ADAM10 promotes pituitary adenoma cell migration by regulating cleavage of CD44 and L1

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Abstract

ADAM10 is a metalloproteinase that regulates invasiveness in many tumors. Here, we found that *ADAM10* expression correlates with the invasiveness of pituitary adenomas and contributes to invasion by cleaving L1 and CD44. In high-grade pituitary adenoma patients, *ADAM10* expression levels were found to be elevated compared with low-grade pituitary adenomas. In a phorbol 12-myristate 13-acetate (PMA)-stimulated pituitary adenoma cell line, AtT-20 cells, we found that the cleavage of L1 was correspondingly enhanced with the increased interaction between Src and Shc. Increases in PMA-induced L1 cleavage and the phosphorylation of residue 418 of Src (418Src) were promoted by overexpression of *ADAM10*. Inversely, knockdown of *Adam10* suppressed PMA-induced L1 cleavage and the phosphorylation of Src, which was blocked by the Src inhibitor PP2 and the MEK inhibitor PD98059. On the other hand, calcium flux activation in AtT-20 cells resulted in increased CD44 cleavage, with reduction of the interaction between calmodulin and ADAM10. The induction of enhanced CD44 cleavage by calcium flux activation was inhibited by knockdown of *Adam10*. In addition, *Adam10* knockdown repressed AtT-20 cell migration, which was reversed by CD44EXT (CD44 ectodomain cleavage). Collectively, these data indicated that ADAM10 facilitated cell migration through modulation of CD44 and L1 cleavage.

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Introduction

Human pituitary adenomas are tumors that occur in the pituitary gland and represent ~10–15% of intracranial neoplasms. Pituitary adenomas are often considered to be benign tumors (Daniel *et al.* 2000). However, a proportion of these tumors do invade the surrounding tissues, including the sphenoid sinus, the cavernous sinus, and even the brain (Qian *et al.* 2007). Invasion of the cavernous sinus space occurs in 6–10% of all pituitary adenomas and has been shown to be the most common cause of incomplete tumor resection. Compared with noninvasive adenomas, tumors that invade the cavernous sinus show significantly higher proliferation rates (Knosp *et al.* 1993). In addition, a surgical cure becomes increasingly difficult with a higher grading score of the tumor. Cavernous sinus invasion implies an increased risk of intraoperative or postoperative cerebrospinal fluid leakage and of damage to the nerves in the cavernous sinus. However, the mechanisms underlying the invasiveness and malignancy of pituitary adenomas are poorly understood. Substantial evidence has indicated

that no or only a few mutations of tumor suppressor genes or oncogenes are found in pituitary adenomas. Hence, we hypothesized that the abnormality of some adhesion molecules might be responsible for the aggressive behavior of pituitary adenomas.

The a-disintegrin-and-metalloprotease (ADAM) family is the major protein family that mediates ectodomain shedding with an activity similar to α -secretase (Allinson *et al.* 2003). This family is a group of cell surface proteins that contains multiple domains. One of these domains is the metalloprotease domain, which can induce ectodomain shedding and extracellular matrix (ECM) cleavage. Another domain, the disintegrin domain, has been implicated in cell adhesion (Blobel 1997, White 2003). Ectodomain shedding has been described as the process by which membrane-attached cell surface proteins release their soluble ectodomains as a result of proteolysis at their extracellular sites proximal to the cell membrane. These released molecules can act as crucial regulators in many physiological and pathological events. The ADAM family is well known as the major sheddase and can shed a large variety of cell surface proteins,

including growth factors, cytokines/cytokine receptors, and cell adhesion molecules (Mochizuki & Okada 2007, Moss *et al.* 2008). Many of these substrates are involved in cancer. Consistent with this phenomenon, the upregulated expression of the members of the ADAM family has been observed in many cancers at both the mRNA level and the mature active protein level (Arribas *et al.* 2006, Santiago-Josefat *et al.* 2007). ADAMs have been regarded as a potential target for tumor therapy in breast cancer and small-cell lung cancer (Borrell-Pages *et al.* 2003, Zhou *et al.* 2006, Kenny & Bissell 2007). ADAM10 belongs to the membrane-anchored ADAM group and is ubiquitously expressed throughout most tissues (Seals & Courtneidge 2003, Mochizuki & Okada 2007, Moss *et al.* 2008, Parkin & Harris 2009). The proteinase activity of ADAM10 contributes to Alzheimer's disease (Lammich *et al.* 1999, Allinson *et al.* 2003), inflammation, and cancer (Crawford *et al.* 2009, Pruessmeyer & Ludwig 2009). Several reports have shown increased expression of *ADAM10* in various types of cancer, including prostate cancer, colon carcinoma, and squamous cell carcinoma (Wu *et al.* 1997, Yoshimura *et al.* 2002, Fogel *et al.* 2003, McCulloch *et al.* 2004, Gavert *et al.* 2005, Ko *et al.* 2007). ADAM10 targets a series of adhesion molecules and the ECM, enhances the invasive properties of tumor cells, and facilitates tumor dissemination, which is the crucial step in cancer development and tumor progression (Millichip *et al.* 1998, Egeblad & Werb 2002). However, whether ADAM10 is involved in pituitary adenomas remains unknown.

