Published in final edited form as:

Cancer Sci. 2009 September ; 100(9): 1597–1604. doi:10.1111/j.1349-7006.2009.01221.x.

Tumorigenicity of cortical astrocyte cell line induced by the protease ADAM17

Mark Katakowski¹, Feng Jiang¹, XuGuang Zheng¹, Jorge A. Gutierrez^{2,3}, Alexandra Szalad⁴, and Michael Chopp^{1,4,5}

¹Department of Neurology, Henry Ford Hospital, Detroit, Michigan, USA

²Department of Hermelin Brain Tumor Center, Henry Ford Hospital, Detroit, Michigan, USA

³Department of Pathology, Henry Ford Hospital, Detroit, Michigan, USA

⁴Department of Physics, Oakland University, Rochester, Michigan, USA

Abstract

The metalloprotease ADAM17 (a.k.a. TACE) plays a pivotal role in the cleavage and activation of membrane-anchored receptor ligands. More recently, it has been revealed that ADAM17 is a potent sheddase of the epidermal growth factor (EGF) family of ligands and regulates epidermal growth factor receptor (EGFR) activity in a variety of tumors. EGFR is a key component of autonomous growth signaling in several tumors, and correlates with the malignancy grade of astrocytoma. In this study, we tested the hypothesis that over-expression of ADAM17 in cortical astrocytes derived from normal brain would induce a progression towards a malignant phenotype. Over-expression of human ADAM17 (hADAM17) in the CTX-TNA2 cortical astrocyte cell line resulted in non-adherent growth, increased proliferation, invasiveness, production of angiogenic factors, and expression of genes associated with immature and/or neoplastic cells. hADAM17 up-regulated EGFR and AKT phosphorylation, and increased proliferation and cell invasion were significantly dependent upon EGFR activity. When implanted in the nude mouse brain, CTX-TNA2 cells induced low histological grade, benign intraventricular gliomas. In contrast, the same astrocytes with hADAM17 formed large malignant gliomas. Taken together, these findings suggest that unregulated ADAM17 activity induces functional changes in astrocytes that significantly advance the malignant phenotype.

The disintegrin and metalloproteinase ADAM17 is the most extensively studied of the ADAM family of proteins. Also known for its proteolytic release of tumor necrosis factor alpha (TNF- α), ADAM17 processes amyloid precursor protein (APP) as an α -secretase, and is a potent sheddase for several epidermal growth factor receptor (EGFR)-binding ligands, including transforming growth factor-alpha (TGF- α), heparin-binding epidermal growth factor (HB-EGF), and amphiregulin.(1)

Alterations of numerous independent pathways may contribute to tumorigenesis. However, whether a predictable cascade of alterations occurs in the early steps of oncogenic transformation is unknown. Even so, autonomous maintenance of growth signaling is a trait of neoplastic cells, acquired during initial the process of transformation to a malignant phenotype.(2) In normal tissue, an absence of growth factors causes cells to withdraw from the cell cycle and enter quiescence.(2) Loss of such growth inhibition is a hallmark of cancer cells, often due to continuous autocrine production of growth factors.(2)

Epidermal growth factor receptor (EGFR) receptor amplification is frequent in tumors of multiple tissues, including non-small cell lung cancer and glioblastoma.(3-5) Mutations resulting in constitutive activation of EGFR common in these tumors as well.(5,6) Expression of wild-type EGFR ligands, such as TGF- α or HB-EGF, is often increased in gliomas resulting in an autocrine loop that contributes to the self-sufficient growth of brain tumor cells.(7,8) Indeed, even in gliomas expressing the constitutively active mutant EGFRvIII, inhibition of wild-type EGFR-binding HB-EGF is sufficient to reduce EGFRvIII-induced proliferation, likely due to an EGFRvIII-wild-type EGFR autocrine mechanism.(9)

Interest has grown in enzymes involved in proteolytic regulation of the EGFR signaling pathway and how these proteases are regulated. ADAM17 is highly expressed in cancers of the breast, brain, lung, ovary, colon, and pancreas,(10-14) and sheds several ligands that bind and activate EGFR.(1) Thus, it is not surprising several recent investigations identify ADAM17 proteolytic shedding of EGFR-binding pro-ligands to be a key component of malignancy in tumors characterized by aberrant EGFR activity.(13,15,16)

ADAM17 is up-regulated in tumor and could therefore conceivably contribute to tumorigenesis, particularly if aberrant activity amplifies shedding of EGFR ligands with known roles in cancer, such as TGF- α , HB-EGF, and amphiregulin.(8,16,17)

In this study, we report that over-expression of active human ADAM17 in the astrocyte cell line CTX-TNA2 increases cell proliferation, invasiveness, and activation of the phosphoinositide-3 kinase/protein kinase B (PI3K/AKT) pathway, by an EGFR-dependent mechanism. hADAM17-expressing clones displayed non-adherent growth, increased invasiveness, expression of development/oncogenic-related genes, and enhanced production of soluble angiogenic factors. When implanted into the brains of athymic nude mice, CTX-TNA2 astrocytes transfected with the empty pcDNA3.1+ control plasmid gave origin to small, intraventricular, low histological grade glial neoplasms. However, the same astrocytes over-expressing hADAM17 transformed into poorly differentiated neoplasms with features characteristic of a malignant glioma. These findings imply that ADAM17 deregulation can lead to broad effects that advance the malignant phenotype and may contribute to oncogenic transformation of precancerous cells.

Materials and Methods

Growth curve

A total of 1500 cells were plated in each well of a 96-well plate containing DMEM with FBS at a concentration of 10%. Every 24 h, total adherent and non-adherent cells in each well were quantified using a hemacytometer. AG1487 (Biosource, Carlsbad, CA, USA) and TAPI-2 (Peptides International, Louisville, KY, USA) were each added at a concentration of 10 μ M. Cell counts of three wells per time-point per group were averaged.

Non-adherent cell growth

50 μ L of pre-warmed 2% noble agar was added to each well of a 96-well plate and allowed to solidify. A total of 1500 cells were plated in each well containing DMEM with FBS at a concentration of 10% or Neuralbasal-A medium containing 2% B27 serum supplement. After 5 days, MTT in PBS was added at 0.5 mg/mL and cells were incubated for 4 h at 37°C. Medium was harvested, 180 μ L 10% SDS was added to each well, and the plate was incubated at 37°C overnight. Absorbance was read at 540 nm.

Secretase activity enzyme-linked immunosorbent assay

To measure alpha enzymatic activity, 2×10^5 cells were lysed and processed with either an α -secretase Activity Kit (R&D Systems, Minneapolis, MN, USA) per the manufacturer's instructions. Fluorescence was read using a Fusion Multiplate reader (Packard Bioscience, Shelton, CT, USA).

