)
Conversely, RHO/ROCK activity suppresses RAC1 and
promotes MAT.\(^9\) The ability of cancer cells to change their
migratory phenotype has important consequences for
therapeutic strategies, as targeting one arm of the
migratory pathway may lead to increased invasion
through the alternate migratory phenotype.

Epithelial cell transforming factor 2 (ECT2) is a GEF,
which is typically expressed in the nucleus during inter-
phase until mitosis, where it localizes with the central
spindle and activates RHOA, enabling the establishment
and ingress of the cleavage furrow.\(^12\) Using a GEF-
targeted microarray, we and others have previously iden-
tified that ECT2 was elevated in astrocytomas.\(^{13,14}\) Knockdown of ECT2 by small-interfering RNA (siRNA)
resulted in binucleated cells and abrogation of migration
and invasion.\(^{13,14}\) Interestingly, ECT2 was originally iden-
tified by its transforming ability in mouse fibroblasts.
ECT2-mediated transformation was linked to an N-termi-
nal truncation and/or loss of the nuclear localization sig-
nal which is typically expressed in the nucleus during inter-
phase until mitosis.\(^{15}\) Furthermore, mislocalization of ECT2 to the cytoplasm is
an important tumorigenic event in lung cancer.\(^{16}\) Interest-
ingly, knockdown of ECT2 using short hairpin RNA (shRNA)
was able to uncouple the cell cycle effects of ECT2 from its
oncogenic role in the cytoplasm.\(^{16}\) The mechanism by which decreased ECT2 expression diminishes migration and
invasion in astrocytoma cells has yet to be elucidated.
Accordingly, we have performed a series of experiments to
increase our knowledge of the role of ECT2 in cellular pro-
cesses beyond its known role in cytokinesis.

Materials and Methods

Astrocytes and Astrocytoma Cell Lines

The permanent, well-characterized human astrocytoma
cell lines U251, U343, U118, SNB19, A341, U87, and T98
and the human embryonic kidney cell line, 293T, were main-
tained in Dulbecco’s modified Eagle’s medium ( Gibco,
Invitrogen, Carlsbad, CA) with 10% fetal bovine serum
(Gibco). Normal human astrocytes (N-HAs; Lonza, Basel,
Switzerland) were maintained in astrocyte basal media
(Lonza) supplemented with AGM Bullet Kit (rhEGF, insulin,
ascorbic acid, l-glutamine, fetal bovine serum, and GA-
1000) (Lonza). Human astrocyte progenitors O2A were ob-
tained from ScienCell and maintained in Oligodendrocyte
Precursor Cell Medium from the manufacturer.

Antibodies

Polyclonal anti-ECT2 antibody (sc-1005; Santa Cruz Bio-
technology, Santa Cruz, CA) was diluted 1:200 for West-
ern blot analysis and 1:100 for immunofluorescence (IF).
Mouse anti-Rac antibody 610651 (BD Biosciences, San
Jose, CA) was diluted 1:500 for IF. Mouse anti-CDC42
ab41429 (Abcam, Cambridge, UK) was diluted 1:100 for IF.
Rabbit anti-RASAL2 19150 (Novus Biologicals, Little-
ton, CO) was used for immunoprecipitation and Western
blot analysis (1:5000). Polyclonal goat anti-RASAL2 S-17
(Santa Cruz Biotechnology) was used for IF at 1:500.
Monoclonal anti–b-actin A5441 (Sigma-Aldrich, St. Louis,
MO) was diluted 1:1000 for both IF and Western blot
analyses. Mouse anti-transferrin (13–6800; Invitrogen)
was diluted 1:2000 for Western blot analysis.

Tissue Microarray

Immunohistochemical staining was performed on the
commercially available brain tumor tissue microarray
GL803 that consists of 55 astrocytic tumor specimens
and 5 normal brain specimens. The patient population
included 22 male and 33 female tumor specimens, with a
mean patient age of 41.7 years (range, 4 to 68 years),
and 3 male and 2 female normal control specimens, with a
mean patient age of 45.8 years (range, 30 to 52 years).
The tissue microarray was stained as previously de-
scribed and verified using anti-ECT2 antibody at a dilu-
tion of 1:500.\(^17\) Immunohistochemical samples were
scored using a three-point scale with 0 indicating no
cytoplasmic staining, 1 indicating minimal cytoplasmic
staining, 2 indicating moderate cytoplasmic staining, and
3 indicating intense cytoplasmic staining.

Stable Cell Line Generation

Three ECT2 shRNA pTRIPZ vectors (RHS4740-NM_018098)
and one non-silencing control shRNA pTRIPZ vector
(RHS4743) were obtained from Open Biosystems
(Thermo Fisher Scientific, Waltham, MA). ShRNA was
packed using the Open Biosystems Trans-Lentiviral
Packaging System according to the manufacturer’s in-
structions. Doxycycline (Sigma-Aldrich) at 2 µg/mL was
used to induce shRNA expression [cells were selected
using 2 µg/mL puromycin (Invitrogen)].