In this study, we analyzed a group of pituitary adenomas and found that the expression of *ADAM10* correlated with tumor invasiveness and cleavage of CD44 and L1. CD44 and L1 cleavage significantly increased in high-grade tumors and was regulated by ADAM10 through calcium-dependent and -independent mechanisms. Calcium promoted ADAM10-mediated CD44 cleavage and reduced cell adhesion, at least partially through the depletion of the interaction of calmodulin (CaM) and ADAM10. Furthermore, phorbol 12-myristate 13-acetate (PMA) stimulation led to acceleration of the cleavage of L1, which is mediated by Src signaling. These data indicate that ADAM10 is involved in pituitary adenoma invasiveness by affecting cell adhesion and migration by cleaving CD44 and L1, suggesting that ADAM10 could be a potential target for clinical therapy.

Materials and methods

Immunoblotting

Cells were washed with chilled PBS and lysed with RIPA that contained a phosphatase inhibitor cocktail (Sigma) and protease inhibitors: 10 µg/ml

phenylmethylsulphonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 µg/ml pepstatin. After 30 min of chilled incubation, cell extracts were centrifuged at 15 000 *g* for 15 min at 4 °C. Then the supernatants were transferred, and the protein concentration was measured by the Bradford method. All samples were resuspended in an equal volume of 2× loading buffer. Equal amounts of protein were resolved using SDS–PAGE, transferred to nitrocellulose membranes, and incubated with the indicated antibodies. All immunoblots were visualized using a chemiluminescence detection kit (ECL; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

The antibodies used in the experiments are as follows: anti- α -tubulin (Sigma), anti-ADAM10 (Chemicon, Billerica, MA, USA), anti-L1 (Santa Cruz), anti-CaM (Upstate, Charlottesville, VA, USA), anti-CD44 (Ancell, Bayport, MN, USA), anti-p418Src, anti-Src and anti-Shc (Cell Signaling Technology, Danvers, MA, USA), and HRP-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies (Santa Cruz).

Cell culture and treatment

AtT-20 cells were maintained in F-12K medium (GIBCO-BRL) with 2.5% heat-inactivated fetal bovine serum and 15% horse serum (GIBCO-BRL), streptomycin (100 µg/ml) and penicillin (100 U/ml) at 37 °C in 95% air, and 5% CO₂. The cells were treated with dimethyl sulfoxide (control), PMA (100 ng/ml; Sigma), or ionomycin (5 µM; Calbiochem, San Diego, CA, USA). In some experiments, the cells were preincubated with the Src kinase inhibitor PP2 (preincubated for 1 h, 50 µM; Calbiochem) or the MAP kinase inhibitor PD98059 (preincubated for 1 h, 50 µM; Calbiochem). In other experiments, AtT-20 cells were treated with or without mechanical scraping or 100 µM trifluoperazine (TFP, a CaM inhibitor, for 30 min), after which cells were collected and subjected to further analysis.

Human pituitary tissues and adenomas

All samples were frozen and stored at –80 °C. Tumors were characterized based on their clinical, radiological, histological, and immunohistochemical features. Based on the secreting features, all tumors were classified as functional or nonfunctional. Tumor invasiveness was defined on the basis of preoperative radiological investigation using Knosp's classification (Knosp *et al.* 1993) and was confirmed during surgery. Grade 0–2 tumors were defined as noninvasive, while grade 3–4 tumors were considered to be invasive. Patient data regarding gender, age, tumor diameter, invasiveness, and secreting status are summarized in Table 1. Written informed consent was obtained from each patient.