In vitro invasion assay

Matrigel invasion assays were used to assess astrocyte invasion the presence or absence of the EGFR inhibitor AG1478. Invasion was determined using 24-well BD invasion chambers (8.0 μ m pore size; BD Biosciences, Cowley, UK) as described previously.(18) Cells were stained with CellTracker Green (Molecular Probes, Eugene, OR, USA) and fixed in 4% paraformaldehyde. Three fields of cells on the lower membrane surface were counted in each well at $\times 10$ magnification.

ADAM17 cloning and transfection

Human ADAM17 full-length cDNA in vector pCMV-NEO was purchased from Origene (Rockville, MD, USA). pCMV-NEO-hADAM17 plasmid was amplified in transformed EPI400 Electrocompetent *E. coli* (Epicentre Biotechnologies, Madison, WI, USA). Ampicillin-resistant clones were expanded using CopyCutter induction solution and DNA was isolated and sequenced to verify hADAM17 sequence integrity. We found hADAM17 was unstable in DH4 α *E. coli*, and only use of CopyCutter *E. coli* with pre-harvest plasmid copy induction produced intact hADAM17 cDNA clones. Transfection of hADAM17 or empty pcDNA3.1+ plasmid was done using a Nucleofector Kit (Amaxa, Gaithersburg, MD, USA) per the manufacturer's protocol. 3×10^6 astrocytes were transfected with 5 μ g of plasmid DNA. The pcDNA3.1+ and pCMV-NEO vectors are near identical, and each confers neomycin resistance. Program A-33 was used for electroporation in an Amaxa Nucleofector Device. Media was refreshed after 6 h, and at 24 h 500 μ g/mL neomycin (Invitrogen, Carlsbad, CA, USA) was added. After 1 week in culture, surviving astrocyte colonies were harvested, expanded, and tested for hADAM17 mRNA expression, ADAM17 protein expression, and α -secretase activity. In all *in vitro* studies, three different experiments employing hADAM17-astrocyte cells derived from three unique clones were performed.

Western blot

Western blot was used to detect ADAM17, CD133, SV40 (Abcam, Cambridge, MA, USA), c-Myc (Cell Signaling, Danvers, MA, USA), Musashi1 (Abcam), glial fibrillary acidic protein (GFAP; Dako, Carpinteria, CA, USA), Nestin (Covance, Princeton, NJ, USA), AKT and p-AKT (Cell Signaling), vascular endothelial growth factor-A (VEGF-A), EGFR, p-EGFR, EGF, and β -actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) and Kodak X-omat film (Kodak, New York, NY, USA) exposure were used for visualization. Soluble VEGF and EGF were detected in media conditioned by 24 h incubation in wells (2 mL) seeded with 2×10^5 cells. Concentration of protein in media was achieved using a Millipore centrifugal filter tube (Millipore, Danvers, MA, USA). Protein concentration was quantified using a BCA protein assay kit (Pierce). Densitometric measurements of three blots per group were averaged.

Immunohistochemistry

Coronal sections (8 μ m) were obtained from paraffin-embedded nude mouse brain. Sections were immunostained for GFAP (Dako), β -III-tubulin (Covance), synaptophysin and brain lipid binding protein (BLBP; Chemicon, Danvers, MA, USA), S100 (Dako), EMA, collagen IV, Ki-67 (Abcam), vimentin (Santa Cruz Biotechnology), and cytokeratin.

Real-time RT-PCR

Polymerase chain reaction (PCR) was performed using the SYBR Green real-time PCR system on an ABI 7000 PCR instrument (Applied Biosystems, Foster City, CA, USA). Total RNA was extracted using an Absolutely RNA Miniprep kit (Stratagene, La Jolla, CA, USA). cDNA was prepared from total RNA using oligo(dT), dNTP mix, First-Strand Buffer, DTT, RNaseOUT, and Superscript III (Invitrogen). Primers employed were β -actin: forward, 5'-ccatcatgaagtgtgacgttg-3', reverse, 5'-caatgatcttgatcttcacgttg-3'; hADAM17: forward, 5'-aagatatcaagaatgtttcacgtttg-3', reverse, 5'-tcttcaggtgggtctctgtactaa-3'; nestin: forward, 5'-ggtatcttgaagaaaccaggaga-3', reverse, 5'-gggaatcctgactcacttttcta-3'; GFAP: forward, 5'-acatcgagatcgccacacac-3', reverse, 5'-gcacacctcacatcacatcc-3'; c-Myc: forward, 5'-gaccagatccctgagttgga-3', reverse, 5'-ctcgccgtttcctcagtaag-3'; Notch-1: forward, 5'-tctctcgagagttgtcctagc-3', reverse, 5'-gtggtctaagtaccatcagca-3'; CD133: forward, 5'-gtcaaaaagaccttgattgtt-3', reverse, 5'-tgtacctgtgtgtcattaaggt-3', and Musashi1: forward, 5'-atggtggaatgaagaaagc-3', reverse, 5'-taacatgccaataccagca-3'. Each sample was tested in triplicate, and relative gene expression was determined as previously described.(18)

Polymerase chain reaction (PCR) array

To profile of gene expression associated with transformation and tumorigenesis, we employed an RT² Profiler PCR Array (SuperArray, Frederick, MD, USA). We restricted our analysis to two functional gene groups in the array, those associated with angiogenesis and those associated with invasion and metastasis. cDNA was prepared as described for real-time RT-PCR experiments and PCR was performed using the SYBR Green system on an ABI 7000 PCR instrument.

In vivo tumor model

Male nude mice (18-22 g) were utilized for this study. Procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. A 3-mm diameter craniotomy was made on the right hemisphere anterior to the coronal suture. Using a Hamilton syringe, cells were injected 3.0 mm deep, 1.5 mm to the right and 2.0 mm anterior of the bregma. Mice were implanted with 5×10^5 cells (5- μ L PBS) over a 5-minute interval. CTX-TNA2 cells transfected with empty pcDNA3.1+ plasmid ($n = 5$ mice) or with the pCMV-hADAM17 plasmid ($n = 5$ mice) were implanted. The craniotomy was covered with Horsley's bone wax, and the incision was closed with 4-0 silk sutures (Ethicon, Somersville, NJ, USA). Mice were sacrificed 4 weeks after implantation under anesthesia with i.p. administration of ketamine (80 mg/kg) and xylazine (13 mg/kg). Animals were perfused with 10% formalin following vascular washout with 0.9% saline. Brains were removed, fixed, and cut into 1-mm thick blocks which were embedded in paraffin.