Orthotopic Intracranial Xenografts

Eight-week-old mice were stereotactically injected in the
right frontal lobe (2 mm lateral and 1 mm anterior to the
bregma) with 1 × 10^6 U251 cells stably expressing doxy-
cycline (dox)-inducible non-silencing shRNA (n = 10) or
ECT2 shRNA (n = 25) similar to Kongkham et al.\(^18\) All 10
non-silencing mice and 15 of the ECT2 shRNA mice were
fed a water diet supplemented with 15 µg/mL doxycycline
reconstituted in sterile water and 25% sucrose (Sigma-Al-
drich). Ten of the ECT2 shRNA mice were fed sterile water
without doxycycline. Mice were monitored for evidence of a
symptomatic intracranial mass such as domed skull, coo-
dination deficits, lethargy, or >20% weight loss, at which
time the animals were sacrificed. Whole brain specimens, including the intracranial xenografts, were fixed and processed as previously described.18

Subcellular Fractionation
Astrocytoma cell lines and NHA were lysed and fractionated using Qproteome (Qiagen, Valencia, CA) as per the manufacturer’s instructions.

Immunofluorescence
Cells were plated on glass coverslips, fixed, and then permeabilized with methanol. Analyses of RASAL2 and ECT2 expression were performed on cells that were fixed in 4% paraformaldehyde as described by Smith et al in 2004. Cells were incubated with primary antibodies diluted in 5% goat serum at the dilutions listed above for 2 hours at room temperature. Cells were then washed and incubated with Alexa-Fluor 488 anti-rabbit or anti-mouse secondary antibodies (Invitrogen) and/or Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA) anti-rabbit or anti-mouse secondary antibodies diluted 1:500 in 5% goat serum. Hoechst 3342 (Invitrogen) diluted 1:10,000 in PBS was used to visualize nuclei. Alexa Fluor 647–conjugated phalloidin (Invitrogen) was used to visualize actin. Cells were mounted in DAKO mounting media and visualized in a Zeiss Axiovert 200M Spinning Disk confocal microscope (Carl Zeiss) equipped with a Hamamatsu Back-THinned EM-CCD camera (Hamamatsu Photonics). The Y-27632 compound (Sigma-Aldrich) was used at a concentration of 100 µmol/L.

Three-Dimensional Collagen Culture
U251 glioma cells (1 × 10⁶ cells/mL) were suspended in 1.4 µg/mL Type I Collagen Cultrex gels (R&D Systems, Minneapolis, MN) for 24 to 48 hours and fixed using 4% paraformaldehyde for 30 minutes at room temperature, permeabilized with 0.5% Triton-X for 10 minutes, and then stained with H3343 1:10,000 (Invitrogen) and Alexa-Fluor Texas Red Phalloidin (Invitrogen) 1:1000 for 10 minutes. The gels were imaged as above and deconvolved where indicated with Volocity 3D Image Analysis Software (PerkinElmer).

RAC, RHO, RAS, and CDC42 GTPase Activation Assays
Protein lysate at a concentration of 0.5 mg/mL was analyzed with G-LISA RAC and CDC42 (Cytoskeleton, Denver, CO) assays according to the manufacturer’s directions. Absorbance at 405 nm was then measured using a SpectraMax Microplate Reader (Molecular Devices, Sunnyvale, CA). RAC, CDC42, RAS, and RHO pull-down assays (1 mg/mL protein lysate) were performed using GTPase Activation Assays (Millipore, Billerica, MA) as per the manufacturer’s directions.

Mass Spectrometry
Ten micrograms of total cellular lysate, from native U251 cells, was fractionated using the Qiagen Qproteome kit, and the resulting protein was exchanged into an NP40 (50 mmol/L Tris-HCL, 150 mmol/L NaCl, and 1% NP-40) buffer and subjected to immunoprecipitation with 8 µg of ECT2 or rabbit IgG antibody. Immunoprecipitations were washed and prepared as described by Tong et al.20 Samples were then sent to the Advanced Protein Technology Centre at the Hospital for Sick Children (Toronto) and subjected to liquid chromatography coupled with tandem mass spectrometry in accordance with Jin et al.21 using LC system (Easy-nLC; Proxeon Biosystems, Odense, Denmark) coupled with an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific). These data were then analyzed using SCAFFOLD2 software.
Co-Immunoprecipitation

One microgram of unaltered U251 cellular lysate was incubated with Protein-A agarose beads and 1.5 μg of rabbit polyclonal anti-ECT2 or rabbit polyclonal anti-RASAL2 or rabbit IgG (Santa Cruz Biotechnology) for 4 hours at 4°C. Samples were then subjected to SDS-PAGE and Western blot analysis.

siRNA Transfection

Three siRNAs to RASAL2 were obtained from Sigma-Aldrich: SASI_Hs02_00338422, 00122060, and 00122061. Transfection of pooled siRNA duplexes was performed in 10-cm plates using 15 μL of Lipofectamine RNAiMAX (Invitrogen) and 200 pmols of each siRNA in 10 mL. Cells were plated in invasion and migration chambers 48 hours later and subjected to activation assays and immunofluorescence 72 hours posttransfection.

Invasion and Migration Assays

Approximately 5000 astrocytoma cells were labeled with DILC12 (BD Biosciences) reconstituted in Dulbecco’s modified Eagle’s medium (GIBCO) and plated in the top well of a BD Biosciences Fluoroblok invasion or migration assays in triplicate. The bottom well contained Dulbecco’s modified Eagle’s medium + 10% fetal bovine serum and 5 ng/μL epidermal growth factor (EGF; Invitrogen). Plates were read at 0 and 14 hours using a SPECTRAmax Gemini Microplate Spectrophotometer (Molecular Devices).