Statistical analysis

Student's *t*-test was used for comparing means between two groups, and anova was employed to compare multiple groups. In all experiments, a *P*-value of 0.05 or less was considered statistically significant.

Results

ADAM17 transfection

To verify astrocytes transfected with pCMV-hADAM17 expressed functional ADAM17, cells were harvested and ADAM17 mRNA and protein were measured. Real-time PCR indicated expression of human ADAM17 mRNA, whereas non-transfected or pcDNA1.3+ transfected rat astrocytes had no detectable hADAM17 mRNA after 40 amplification cycles (not shown). Western blot for ADAM17 revealed increased expression of ADAM17 protein after clonal

selection (Fig. 1a). Of note, our ADAM17 antibody reacted with both human and rat ADAM17; however, hADAM17 mRNA was detected in addition to increased ADAM17 protein, indicating hADAM17 transfection. Furthermore, an α -secretase assay verified increased α -proteolytic activity of cell lysates from hADAM17 transfected astrocytes, compared to non-transfected or pcDNA3.1+ transfected control (Fig. 1b, $**P < 0.01$). Similar mRNA, protein expression, and α -secretase activity was confirmed in cell cultures derived from three clones, each derived from a distinct transfection experiment.

Bacterial amplification of the full-length ORF of hADAM17 cDNA consistently produced few hADAM17 plasmid-bearing *E. coli* clones, and all ampicillin-resistant clones tested contained plasmid with mutations within the hADAM17 sequence (our unpublished data). Use of *E. coli* that maintained a low plasmid copy number with induction of plasmid production just prior to harvest produced a mutation-free hADAM17 sequence. These findings are similar to those of Perez *et al.*, who reported that human ADAM17 is unstable in *E. coli* and mutates at a high frequency.(19) Furthermore, although hADAM17-astrocytes were maintained under antibiotic selection at a concentration capable of a total parallel non-transfected cell-kill, hADAM17 mRNA and protein expression was lost after 10-15 passages (unpublished data). hADAM17-astrocytes were used within 2-6 passages after verified transfection.

ADAM17 promotes EGFR/AKT signaling activity

We have previously reported ADAM17 promotes EGFR phosphorylation and downstream PI3K/AKT activity in human glioma cells.(18) Therefore we tested whether hADAM17 transfection in rat astrocytes lead to increased phosphorylation of EGFR and AKT. Western blotting for EGFR, p-EGFR, AKT, and p-AKT revealed increased phosphorylation of EGFR and AKT in hADAM17-astrocytes compared to non-transfected cells or those transfected with the empty pcDNA3.1+ plasmid (Fig. 1c,d). In addition, an increase in soluble EGF was detected in media from hADAM17-astrocytes cultures compared to control (Fig. 1c,d), indicating increased EGF-ligand shedding. hADAM17 transfection did not significantly alter expression of total AKT or EGFR proteins.

ADAM17 enhances proliferation via an EGFR-dependent mechanism

Following hADAM17 transfection, when grown to near-confluence, hADAM17-astrocytes no longer displayed a fibroblast-like morphology, but instead contracted into shiny rounded cells. These cells grew as clusters, which formed non-adherent sphere-like bodies after several days (Fig. 2a). Interestingly, hADAM17-astrocyte sphere formation was nearly abolished by the presence of EGFR inhibitor AG1487 (10 μ M) or ADAM17 inhibitor TAPI-2 (10 μ M, Fig. 2a). As hADAM17-expression altered morphology and growth characteristics, we next tested whether hADAM17 affected astrocyte proliferation. A growth curve assay indicated little difference between hADAM17-astrocytes and controls; a significant difference in cell number was detected only after 6 days in culture (Fig. 2b). However, addition of the EGFR inhibitor AG1487 or ADAM17 inhibitor TAPI-2 decreased growth in hADAM17-astrocyte cultures, but did not significantly alter growth of pcDNA3.1+ controls (Fig. 2b). As cells within the hADAM17-astrocyte group had begun to detach and grow as spheres, we surmised loss of adherence enabled hADAM17-astrocytes to expand more quickly than their adherent counterparts in these conditions. To test this hypothesis, we cultured cells in wells coated with noble agar, to grow cells in non-adherent conditions in DMEM or neurosphere culture medium Neuralbasal-A. After 5 days' culture, we performed a MTT assay test of cell viability. Formazan blue metabolism did not indicate that hADAM17-astrocytes were more viable in non-adherent growth conditions compared to control at 5 days (Fig. 2c). However, when added to cells grown in non-adherent conditions, TAPI-2 lead to a significant decrease in viable hADAM17-astrocytes. AG1487 reduced non-adherent hADAM17-astrocyte growth in DMEM cultures but had no effect in Neuralbasal-A cultures. Neither inhibitor significantly

altered cell viability in non-adherent pcDNA3.1+ control cultures (Fig. 2c). The effects of AG1487 and TAPI-2 suggest ADAM17 and EGFR signaling play a significant role in the non-adherent growth of hADAM17-expressing astrocytes.

ADAM17 enhances invasion via an EGFR-dependent mechanism

Previously, we established that ADAM17 increased hypoxic-induced invasion of glioma cells *in vitro*.(18) Therefore we tested whether hADAM17 could enhance astrocyte invasion using a Matrigel invasion chamber. hADAM17 expression in cortical astrocytes significantly enhanced cell invasion (Fig. 2d,e). Furthermore, as ADAM17-induced invasion in glioma cells was EGFR-dependent, we investigated whether inhibition of EGFR would affect ADAM17-astrocyte invasion. Addition of the EGFR inhibitor AG1487 significantly reduced the number of ADAM17 astrocytes that had penetrated the Matrigel matrix, confirming that invasion was dependent upon EGFR signaling.

ADAM17 promotes pro-invasive and pro-angiogenic gene expression

ADAM17 over-expression in astrocytes leads to non-adherent growth, and increased proliferation and invasion. ADAM17 has been reported to play a role in malignancy of multiple types of tumor, including astrocytoma.(18) To better characterize the effect of ADAM17 in astrocytes, we employed a real-time PCR array to profile the expression of genes associated with tumor invasion and angiogenesis. Expression of 35 genes was determined in pcDNA3.1+ or hADAM17-transfected astrocytes. Of the 15 invasion- and metastasis-related genes measured, four were significantly altered by hADAM17 expression, all which were up-regulated (Table 1a). These four genes (S100 calcium-binding protein A4 [S100], matrix metalloproteinase-9 [MMP-9], mucin-1, and twist gene homolog-1 [Twist-1]) have been reported to promote tumor invasion.(20-22) These data correlated with our finding that hADAM17 expression increases astrocyte invasiveness. Interestingly, of 20 angiogenesis-related genes analyzed, 12 were significantly altered by hADAM17 expression (Table 1b). Eleven of these angiogenesis-related genes were over-expressed (insulin-like growth factor-1, fibroblast growth factor receptor-2, VEGF-C, fibroblast growth factor-1, TNF member-2, C-fos-induced growth factor, VEGF-A, hepatocyte growth factor, transforming growth factor-beta 1, angiopoietin-1, and VEGF-B), each reported as a pro-angiogenic factor.(23,24) Furthermore, mRNA for thrombospondin-1, a known inhibitor of tumor-induced angiogenesis, (25) was significantly decreased after hADAM17 transfection (Table 1b). Taken together, these data indicate ADAM17 over-expression results in broad changes in gene expression. In particular, ADAM17 expression promotes expression of several mRNAs associated with cell invasiveness and angiogenesis.

ADAM17 increases angiogenic factor production and induces tubeformation in endothelial cells

ADAM17 sheds membrane TGF- α which has been reported to promote tumor-induced angiogenesis through the MAP kinase signal transduction pathway by activating transcription factors such as AP-2, which then bind to the promoter regions of genes encoding angiogenesis growth factors such as VEGF.(26) Our PCR array data indicated hADAM17 expression up-regulated multiple genes associated with angiogenesis, including VEGF, and decreased expression of the endogenous angiogenic inhibitor thrombospondin-1. We therefore tested if expression of hADAM17 in astrocytes elicited a functional pro-angiogenic effect. Western blot revealed increased levels of VEGF-A in medium of hADAM17-astrocytes compared to non-transfected or pcDNA3.1+ transfected control (Fig. 3d). In addition, to determine if hADAM17 could induce angiogenesis, mouse endothelial cells plated on Matrigel were exposed to medium conditioned by 24-h culture of astrocytes, hADAM17-astrocytes or those transfected with pcDNA3.1+. In line with an increased production of soluble VEGF, tube-like formation was

increased in endothelial cell cultures treated with hADAM17-astrocyte-conditioned medium (Fig. 3a-c). These results indicate that ADAM17 expression increases production of angiogenic factors in rat astrocytes such as VEGF, resulting in a pro-angiogenic effect.

ADAM17 promotes an undifferentiated phenotype

Adherent-independent proliferation and invasiveness is a hallmark of an immature and/or neoplastic phenotype. Therefore, we tested whether expression of genes related to cellular differentiation were altered by ADAM17 overexpression. We found the transcription factors c-Myc and Musashi-1 were up-regulated at the mRNA and protein level in hADAM17-astrocytes (Fig. 4). Furthermore, as ADAM17 is a primary activator of the Notch component in regulation of the Notch signaling pathway, we also tested whether ADAM17-expression affected Notch expression and/or activity. Western blot and PCR revealed Notch-1 expression was higher in hADAM17-astrocytes compared to control. Expression of the Notch intercellular domain (NICD) was greatly enhanced by ADAM17 over-expression, suggesting increased Notch activity. c-Myc, Musashi-1, and Notch activity are indicators of cell multipotency, often expressed in neural progenitor cells and neoplastic cells.(27-30) hADAM17-astrocytes also expressed higher levels of the stem cell marker CD133,(31) supporting this hypothesis. These data suggest ADAM17 over-expression induced an undifferentiated phenotype in CTX-TNA2 astrocytes.

ADAM17 over-expressing astrocytes form tumors in a nude mouse model of brain tumor

CTX-TNA2 astrocytes over-expressing ADAM17 possessed genetic and phenotypical characteristics of neoplasm. Therefore we tested whether hADAM17-astrocytes could form tumor *in vivo*. hADAM17-expressing astrocytes ('test') or astrocytes transfected with the empty pcDNA3.1+ plasmid ('control') were implanted into the brains of nude mice using an established model of brain tumor. We found after 28 days, the pcDNA3.1+ and hADAM17 developed intracerebral neoplasms. However, the tumors in each group were distinctively different. The control tumors were intraventricular and small, with an average volume of $6.1 \pm 4.6 \text{ mm}^3$, about $4.63 \pm 0.02\%$ of total brain volume (Fig. 5a-c). These control tumors had histological features of a low histological grade, benign glial neoplasm, i.e. rather inconspicuous mitoses, sparse vascularity, and absent necrosis or leptomeningeal spread. In contrast, the tumors originating from the hADAM17 astrocytes were much larger, with an average volume of $92 \pm 39.3 \text{ mm}^3$, comprising $65.8 \pm 29.7\%$ of total brain volume (Fig. 5a-c). In addition, these tumors had morphological features of a high histological grade, malignant neoplasm: anaplastic cellular features, frequent mitoses, prominent vascularity, apoptosis throughout, and widespread dissemination through the leptomeningeal space. The neoplastic transformation of CTX-TNA2 astrocytes observed in this nude mouse model may possibly be related to their transfection with a DNA construct containing the oncogenic early region of the SV40 virus; this early region makes the astrocytes immortal and is known to induce neoplastic malignant transformation. This immortal transformation is under the transcriptional control of the human GFAP promoter.(32) We posited that the expression of the oncogenic SV40 peptide might be primarily responsible for the different neoplastic effect observed in pcDNA3.1+ control astrocytes. With this consideration, we employed western blot to test whether our hADAM17 astrocytes expressed a different level of the SV40 peptide compared to pcDNA3.1+ control. All hADAM17 ($n = 3$) and pcDNA3.1+ ($n = 3$) clonal cell lines tested expressed similar levels of SV40 (Fig. 5d). Immunoperoxidase staining for GFAP and BLBP indicated glial processes and collagen IV reactivity indicated increased microvasculature within hADAM17-astrocytic tumors (Fig. 5e).