Results

Overexpression of ECT2 Correlates with Increased Anaplasia and Diminished Survival

The Rembrandt database at the National Cancer Institute contains clinically annotated expression data on 343 human astrocytomas. We mined the database and determined that ECT2 predicted for both poor survival by Kaplan-Meier analysis and higher-grade malignancy (Figure 1, A and B). These data are also in agreement with the Cancer Genome Atlas Research studies demonstrating that ECT2 is elevated in the majority of primary human glioblastoma multiforme (GBM) samples (see Supplemental Figure S1A at http://ajp.amjpathol.org).

Figure 1. Elevated ECT2 predicts grade and prognosis of primary human astrocytoma and exhibits aberrant cytoplasmic localization. A: Kaplan-Meier curves derived from the Rembrandt database demonstrate that elevated ECT2 (red) expression predicts poor survival in human primary astrocytoma compared to down-regulated (green) or intermediate (yellow) ECT2 expression (P < 0.001).22 B: Of 173 astrocytomas with elevated ECT2, 90% (n = 155) were high-grade lesions (GBM n = 121, Grade III Astrocytoma n = 12 and Grade III Oligodendroglioma n = 13), in contrast to the 57 astrocytomas with low levels of ECT2 where only 40% were high-grade lesions (GBM n = 18, Grade III Astrocytoma n = 4 and Grade III Oligodendroglioma = 1). Statistical analysis using Fisher exact test demonstrates that elevated ECT2 expression predicts grade of astrocytomas (P < 0.001).22 C: Immunohistochemical staining of ECT2 protein expression from human primary astrocytomas demonstrated increased cytoplasmic localization when compared to normal brain. Using a three-point scoring system (3 = high cytoplasmic expression, 2 = moderate cytoplasmic expression, 1 = low cytoplasmic expression, and 0 = no cytoplasmic expression), the average score for Grade III/IV lesions (n = 50) was 2.05 and significantly elevated compared to 1.3 (n = 15) for Grade I/II astrocytomas, (P < 0.01, χ² test). Normal brain had a score of 0.0 (n = 5). Black arrow denotes a blood vessel that is negative for cytoplasmic ECT2. Scale bar = 100 μm. D: Immunohistochemical staining of the brain-tumor interface revealed cytoplasmic ECT2 in cells at the leading edge (low magnification in top panel, high magnification in bottom panel). Scale bar = 500 μm.
ECT2 Exhibits a Cytoplasmic Localization in High-Grade Astrocytomas

Given ECT2 is elevated in astrocytoma and plays a role in invasion, we investigated whether ECT2 mislocalization played a role in astrocytoma pathobiology. Using a human astrocytoma tissue microarray, we determined that ECT2 expression was increased in the cytoplasm of high-grade astrocytomas compared to low-grade astrocytomas and normal brain. Using a three-point scoring scale for immunohistochemical analysis, we show that high-grade astrocytomas had significantly higher cytoplasmic ECT2 than low-grade astrocytomas or normal brain (which had no detectable cytoplasmic ECT2) (Figure 1C). Interestingly, examination of a brain–tumor interface section exhibited cytoplasmic ECT2 localization in cells located at the invasive front (Figure 1D).

ECT2 Knockdown Decreases Astrocytoma Xenograft Formation and Increases Survival in Mice

We generated a stable dox-inducible ECT2 shRNA expressing the U251 astrocytoma cell line. On administration of dox, U251-ECT2 shRNA showed maximal knockdown after 72 hours relative to U251-Non-Silencing (NS) shRNA and U251-ECT2 shRNA – dox controls (Figure 2A).

Given ECT2 has a role in cytokinesis, migration and invasion, we evaluated ECT2 as a target for astrocytoma therapy. We confirmed that U251-ECT2 shRNA + dox astrocytoma cells exhibited no change in proliferation using an MTS assay before implantation into an orthotopic xenograft model (Figure 2B). Although this is in contrast to our previously published data using transient siRNA to ECT2, this is likely attributed to transient versus stable knockdown preclinical efficacy of ECT2 knockdown xenografts. Knockdown of ECT2 by shRNA resulted in significantly increased survival compared to controls in Kaplan-Meier analysis (Figure 2C). Six of 15 mouse xenografts expressing U251-ECT2 shRNA + dox formed tumors, whereas 9 of the 15 remaining mice were tumor free at the 90-day implantation time point. Immunohistochemistry of tumors dissected from U251-ECT2 shRNA + dox–expressing xenograft mice showed diminished ECT2 expression compared to both control cohorts (Figure 2D). Interestingly, U251-ECT2 shRNA + dox tumors showed discrete margins at the brain–tumor interface (distance of invasion from tumor bulk: 106 μm/L ± 47) compared to both control groups (U251-NS shRNA + dox: 367 ± 206 μm/L and U251-ECT2 shRNA – dox: 296 ± 94 μm/L), indicative of a diminished invasive pattern at the brain–tumor interface (Figure 2, D and E). High magnification of invasive cells in the tumors derived from the control groups showed aberrant cytoplasmic ECT2 localization (Figure 2F). Microscopic examination of the tumors derived from xenografts did not exhibit an increase in binucleation, confirming previous data from Justilien et al16 that stable ECT2 shRNA knockdown uncouples the cytokinetic function of ECT2 from its cytoplasmic role.