Discussion

The ADAM proteins belong to the superfamily of Zn-dependent metalloproteinases and are best known as ectodomain sheddases, a function of their metalloprotease domains.(32) ADAM17 is an extensively studied member of the ADAM family, and is reported to shed a variety of cell-surface molecules, including cytokines, adhesion molecules, and growth factors.(33-35) Of particular interest, ADAM17 is the primary sheddase for multiple EGFR-binding pro-ligands.(1) EGFR ligand-binding results in receptor self-dimerization, autophosphorylation, and consequent activation of the Ras/MAPK/ERK and PI3K/AKT signaling pathways.(36) The prototype of a family of tyrosine kinases, EGFR participates in the control of proliferation, invasion, and cell survival, as well as in the development of tumors of epidermal origin.(7,8,36,37) ADAM17 plays a central role in the shedding of EGF ligands and is required for activation of EGFR-dependent maximal tumor growth *in vivo*.(16) In particular, ADAM17-released amphiregulin promotes proliferation and motility of head and neck squamous cell carcinoma,(38) and amphiregulin and/or HB-EGF activated by ADAM17 stimulated EGFR and consequent activation of MAPK/ERK1/2 and prosurvival AKT signaling in squamous cell carcinoma or lung cancer.(11) In renal carcinoma, ADAM17 supports acquisition of several tumor cell capabilities via release of EGFR-activating TGF- α , and silencing of ADAM17 corrected these critical features, including growth autonomy, tumor inflammation, and tissue invasion.(39) Recently, we demonstrated ADAM17 enhances hypoxic-induced invasiveness of glioma cells through the EGFR/PI3K/AKT signaling pathway.(18) Considering EGFR overexpression is characteristic of many tumors including astrocytoma, we posited that ADAM17 proteolysis could induce selfsufficient growth-signaling, and promote a malignant phenotype in astrocytes, possibly via autocrine EGFR activation.

ADAM17 over-expression in astrocytes resulted in increased α -secretase activity, release of EGF, and phosphorylation of EGFR and its downstream target AKT. ADAM17 stimulates EGFR/AKT,(11,18) suggesting transfected hADAM17 had a functionally appropriate effect. Once hADAM17-expressing astrocyte clones were grown to ~50% of confluence, cells began to cluster and to detach as spheroids; however, EGFR or ADAM17 antagonists inhibited sphere formation. Growth curve and non-adherent viability assays indicated hADAM17 astrocytes continued to proliferate as cellular spheres, and that this nonadherent growth was in part dependent upon ADAM17 activity. Non-adherent growth is a classic characteristic of progenitor cells and poorly differentiated tumor.(31) The CTX-TNA2 cortical astrocyte cell-line was derived from day 1 rats, and thus represents an immature cell.(40) Nevertheless, control or pcDNA3.1+ transfected cells did not display non-adherent growth whereas all verified hADAM17-expressing clones did, indicating that non-adherent growth was a function of hADAM17 expression.

In addition to loss of adherence, CTX-TNA2 rat astrocytes over-expressing hADAM17 demonstrated increased cell invasion, which could be significantly reduced by inhibition of EGFR. This is reasonable in light of our finding that hADAM17 expression up-regulated EGFR phosphorylation. Treated with EGF, astrocytes generate extracellular fibronectin and synthesize integrin β 1, which induces migration in the direction of fibronectin deposits.(41) In addition, several genes related to cell migration are up-regulated by EGFR activation in astrocytes.(41) ADAM17 expression is a key component of hypoxic-induced glioma invasion, primarily via activation of EGFR/AKT.(18) Similarly, small interfering RNA to ADAM17/TACE dramatically reduced invasion of pancreatic ductal adenocarcinoma *in vitro*.(14) Our data indicates that hADAM17 over-expression leads to the increased motility and invasiveness of astrocytes. Constitutively active EGFR promotes tumor motility and invasiveness,(37) and we found hADAM17 invasiveness was significantly EGFR-dependent. Considering that ADAM17 also activates not only EGFR, but invasion-related proteins such as metastasis-

associated C4.4A and Notch,(42-44) we suggest ADAM17 up-regulation could play a pivotal role in the acquisition of motile and invasive properties, a fundamental step in malignant transformation.

hADAM17 over-expression resulted in significant changes in astrocyte growth and invasion, suggesting a progression towards a malignant state. To better characterize the effect of ADAM17 in tumorigenesis, we employed a PCR array to test the expression profiles of a number of genes involved in oncogenic invasion and angiogenesis. Here we found the invasion-related genes *S100*, *MMP-9*, *Mucin-1*, and *Twist-1* were up-regulated by hADAM17 transfection, each which have been reported to promote tumor cell invasion.(20-22) In addition, several pro-angiogenic genes were significantly up-regulated by hADAM17, including the *VEGFs A, B, and C*. hADAM17 over-expression decreased thrombospondin-1 mRNA, indicating a disinhibition effect of ADAM17 upon angiogenesis as well. We did not test how ADAM17 mediated the expression of these genes. However, ADAM17-shed TGF- α has been reported to promote angiogenic gene transcription including VEGF via MAP kinase activation of the transcription factor AP-2, suggesting one pro-angiogenic possibility.(26) Additionally, HB-EGF, an ADAM17 substrate, can also induce angiogenesis via activation of PI3K, MAPK, and eNOS in a VEGF-independent fashion.(45) Recently, we found ADAM17 over-expression increased VEGF production in MDA-MB-231 breast cancer cells.(46) Taken together, these findings indicate hADAM17 over-expression results in broad changes in gene expression, with an overall bias towards increased invasiveness and angiogenesis. On a functional level, hADAM17-astrocytes produced substantially more soluble VEGF-A than control cultures, and hADAM17-astrocyte conditioned media induced greater endothelial tube formation *in vitro*. Therefore, in addition to non-adherent growth and invasion, ADAM17 expression provoked a pro-angiogenic effect from astrocytes, satisfying another requirement for autonomous tumor growth.

Over-expression of hADAM17 caused CTX-TNA2 rat astrocytes to acquire multiple characteristics commonly associated with immature or neoplastic cells. Western blot revealed hADAM17-astrocytes expressed significantly higher levels of the NICD, indicating enhanced Notch activity. Notch activation contributes to Ras-induced transformation of glial cells and promotes glioma growth and survival.(47) As an α -secretase, ADAM17 is a component of Notch activation, cleaving the extracellular region of the Notch receptor, preceding the proteolytic release of the intracellular domain by γ -secretase.(43,48) Furthermore, the NICD increases expression of the c-Myc proto-oncogene, by directly binding to its promoter.(49) Indeed, c-Myc mRNA and protein expression were significantly increased in hADAM17 expressing astrocytes compared to control. It would therefore be interesting to test if increased c-Myc following ADAM17-overexpression was due to activation of Notch by ADAM17. In addition, hADAM17 promoted Musashi-1 expression in astrocytes, a DNA-binding protein highly expressed in neural stem cells that positively correlates with malignancy of astrocytoma.(27,50) Musashi-1 can enhance Notch signaling through translational repression of its target mRNA Numb.(50) Regulation of Musashi-1 expression by ADAM17 has not been reported. The mechanisms underlying ADAM17 induction of Musashi-1 and c-Myc expression and of Notch activity were not elucidated. Nevertheless, these data confirm progression towards a neoplastic state resulting from hADAM17 over-expression.

The pathological findings in our *in vivo* nude mouse model of brain tumor correlate with the *in vitro* findings that we have reported above. The CTX-TNA2 astrocytes transfected with the empty pcDNA3.1+ plasmid (control) developed small, intraventricular, low histological grade glial neoplasms. In stark contrast, the same astrocytes, but over-expressing hADAM17, transformed into large malignant gliomas, with cellular anaplasia, brisk mitoses, dense vascularity, and leptomeningeal spread. This different neoplastic potential was probably not

primarily related to the oncogenic SV40 peptide of the CTX-TNA2 astrocyte, because we found that both tumor types, benign and malignant, had similar expression of the SV40 peptide.

Our study indicates that over-expression of the ADAM17 protease promotes a malignant phenotype in a cortical astrocyte cell line. These findings are compatible with increasing evidence that ADAM17 promotes autocrine growth signaling in tumor, principally through activation of EGFR-binding ligands. ADAM17 activation resulted in increased invasion, non-adherent growth, and production of angiogenic factors, suggesting that the protease represents a molecular switch for multiple mechanisms fundamental to malignant growth. A role for ADAM17 in EGFR shedding exists in multiple cancers, and ADAM17 inhibition phenotypes EGFR inhibition.(10,15) In fact, highly malignant EGFR-dependent renal carcinoma cells failed to form tumors *in vivo* the absence of ADAM17, confirming a essential role in tumorigenesis.(39) Our *in vivo* is consistent with the above findings, the malignancy promoting effects of ADAM17. This effect may perhaps be a constitutive autocrine activation of trophic ligands, enabling cells to overcome mechanisms that inhibit autonomous growth. Thus, up-regulation of ADAM17 activity represents a potential gateway in the transformation from a precancerous or low histological grade to a malignant phenotype.

Acknowledgment

This work was supported by NIH grants PO1 CA043892 and RO1 CA100486.

References

1. Blobel CP. ADAMs: key components in EGFR signalling and development. *Nat Rev Mol Cell Biol* 2005;6:32–43. [PubMed: 15688065]
2. Brattain MG, Howell G, Sun LZ, Willson JK. Growth factor balance and tumor progression. *Curr Opin Oncol* 1994;6:77–81. [PubMed: 8204695]
3. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004;304:1497–500. [PubMed: 15118125]
4. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004;350:2129–39. [PubMed: 15118073]
5. Frederick L, Wang XY, Eley G, James CD. Diversity and frequency of epidermal growth factor receptor mutations in human glioblastomas. *Cancer Res* 2000;60:1383–7. [PubMed: 10728703]
6. Hirsch FR, Varella-Garcia M, Bunn PA Jr. et al. Epidermal growth factor receptor in non-small-cell lung carcinomas: correlation between gene copy number and protein expression and impact on prognosis. *J Clin Oncol* 2003;21:3798–807. [PubMed: 12953099]
7. Tang P, Steck PA, Yung WK. The autocrine loop of TGF- α /EGFR and brain tumors. *J Neurooncol* 1997;35:303–14. [PubMed: 9440027]
8. Ongusaha PP, Kwak JC, Zwible AJ, et al. HB-EGF is a potent inducer of tumor growth and angiogenesis. *Cancer Res* 2004;64:5283–90. [PubMed: 15289334]
9. Ramnarain DB, Park S, Lee DY, et al. Differential gene expression analysis reveals generation of an autocrine loop by a mutant epidermal growth factor receptor in glioma cells. *Cancer Res* 2006;66:867–74. [PubMed: 16424019]
10. Kenny PA, Bissell MJ. Targeting TACE-dependent EGFR ligand shedding in breast cancer. *J Clin Invest* 2007;117:337–45. [PubMed: 17218988]
11. Hart S, Fischer OM, Ullrich A. Cannabinoids induce cancer cell proliferation via tumor necrosis factor α -converting enzyme (TACE/ADAM17)-mediated transactivation of the epidermal growth factor receptor. *Cancer Res* 2004;64:1943–50. [PubMed: 15026328]
12. Kodama T, Ikeda E, Okada A, et al. ADAM12 is selectively overexpressed in human glioblastomas and is associated with glioblastoma cell proliferation and shedding of heparin-binding epidermal growth factor. *Am J Pathol* 2004;165:1743–53. [PubMed: 15509542]