Figure 2. Loss of ECT2 increases xenograft survival. A: Western blot of a stable U251-ECT2 shRNA tetracycline inducible cell line. Dox-treated cells demonstrated maximal ECT2 depletion at 72 hours compared to U251-NS + dox and U251-ECT2 shRNA + dox controls. B: MTS assay of tetracycline-inducible U251 cell lines. U251-NS shRNA + dox (yellow), U251-ECT2 shRNA – dox (blue), or U251-ECT2 shRNA + dox (red) cells exhibited no change in MTS parameters during the 72 hours examined. Cells were plated in the MTS assay 72 hours post-dox induction. C: Kaplan-Meier survival curves of orthotopic mouse tumor xenografts of U251-ECT2 shRNA + dox tumors. U251-ECT2 shRNA + dox (red) cells exhibited no change in MTS parameters during the 72 hours examined. Cells were plated in the MTS assay 72 hours post-dox induction. Compared to both control cohorts. D: Top panel: Immunohistochemical analysis of tumors dissected from U251-ECT2 shRNA + dox–expressing xenograft mice showed diminished ECT2 expression compared to both control cohorts (Figure 2D). Interestingly, U251-ECT2 shRNA + dox tumors showed discrete margins at the brain–tumor interface (distance of invasion from tumor bulk: 106 μm/L ± 47) compared to both control groups (U251-NS shRNA + dox: 367 ± 206 μm/L and U251-ECT2 shRNA – dox: 296 ± 94 μm/L), indicative of a diminished invasive pattern at the brain–tumor interface (Figure 2, D and E). High magnification of invasive cells in the tumors derived from the control groups showed aberrant cytoplasmic ECT2 localization (Figure 2F). Microscopic examination of the tumors derived from xenografts did not exhibit an increase in binucleation, confirming previous data from Justilien et al16 that stable ECT2 shRNA knockdown uncouples the cytokinetic function of ECT2 from its cytoplasmic role.

AJP August 2012, Vol. 181, No. 2
Astrocytoma Cell Lines Demonstrate Aberrant Cytoplasmic ECT2 Expression

To further delineate the role of ECT2 mislocalization in invasion and migration, we sought to characterize the localization of ECT2 in tumor cells. Using immunofluorescence analysis, cytoplasmic ECT2 expression was found in a panel of astrocytoma cell lines and was localized predominantly to the nucleus and membrane ruffles. This expression pattern was observed in all astrocytoma cell lines examined: U251 and T98-G (Figure 3A), as well as U87, U343, U118, U373, and A172 (data not shown). The cytoplasmic localization of ECT2 in astrocytoma cell lines was markedly different compared to ECT2 localization in NHAs, which exhibited nuclear and perinuclear expression (Figure 3A). As expected, following subcellular fractionation, ECT2 was significantly enriched in the cytoplasmic fraction of astrocytoma cells, but not in NHAs (Figure 3B, upper panel). This enrichment may be explained in part by increased expression alone as ECT2 is also increased in the nuclear fragment of many astrocytoma cells lines, but not all (Figure 3B, lower panel).

ECT2, RAC1, and CDC42 Colocalize at the Leading Edge of Migrating Astrocytoma Cells

ECT2 typically activates RHOA during cytokinesis, and to determine whether ECT2 was modulating RHO activity, we tested the status of RHO activation of the U251-ECT2 shRNA cells and observed that there was no change in RHO activity with stable knockdown of ECT2 (Figure 3C).

Since ECT2 did not appear to be altering RHO activity, we checked the status of related Rho family proteins RAC and CDC42. ECT2 localized within membrane ruffles at the leading edge of the invasive front 3 hours after inducing a wound in a confluent culture of U251 (Figure 3, D and E, upper panels). The ECT2 localized at the leading edge of migrating cells and colocalized with the mesenchymal pro-migratory small cytoskeletal GTPases RAC1 and CDC42 (Figure 3, D and E, upper panels). These experiments were repeated in the astrocytoma cell line T98G with similar findings (data not shown).

To determine whether the colocalization of ECT2 with RAC1 and CDC42 had functional significance, we used the stable inducible ECT2 shRNA system to assess the activity of Rho family GTPases. Seventy-two hours following knockdown of ECT2, the activity of RAC1 and CDC42 were significantly decreased compared to NS or dox (−) controls (Figure 3, D and E, lower panels).

Ectopic Overexpression of Wild-Type ECT2 Results in Cytoplasmic Localization in NHAs

Given that Rac1 and CDC42 activity is decreased and invasion is diminished following ECT2 knockdown, we investigated the consequence of ectopic wild-type ECT2 overexpression in NHAs.