13. Blanchot-Jossic F, Jarry A, Masson D, et al. Up-regulated expression of ADAM17 in human colon carcinoma: co-expression with EGFR in neoplastic and endothelial cells. *J Pathol* 2005;207:156–63. [PubMed: 16041691]
14. Ringel J, Jesnowski R, Moniaux N, et al. Aberrant expression of a disintegrin and metalloproteinase 17/tumor necrosis factor-alpha converting enzyme increases the malignant potential in human pancreatic ductal adenocarcinoma. *Cancer Res* 2006;66:9045–53. [PubMed: 16982746]
15. Kenny PA. Tackling EGFR signaling with TACE antagonists: a rational target for metalloprotease inhibitors in cancer. *Expert Opin Ther Targets* 2007;11:1287–98. [PubMed: 17907959]
16. Borrell-Pages M, Rojo F, Albanell J, Baselga J, Arribas J. TACE is required for the activation of the EGFR by TGF-alpha in tumors. *Embo J* 2003;22:1114–24. [PubMed: 12606576]
17. Berasain C, Castillo J, Perugorria MJ, Prieto J, Avila MA. Amphiregulin: a new growth factor in hepatocarcinogenesis. *Cancer Lett* 2007;254:30–41. [PubMed: 17321672]
18. Zheng X, Jiang F, Katakowski M, et al. Inhibition of ADAM17 reduces hypoxia-induced brain tumor cell invasiveness. *Cancer Sci* 2007;98:674–84. [PubMed: 17355261]
19. Perez L, Kerrigan JE, Li X, Fan H. Substitution of methionine 435 with leucine, isoleucine, and serine in tumor necrosis factor alpha converting enzyme inactivates ectodomain shedding activity. *Biochem Cell Biol* 2007;85:141–9. [PubMed: 17464354]
20. Luo GQ, Li JH, Wen JF, Zhou YH, Hu YB, Zhou JH. Effect and mechanism of the Twist gene on invasion and metastasis of gastric carcinoma cells. *World J Gastroenterol* 2008;14:2487–93. [PubMed: 18442194]
21. Saleem M, Kweon MH, Johnson JJ, et al. S100A4 accelerates tumorigenesis and invasion of human prostate cancer through the transcriptional regulation of matrix metalloproteinase 9. *Proc Natl Acad Sci USA* 2006;103:14825–30. [PubMed: 16990429]
22. Schroeder JA, Adriance MC, Thompson MC, Camenisch TD, Gendler SJ. MUC1 alters beta-catenin-dependent tumor formation and promotes cellular invasion. *Oncogene* 2003;22:1324–32. [PubMed: 12618757]
23. Cao Y, Liu Q. Therapeutic targets of multiple angiogenic factors for the treatment of cancer and metastasis. *Adv Cancer Res* 2007;97:203–24. [PubMed: 17419947]
24. Ryuto M, Ono M, Izumi H, et al. Induction of vascular endothelial growth factor by tumor necrosis factor alpha in human glioma cells. Possible roles of SP-1. *J Biol Chem* 1996;271:28220–8. [PubMed: 8910439]
25. Lawler J. Thrombospondin-1 as an endogenous inhibitor of angiogenesis and tumor growth. *J Cell Mol Med* 2002;6:1–12. [PubMed: 12003665]
26. Gille J, Swerlick RA, Caughman SW. Transforming growth factor-alpha-induced transcriptional activation of the vascular permeability factor (VPF/VEGF) gene requires AP-2-dependent DNA binding and transactivation. *Embo J* 1997;16:750–9. [PubMed: 9049304]
27. Ma YH, Mentlein R, Knerlich F, Kruse ML, Mehdorn HM, Held-Feindt J. Expression of stem cell markers in human astrocytomas of different WHO grades. *J Neurooncol* 2008;86:31–45. [PubMed: 17611714]
28. Fan X, Eberhart CG. Medulloblastoma stem cells. *J Clin Oncol* 2008;26:2821–7. [PubMed: 18539960]
29. Rao G, Pedone CA, Coffin CM, Holland EC, Fults DW. c-Myc enhances sonic hedgehog-induced medulloblastoma formation from nestin-expressing neural progenitors in mice. *Neoplasia* 2003;5:198–204. [PubMed: 12869303]
30. Wang J, Wang H, Li Z, et al. c-Myc Is Required for Maintenance of Glioma Cancer Stem Cells. *PLoS ONE* 2008;3:e3769. [PubMed: 19020659]
31. Inagaki A, Soeda A, Oka N, et al. Long-term maintenance of brain tumor stem cell properties under at non-adherent and adherent culture conditions. *Biochem Biophys Res Commun* 2007;361:586–92. [PubMed: 17673180]
32. Primakoff P, Myles DG. The ADAM gene family: surface proteins with adhesion and protease activity. *Trends Genet* 2000;16:83–7. [PubMed: 10652535]
33. Ding X, Yang LY, Huang GW, Wang W, Lu WQ. ADAM17 mRNA expression and pathological features of hepatocellular carcinoma. *World J Gastroenterol* 2004;10:2735–9. [PubMed: 15309730]

34. Bauvois B. Transmembrane proteases in cell growth and invasion: new contributors to angiogenesis? *Oncogene* 2004;23:317–29. [PubMed: 14724562]
35. Matthews V, Schuster B, Schutze S, et al. Cellular cholesterol depletion triggers shedding of the human interleukin-6 receptor by ADAM10 and ADAM17 (TACE). *J Biol Chem* 2003;278:38829–39. [PubMed: 12832423]
36. Wells A. EGF receptor. *Int J Biochem Cell Biol* 1999;31:637–43. [PubMed: 10404636]
37. Lal A, Glazer CA, Martinson HM, et al. Mutant epidermal growth factor receptor up-regulates molecular effectors of tumor invasion. *Cancer Res* 2002;62:3335–9. [PubMed: 12067969]
38. Gschwind A, Hart S, Fischer OM, Ullrich A. TACE cleavage of proamphiregulin regulates GPCR-induced proliferation and motility of cancer cells. *Embo J* 2003;22:2411–21. [PubMed: 12743035]
39. Franovic A, Robert I, Smith K, et al. Multiple acquired renal carcinoma tumor capabilities abolished upon silencing of ADAM17. *Cancer Res* 2006;66:8083–90. [PubMed: 16912185]
40. Radany EH, Brenner M, Besnard F, Bigornia V, Bishop JM, Deschepper CF. Directed establishment of rat brain cell lines with the phenotypic characteristics of type 1 astrocytes. *Proc Natl Acad Sci USA* 1992;89:6467–71. [PubMed: 1378628]
41. Liu B, Neufeld AH. Activation of epidermal growth factor receptors in astrocytes: from development to neural injury. *J Neurosci Res* 2007;85:3523–9. [PubMed: 17526018]
42. Esselens CW, Malapeira J, Colome N, Moss M, Canals F, Arribas J. Metastasis-associated C4.4A, a GPI-anchored protein cleaved by ADAM10 and ADAM17. *Biol Chem* 2008;389:1075–84. [PubMed: 18979631]
43. Delwig A, Rand MD. Kuz and TACE can activate Notch independent of ligand. *Cell Mol Life Sci* 2008;65:2232–43. [PubMed: 18535782]
44. Sahlgren C, Gustafsson MV, Jin S, Poellinger L, Lendahl U. Notch signaling mediates hypoxia-induced tumor cell migration and invasion. *Proc Natl Acad Sci USA* 2008;105:6392–7. [PubMed: 18427106]
45. Mehta VB, Besner GE. HB-EGF promotes angiogenesis in endothelial cells via PI3-kinase and MAPK signaling pathways. *Growth Factors* 2007;25:253–63. [PubMed: 18092233]
46. Zheng X, Jiang F, Katakowski M, Zhang ZG, Lu QE, Chopp M. ADAM17 promotes breast cancer cell malignant phenotype through EGFR-PI3K-AKT activation. *Cancer Biol Ther* 2009;8
47. Kanamori M, Kawaguchi T, Nigro JM, et al. Contribution of Notch signaling activation to human glioblastoma multiforme. *J Neurosurg* 2007;106:417–27. [PubMed: 17367064]
48. Brou C, Logeat F, Gupta N, et al. A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE. *Mol Cell* 2000;5:207–16. [PubMed: 10882063]
49. Hsu KW, Hsieh RH, Lee YH, et al. The activated Notch1 receptor cooperates with alpha-enolase and MBP-1 in modulating c-myc activity. *Mol Cell Biol* 2008;28:4829–42. [PubMed: 18490439]
50. Okano H, Kawahara H, Toriya M, Nakao K, Shibata S, Imai T. Function of RNA-binding protein Musashi-1 in stem cells. *Exp Cell Res* 2005;306:349–56. [PubMed: 15925591]

Fig. 1. Human ADAM17 (hADAM17) increases phosphorylation of epidermal growth factor receptor (EGFR) and AKT in astrocytes. (a) Western blot: transfection with hADAM17 increased ADAM17 expression compared to control. Upper and lower bands indicate predicted pro-ADAM17 and ADAM17, respectively. (b) hADAM17 significantly increased α -secretase activity (** $P < 0.01$ compared to pcDNA3.1+ transfected control). (c and d) hADAM17 increased phosphorylation of EGFR and AKT, and increased soluble EGF (lane 1, control; lane 2, pcDNA3.1+; lane 3, hADAM17). (c) Representative blot. (d) Densitometric analysis indicated increased p-EGFR and p-AKT in hADAM17 astrocytes, and more soluble EGF in hADAM17-astrocytes conditioned medium, compared to pcDNA3.1+ control (* $P < 0.05$).