**Figure 3.** ECT2 is aberrantly expressed in the cytoplasm of astrocytoma cells. A: Immunofluorescence analysis revealed that ECT2 is cytoplasmic and is enriched within the membrane ruffles in two astrocytoma cell lines U251 and T98 compared to NHA, which exhibit nuclear and perinuclear ECT2 expression. B: Upper panel: Cytoplasmic subcellular fractionation of NHAs and astrocytoma cell lines showed increased ECT2 protein in the cytoplasm of astrocytoma cells compared to NHAs. Lower panel: Nuclear fraction of NHAs and astrocytoma cell lines showing ECT2 protein levels vary but are generally increased in astrocytoma cells. Nuclear HDAC1 was used as a marker of the nuclear fraction and uncleaved caspase-3 as a marker of the cytoplasmic fraction. C: RHO activity measured using a RHO-A, -B, -C pull-down assay demonstrated low levels of endogenous RHO activity in all three cell conditions and no change in RHO activity following ECT2 knockdown. D and E: Top panels: ECT2 (green) colocalizes with RAC1 (D, red) or CDC42 (E, red) at the leading edge of parental U251 migratory cells 3 hours after inducing a wound on the confluent cell monolayer. White arrow shows ECT2 at the leading edge. Diagonal white line denotes wound location. D and E: Bottom panels: Knockdown of ECT2 exhibited diminished RAC1 activity: U251-NS shRNA + dox = 0.554 ± 0.065; U251-ECT2 shRNA = dox = 0.547 ± 0.031; and U251-ECT2 shRNA + dox = 0.393 ± 0.066 (P < 0.05, Student’s t-test). Similarly, ECT2 knockdown diminished CDC42 activity: U251-ECT2 NS shRNA + dox = 0.876 ± 0.08; U251-ECT2 shRNA = dox = 0.816 ± 0.06; and U251-ECT2 shRNA + dox = 0.600 ± 0.065. (*P < 0.05, Student’s t-test).
Ectopic GFP-ECT2 expression was sufficient to induce aberrant cytoplasmic localization in normal human astrocytes (NHAs), however, the morphology of NHA GFP-ECT2 cells remained unchanged, and retained a morphology similar to GFP-EV controls. Expression of GFP-ECT2 in U87 and U251 astrocytoma cell lines resulted in cell rounding, cortical instability, and bleb formation compared to GFP-EV. White arrow demarks Annexin-V debris (dark blue); however, cells remained negative for Annexin-V.

Ectopic expression of ECT2 in U251 astrocytoma cells did not result in cleaved caspase-3 expression (an indicator of apoptosis) at either 0.25 μg or 1.0 μg of transfected GFP-ECT2. Positive control was Jurkat cells treated with cytochrome-C. Negative control was untransfected Jurkat cells.
observations in non-astrocytoma cell lines by Saito et al., suggesting cytoplasmic mislocalization may be unique to astrocytoma cells (see Supplemental Figure S1B at http://ajp.amjpathol.org). We investigated whether cytoplasmic expression of ECT2 was an acquired oncogenic phenotype of astrocytoma cells or was an inherent property of astrocytes or astrocyte progenitors, by comparing expression levels of ECT2 in normal progenitor and transformed astrocytes. Interestingly, Type 2A oligoastrocyte progenitor cells, which can differentiate into oligodendrocytes/or Type 2A astrocytes, exhibited cytoplasmic expression of ECT2 with enhanced expression at the cortical membrane, suggesting that cytoplasmic ECT2 expression may be an important property of astrocytes during development and differentiation (see Supplemental Figure S1C at http://ajp.amjpathol.org).

Ectopic Overexpression of Wild-Type GFP-ECT2 Induces MAT in Astrocytoma

In contrast to NHAs, overexpression of wild-type GFP-ECT2 in U251 and U87 astrocytoma cell lines results in cell rounding, dynamic cortical instability, and formation of membrane blebs reminiscent of an amoeboid phenotype (Figure 4A). Time-lapse live-cell imaging of cells expressing GFP-ECT2 demonstrated an amoeboid morphology. These cells were capable of migrating at a rate of 5 to 10 μm/min, velocities similar to that of amoeboid cells, but not mesenchymal cells, which typically migrate at 0.5 μm/min (Figure 4B) (reviewed by Friedl and Wolf [24]). ECT2 expression was enhanced at the trailing edge of amoeboid cells and at sites of bleb retraction after cellular expansion, sites previously identified as RHOA-positive regions during amoeboid invasion [25] (Figure 4B, lower panel).

Morphological transformation to an amoeboid phenotype was quantified in U87 astrocytoma cells overexpressing GFP-ECT2 in fixed samples. GFP-ECT2–positive cells were enumerated, and 46% exhibited a morphology indicative of MAT in normal progenitor and transformed astrocytes. Interpreting Type 2A astrocytoma cell line 293T led to cells that retained a nuclear localization of ECT2. Following forced cytoplasmic expression of ECT2 with a mutated nuclear localization signal, we observed cytoplasmic expression of ECT2 with formation of lamellipodia suggestive of Rac1 activation and not RHO activation (see Supplementary Figure S1B at http://ajp.amjpathol.org).

Astrocytoma Cells Form Amoeboid-Type Cells in a Collagen Matrix

ECT2 Interacts with RASAL2, and siRNA to RASAL2 Results in Loss of RHO Activity

To address the mechanism underlying the role of ECT2 in MAT, we performed mass spectrometry to identify cytoplasmic interacting proteins on the cytoplasmic fraction of unaltered U251 protein lysates immunoprecipitated with ECT2 antibody (Figure 6A). In this screen, the novel RAS-GAP, RASAL2, was identified, and the interaction was validated by endogenous, reciprocal co-immunoprecipitation (Figure 6B). The yield of ECT2 in the reciprocal RASAL2 immunoprecipitation is low, which is either an issue of antibody efficiency, given that endogenous proteins are examined, or alternatively, a separate pool of RASAL2 protein is unassociated with ECT2, resulting in the decreased yield of ECT2 during co-immunoprecipitation. In support of this direct interaction between ECT2 and RASAL2, immunofluorescence demonstrated that ECT2 and RASAL2 colocalize at the leading edge of cells (Figure 6B). Knockdown of RASAL2 using siRNA resulted in no change in RAS activity (Figure 6C, left). However,
Amoeboid Cells in a Type I Collagen Gel

We revealed that loss of ECT2 significantly diminished the amoeboid phenotype (Student’s t-test, U251-ECT2 shRNA vs. U251-NS shRNA). Immunofluorescence analysis and 3D reconstruction of two parental U251 astrocytoma cells stained with phalloidin (red) and H3342 (blue) and plated in a 1.4 mg/ml Type I collagen gel. Images were deconvolved using Volocity software and represent a mesenchymal (right cell) and an amoeboid cell (left cell). The nucleus of the amoeboid cell is deformed around the collagen matrix (white arrow). B: Top panel: Immunofluorescence of cells induced to express U251-ECT2 shRNA + dox versus U251-NS shRNA + dox in a type I collagen gel and stained with phalloidin (green) demonstrated a decreased amoeboid phenotype (Student’s t-test, P < 0.05). The assay was repeated three times. *P < 0.05. C: Lower panel: Immunofluorescence of cells induced to express U251-ECT2 shRNA + dox expressing cells. DsRed is a reporter construct to indicate shRNA expression. U251-ECT2 shRNA dox expressing cells. The tumors with decreased ECT2 expression resulted in increased RHO activity, consistent with RASAL2 either having a direct RHO-GAP function in astrocytoma cells or indirectly altering RHO activity levels (Figure 6C, left). We also observed a moderate decrease in RAC1 activity following the loss of RASAL2. In support of RASAL2 altering RHOA activity, immunofluorescence of U251 cells following RASAL2 knockdown exhibited an increase in an amoeboid morphology (Figure 6C, right). If RASAL2 physically interacts with and modulates ECT2 activity, then loss of ECT2 would be expected to disrupt RASAL2 localization at the leading edge. Indeed, when ECT2 is knocked down by shRNA, the actin-rich lamellipodia are lost, consistent with diminished RAC1 activity. Under these circumstances, RASAL2 localization at the cortical membrane is disrupted (Figure 6D). If RASAL2 is modulating ECT2 activity, then loss of this physical interaction by RASAL2 shRNA should affect astrocytoma invasion, and indeed, this is precisely what we observed (Figure 6E).

**Discussion**

In this study, we have shown that elevated ECT2 expression correlates with both a poor prognosis and high-grade malignancy. Previously, we showed that loss of ECT2 expression resulted in diminished invasion and migration in astrocytoma cells lines. However, it is yet unknown whether ECT2’s role in invasion is distinct from its functional role in cytokinesis in astrocytoma. Here, we show that ECT2 is aberrantly localized to the cytoplasm in astrocytoma cells, and that cytoplasmic expression of ECT2 is associated with increasing astrocytoma anaplasia. In addition, ECT2 specifically localizes to the cytoplasm of astrocytoma cells at the normal brain–tumor interface. Aberrant ECT2 localization suggests that ECT2 plays a role in astrocytoma migration and invasion distinct from its function in cytokinesis. The mechanism by which ECT2 is aberrantly localized to the cytoplasm may be due to overexpression as our data suggest; however, other modes of aberrant localization, such as changes in degradation pathways, cannot be excluded. ECT2 is known to undergo ubiquitination, which alters function and could alter localization, and deserves to be examined.

ECT2 is an attractive target for cancer therapeutics, given its apparent dual role in cell division and migration/invasion. We have shown that mice with orthotopic tumor xenografts with decreased ECT2 expression mediated by shRNA knockdown exhibit a significant increase in survival. Knockdown of ECT2 did not increase the number of binucleated cells in xenografts expressing ECT2 shRNA, demonstrating it is possible to uncouple the cytokinetic role of ECT2 from its other cellular roles. In previous studies, we showed that knockdown of ECT2 using transient siRNA did effect cell cycle; however, in agreement with data from Justilien et al., stable knockdown of ECT2 with shRNA did not result in similar abnormalities in cell cycle control. We attribute this to different transfection methods (siRNA versus shRNA) or to development of compensatory cytokinetic mechanisms in the stable knockdown cells. The tumors with decreased ECT2 ex-
expression also showed diminished invasive capabilities, which, combined with our in vitro data, supports the notion that ECT2 has an independent role in invasion of astrocytoma tumors.

In support of the data in primary astrocytoma cells, astrocytoma cell lines exhibited aberrant localization of ECT2 to the cortical membrane, which was not observed in NHAs. In addition, ECT2 colocalized with the pro-migratory mesenchymal Rho GTPases RAC1 and CDC42, at the leading edge of migrating astrocytoma cells. Loss of ECT2 expression resulted in diminished activation of both RAC1 and CDC42, suggesting that ECT2 is capable of mediating GTP exchange on RAC1 and CDC42 in astrocytoma cells during mesenchymal migration and invasion. ECT2 can variably activate RAC1, RHOA, and CDC42, depending on the cellular context. In fibroblasts, forced cytoplasmic expression through mutation of the NLS resulted in aberrant activation of RHOA and RAC1, but not CDC42. In a lung carcinoma model system, Justilien et al also found that ECT2 was abnormally localized to the cytoplasm and capable of activating RAC1, but not CDC42 or RHOA. The promiscuity of ECT2 in affecting multiple members of the Rho GTPase pathway is likely modulated by phosphorylation and the temporal or spatial expression of co-regulatory proteins.