Fig. 2.

Effects of human ADAM17 (hADAM17) upon astrocyte growth and invasion *in vitro*. (a) As cell density increased after passage, hADAM17 astrocytes clustered and grew as non-adherent cellular spheres. Addition of epidermal growth factor receptor (EGFR) inhibitor AG1487 (10 μ M) or ADAM17 inhibitor TAPI-2 (10 μ M) abrogates sphere formation of hADAM17 astrocytes. (b) A growth curve assay indicates an ADAM17/EGFR-dependent growth advantage of hADAM17-astrocytes compared to pcDNA3.1+ control (* P < 0.05, ** P < 0.01; AG1487; # P < 0.05, ## P < 0.01: TAPI-2). (c) MTT assay revealed cell viability of hADAM17 astrocytes in non-adherent culture was ADAM17- and EGFR-dependent (* P < 0.05, ** P < 0.01 compared to hADAM17-transfected). (d and e) hADAM17 increased invasiveness via an EGFR-dependent mechanism. (d) Astrocyte invasion at $\times 4$ objective. (e) Quantitative analysis of invasion (** P < 0.01 compared to control, ## P < 0.01 compared to hADAM17).

Fig. 3. Endothelial tube-formation assay. (a-c) Representative images of mouse endothelial cells cultured in medium conditioned by astrocytes (a), pcDNA3.1+ astrocytes (b), or human ADAM17 (hADAM17) astrocytes (c). (d) Western blot indicated higher levels of vascular endothelial growth factor (VEGF)-A in medium conditioned by hADAM17 astrocytes (representative blot).

Fig. 4.

Western blot and real-time PCR. (a) hADAM17 increases c-Myc, Musashi1, Notch intercellular domain (NICD), and CD133 protein expression (representative blot). (b) Human ADAM17 (hADAM17) increased c-Myc, Musashi-1, and CD133 mRNA expression compared to pcDNA3.1+ transfected astrocytes (* $P < 0.05$). GFAP, glial fibrillary acidic protein.

Fig. 5.

Human ADAM17 (hADAM17) astrocytes formed gliomas when implanted in nude mouse brain. The tumors were small, intraventricular, and low histological grade in pcDNA3.1+ implanted mice, and massive, high histological grade, and infiltrative in hADAM17 implanted mice. The neoplasms were gliomas, possibly of radial glia origin. (a) H&E-stained coronal sections from nude mice implanted with pcDNA3.1+ or hADAM17-astrocytes. c1-c4 indicate coronal slice location. (b) Higher magnification images of pcDNA3.1 (c3) and hADAM17 (c4) tumors shown in (a) demonstrating increased cellularity, nuclear anaplasia, numerous mitoses, and infiltration of the leptomeninges in the high grade neoplasm (c4). (c) Tumor volume in pcDNA3.1+ and hADAM17 astrocyte implanted mice ($*P < 0.05$). (d) Western blot of SV40 in pcDNA3.1- and hADAM17 astrocyte cells. (e) Immunoperoxidase stain demonstrating glial processes (glial fibrillary acidic protein, GFAP; and brain lipid binding protein, BLBP) and increased microvasculature (vessel-associated collagen IV).

Table 1
Gene expression significantly altered by human ADAM17 in astrocytes ($P < 0.05$ for all statistics)

(a) Invasion- and metastasis-related genes			
Symbol	Gene	GenBank	hADAM17/pcDNA3.1 mRNA
S100A4	S100 calcium-binding protein A4	NM_012618	3.8 ± 0.39
MMP-9	Matrix metalloproteinase-9	NM_031055	2.98 ± 0.19
MUC-1	Mucin-1, transmembrane	XM_342281	1.96 ± 0.27
Twist-1	Twist gene homolog-1	NM_053530	1.75 ± 0.10
Other mRNAs detected ($P > 0.05$): Expressed in non-metastatic cells-1, Kiss-1 metastasis suppressor, matrix metalloproteinase-2, met proto-oncogene, metastasis associated gene family member-2, metastasis associated-1, serine peptidase inhibitor clade E member-1 (PAI1), serine proteinase inhibitor clade B member-2, tissue inhibitor of metalloproteinase-1, urokinase plasminogen activator receptor, urokinase plasminogen activator.			
(b) Angiogenesis-related genes			
Symbol	Gene	GenBank	hADAM17/pcDNA3.1 mRNA
IGF-1	Insulin-like growth factor-1	NM_178866	6.16 ± 0.13
FGFR-2	Fibroblast growth factor receptor-2	XM_341940	6.11 ± 1.0
VEGF-C	Vascular endothelial growth factor-C	NM_053653	4.90 ± 0.32
FGF-1	Fibroblast growth factor-1	NM_012846	4.56 ± 0.43
TNF	Tumor necrosis factor (TNF superfamily, member 2)	NM_012675	4.54 ± 0.70
FIGF	C-fos-induced growth factor	NM_031761	4.48 ± 0.6
VEGF-A	Vascular endothelial growth factor-A	NM_031836	3.90 ± 0.48
HGF	Hepatocyte growth factor	NM_017017	3.57 ± 0.45
TGFB-1	Transforming growth factor-beta 1	NM_021578	3.52 ± 0.46
ANG-1	Angiopoietin-1	NM_053546	3.46 ± 0.27
VEGF-B	Vascular endothelial growth factor-B	NM_053549	2.24 ± 0.11
THBS-1	Thrombospondin-1	NM_001013062	0.16 ± 0.08
Other mRNAs detected ($P > 0.05$): Endothelial growth factor receptor, endothelial-specific receptor tyrosine kinase, interferon-alpha 1, interferon-beta 1 (fibroblast), platelet-derived growth factor B-polyptide, platelet-derived growth factor-alpha, procollagen type XVIII-alpha 1 (endostatin).			