The molecular mechanism resulting in the mislocalization of ECT2 in the cytoplasm may provide further clues to the function of ECT2 in cancer progression. Our data support the concept that overexpression of ECT2 occurs in malignant gliomas, and given that some glioma cell lines have both elevated nuclear and cytoplasmic fractions, suggest that overexpression alone might account for a portion of the aberrantly expressed ECT2. Indeed, overexpression of ECT2 alone is sufficient to induce cytoplasmic localization of ECT2 in NHAs. The increased

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Figure 6. RASAL2 physically interacts with ECT2 at the cortical membrane and functions as a RHO-GAP. A: Candidate list of putative ECT2 cytoplasmic interacting partners identified following endogenous ECT2 or IgG immunoprecipitation (IP) and liquid chromatography coupled with tandem mass spectrometry. Candidates listed were only present in ECT2 IP and not the IgG control. B: Reciprocal endogenous co-immunoprecipitation of ECT2 and RASAL2 validated the interaction. Immunofluorescence labeling of endogenous ECT2 (red) and RASAL2 (green) colocalized at the leading edge of mesenchymal astrocytoma cells. C: Left panel: RAS, RHO, and RAC pull-down activation assays following RASAL2 knockdown versus NS knockdown controls showed that loss of RASAL2 resulted in RHO activation and slightly decreased RAC1 activity, whereas no change was detected in RAS activity. Blots shown are representative of blots from three independent experiments. Right panel: Immunofluorescence analysis of U251 cells stained with phalloidin (blue) and transfected with RASAL2 siRNA or NS siRNA demonstrate that loss of RASAL2 results in formation of amoeboid cells with cortical blebs (white arrowheads). D: Immunofluorescence analysis of U251 astrocytoma cells immunolabeled for RASAL2 (green) and phalloidin (blue) and expressing either U251-NS shRNA + dox, U251-ECT2 shRNA + dox, or U251-NS shRNA + dox. ECT2 knockdown results in a loss of actin-rich lamellipodia and disrupts RASAL2 foci at the cortical edge of astrocytoma cells. DsRed is a reporter construct to indicate positive siRNA expression. Scale bar in all images represents 10 μm. E: Boyden chamber invasion assay using NS and RASAL2 siRNA in U251 cells. Knockdown of RASAL2 using siRNA resulted in a significant increase in invasion compared to NS siRNA, 200.8 ± 28.7 relative fluorescence units (RFU) versus 112.7 ± 11.8 RFU (P-value < 0.01). Experiment repeated in triplicate.
cytoplasmic staining of ECT2 following ectopic expression in NHAs is distinct from the non-astrocyte 293T cells, and there are previous reports in other non-astrocytic cells in which mutation of the NLS was required for cytoplasmic localization and transformation phenotype. To date, no mutations have been identified in the ECT2 gene in astrocytomas. Interestingly, astrocyte progenitors also express cytoplasmic ECT2, although the functional significance of this is unknown. Given that our studies revealed a function for ECT2 in migration and invasion, it is plausible that ECT2 is used in progenitor astrocyte migration during neuroembryogenesis. Although NHAs do not express cytoplasmic ECT2, it would be interesting to investigate whether reactive astrocytes, which reacquire the ability to migrate into areas of brain trauma, regain cytoplasmic ECT2 expression. Taken together, cytoplasmic localization of ECT2 may be an end result of ECT2 overexpression in astrocytomas. In astrocytomas, there may be an aberrant adaptation of the normal developmental migratory role for ECT2 to enhance astrocytoma invasion.

Astrocytoma cells ectopically expressing GFP-ECT2 exhibited a phenotype characterized by cortical instability and blebs. Time-lapse imaging demonstrated that a proportion of cells used these blebs to migrate at velocities similar to amoeboid cells. This phenotype was not observed in either GFP-EV controls or NHAs expressing GFP-ECT2, both of which maintained a mesenchymal morphology. Amoeboid morphology is known to be mediated by the RHO/ROCK pathway in other cell types (reviewed by Sahai and Marshall and Pankova et al). During amoeboid invasion, the cortex expands through the matrix and then requires RHO/ROCK activation at the bleb cortex to coordinate actin and myosin contractility and bleb retraction (reviewed by Fackler and Grosse). To date, no mutations have been identified in the ECT2 gene in astrocytomas. Interestingly, nontransfected U251 astrocytoma cells were capable of forming amoeboid cells when plated in a three-dimensional (3D) type I collagen matrix, and these cells were reminiscent phenotypically of ectopic GFP-ECT2–expressing cells. Loss of ECT2 in this 3D context resulted in diminished capacity for MAT, indicating ECT2 is important in this process. Interestingly, loss of ECT2 in the 2D mesenchymal context resulted in loss of the actin-rich lamellipodia and diminished RAC1 activation. We did not, however, observe MAT in mesenchymal glioma cells with ECT2 knockdown and loss of RAC1 activation cells, this suggests that ECT2 may be key player in MAT in glioma cells. These data demonstrate that ECT2 serves different roles depending on whether the cell assay system is in a two-dimensional versus a three-dimensional context.

ECT2 is a known activator of RHOA during cytokinesis; however, cytoplasmic ECT2 will modulate mesenchymal migration through increased RAC1 and CDC42 activity in a 2D context. Given that RAC and RHO work antagonistically to promote mesenchymal or amoeboid migration, respectively, we hypothesized that cytoplasmic ECT2 may be shunted toward activating RAC1 and CDC42 to promote mesenchymal migration through repression of RHOA activity. Indeed, in the mesenchymal 2D context, RHO activation assays showed low basal activity levels in astrocytoma cells.

Using a mass spectrometry approach, we determined for the first time that cytoplasmic ECT2 interacts with a novel RHO-GAP, RASAL2. RASAL2 was initially identified as a RAS-GAP due to its ability to complement the loss of a defective RAS-GAP gene in yeast and has been implicated in cancer. In astrocytomas, however, RASAL2 either directly or indirectly alters RHO activity. This may be due to intrinsic RHO-GAP activity (RASAL2 has a RHO-GAP domain) or by indirectly altering RHO activity through interactions with other proteins in the RHOA pathway.
Currently undertaking studies to examine how RASAL2 exerts the observed effects on the RHO pathway.

Given the physical association between RASAL2 with ECT2, the mesenchymal phenotype, and the fact that RASAL2 plays a role in RHO inactivation, we hypothesized that RASAL2 localizes to the membrane ruffles of mesenchymal glioma cells to modulate ECT2 ability. We suspect that in mesenchymal cells, the association of ECT2 and RASAL2 leads to a higher degree of ECT2 activating RAC1, as RASAL2 would negate any ECT2 activation of RH0A (Figure 7, left panel). Loss of ECT2 did not lead to MAT because ECT2 may also be required to activate the amoeboid phenotype in glioma cells. We are investigating the possibility that ectopic ECT2 overcomes the interaction with RASAL2, allowing ECT2 to activate RH0A, and results in MAT (Figure 7, right panel). This hypothesis is supported by our data that show that loss of RASAL2 also leads to MAT. Therefore, the interaction and activity of RASAL2 with ECT2 may define in part the invasive phenotype of glioma cells (Figure 7). It is interesting to note in data generated through the Cancer Genome Atlas Research GBM studies that the ECT2/RASAL2 ratio is high in primary GBM specimens, which may be a key requirement to promote MAT in astrocytomas (see Supplemental Figure S1A at http://ajp.ajpamathol.org). Our data support the ratio of ECT2 to RASAL2 as an important factor in MAT; however, mutational studies of the binding regions of ECT2 and RASAL2 would be key to further delineating the functional significance of this interaction. Increase in ECT2 expression or knockdown of RASAL2 expression both lead to MAT and increased invasive capability of cells (Figure 7). How a 3D organ or tissue context alters this ratio remains to be elucidated, but will be key in understanding the roles of RASAL2 and ECT2 in vivo. Known factors influencing MAT in other cancer subtypes involve deregulating the balance of Rho family GTPases, inhibition of matrix metalloproteinases, and growth within a 3D substrate (reviewed by Pankova et al and by Wolf et al). It will be important to determine how these factors for MAT affect both ECT2 and RASAL2 activity levels.

Few studies have described the process of amoeboid invasion and migration in human astrocytomas, but the role of MAT in cancer invasion is becoming increasingly recognized. Interestingly, a recent paper showed that shRNA-meditated loss of SOX-2 was sufficient for MAT in glioma cells. Amoeboid invasion is a rapid single-cell type of invasion that does not rely on degradation of the extracellular matrix, as opposed to mesenchymal invasion, which is slow and requires degradation of the extracellular matrix before cellular movement. Amoeboid invasion may explain why single or small clusters of glioma cells are found in the contralateral hemispheres of humans afflicted with GBM on autopsy, far from the primary lesion. It is plausible that mesenchymal migration may predominate at the local invasive front whereas single-cell amoeboid invasion would allow for rapid dissemination and seeding of cells throughout the central nervous system because amoeboid invasion does not require breakdown of the brain matrix. It would be interesting to determine the extracellular cues that may trigger MAT in vivo. Taken together with our data on the pro-invasive role of ECT2, and ECT2 expression correlating with higher-grade glioma, it is plausible that higher ECT2 expression leads to a more pathogenic invasive glioma cell via amoeboid invasion.

Our results indicate that astrocytoma cells are capable of migrational plasticity mediated through ECT2 and RASAL2. It will be important to understand the role of MAT in context of invasion; although our studies show that ECT2 and RASAL2 play a role in MAT, further studies are needed to determine the importance of this phenotype switch in the overall scheme of glioma invasion. We are currently undertaking studies to determine the extent to which this amoeboid phenotype switch alters invasion characteristics of glioma cells. Our data were limited in determining the overall invasive abilities of the amoeboid phenotype due to low transfection efficiencies.

If astrocytoma cells are capable of the same plasticity in vivo, there are important implications for therapeutic strategies targeting invasion. Small molecular inhibitors to the Rho superfamily are currently under development as cancer therapeutics (reviewed by Vigil et al). Therefore, the targeting of one migratory mechanism may result in increased invasion through an alternate phenotype. In future studies, intra vital imaging of orthotopic astrocytoma xenografts combined with multiphoton laser scanning microscopy may yield important insights into the predominant modes of astrocytoma invasion within the microcellular environment in vivo, similar to other studies in other tumors (reviewed by Provenzano et al).

Our data support the concept that ECT2 has a role in both mesenchymal and amoeboid migration. The fact that ECT2 targets both amoeboid and mesenchymal migratory phenotypes, as well as the cell cycle, makes it an attractive candidate for therapeutic strategies because other Rho family GTPase targets may increase invasiveness by permitting cells to undergo MAT. Our future studies will involve understanding the differential signaling pathways involved in ECT2 modulation when cells are plated in a 3D matrix, and we will explore the use of therapeutic siRNA delivery to astrocytoma xenograft models, given that targeting the Rho family GTPases may have an important role in future cancer therapeutics.

References