Table 1 The relationship between ADAM10, p-418Src, or p-ERK1/2 and clinicopathological features in pituitary adenomas ($n=90$). Pearson's χ^2 test

Variable	ADAM10			p-418Src			p-ERK1/2		
	High	Low	P value	High	Low	P value	High	Low	P value
Age (years)									
≤ 50	29	30	0.608	32	27	0.957	29	30	0.824
> 50	17	14		17	14		16	15	
Gender									
Male	15	11	0.426	11	15	0.141	12	14	0.642
Female	31	33		38	26		33	31	
Size (cm)									
≤ 1	10	19	0.054	14	15	0.681	11	18	0.285
1–3	11	11		12	10		12	10	
≥ 3	25	14		23	16		22	17	
Invasiveness									
Invasive	32	19	0.012	33	18	0.025	28	23	0.288
Noninvasive	14	25		16	23		17	22	
Secreting									
Functional	28	32	0.233	34	26	0.549	28	32	0.371
Nonfunctional	18	12		15	15		17	13	
Functional (PRL, 22 cases)	10	12	0.886	14	8	0.407	7	15	0.079
Functional (GH, 38 cases)	18	20		20	18		21	17	

Immunohistochemistry

Paraffin sections were obtained from the indicated patients. The sections were deparaffinized, and the epitopes were unmasked. After 30 min of blocking with serum, the sections were incubated with anti-ADAM10 antibody, anti-P-418Src antibody, and anti-ERK1/2 antibody (Cell Signaling) overnight at 4 °C. The sections were then incubated with a biotinylated anti-rabbit antibody (Santa Cruz) for 30 min at 37 °C. Immunohistochemical staining was performed by the avidin–biotin complex method as the standard procedure.

Immunocytochemistry

AtT-20 cells grown on 35 mm culture dishes were fixed with 4% PFA for 15 min and incubated in 0.2% Triton X-100 in PBS for 5 min. After being washed with PBS, the cells were incubated in primary antibodies diluted in PBS containing 0.2% BSA for 60 min at room temperature, washed three times in PBS, and incubated for 60 min at room temperature with the appropriate secondary antibodies linked to Cy5 diluted in PBS containing 0.2% BSA. After being washed with PBS, the samples were mounted in 80% glycerol and visualized using a confocal microscope (model Fluoview; Olympus).

Co-immunoprecipitations

Homogenized tumor tissue or cultured cells were washed with ice-cold PBS and lysed for 30 min on ice using immunoprecipitation buffer (25 mM HEPES,

pH 7.4, 10% glycerol, 150 mM NaCl, 0.1 mM EGTA, 0.5 mM dithiothreitol, and 0.5% Triton X-100) containing protease (Roche) and phosphatase inhibitor (Sigma) mixtures. The lysates were forced through a 26-gage needle four times and cleared by centrifugation at 16 000 *g* for 10 min at 4 °C. Equal amounts of lysate were incubated with 2 µg of the indicated antibodies together with 20 µl protein A-Sepharose slurry overnight at 4 °C by continuous agitation. Immunocomplexes were washed four times in immunoprecipitation buffer, and precipitated proteins were eluted by boiling in Laemmli buffer. Proteins were separated using SDS–PAGE, transferred to polyvinylidene difluoride membranes, and incubated with antibodies. All immunoblots were visualized using a chemiluminescence detection kit (ECL; Santa Cruz Biotechnology). The antibodies used in the experiments are as follows: anti- α -tubulin (Sigma), anti-ADAM10 (Chemicon), anti-L1 (Santa Cruz), anti-CaM (Upstate), anti-CD44 (Ansell), and anti-Src and anti-Shc (Cell Signaling).

Plasmid construction and transfection

The sequence of ADAM10 was inserted between the KpnI and XhoI sites of the pENTR1A dual selection vector (Invitrogen) and then subcloned into pLenti6/V5-DEST (Invitrogen). The sequences of L1 and CD44 were inserted between the EcoRI and XhoI sites of the pENTR1A-GFP dual selection vector and then subcloned into pLenti6/V5-DEST. CD44EXT was

generated by PCR from full-length CD44 cDNA and then subcloned into pLenti6/V5-DEST. The sequences (letters in bold indicate siRNA sequence after splicing) of shADAM10, 5'-CACCGAGATACATTAAAG-ATCGACGAA TCGATCTTTAATGTATCTCC-3' and 5'-AAAAGGAGATACATTAAAGATCGATTCTCGTC-GATCTTTAATGTATCTC-3', were synthesized, annealed, and ligated into the vector pENTER/U6 (Invitrogen). Then, the pENTER/U6-ADAM10^{shRNA} plasmid was mixed with pLenti6/BLOCK-iT-DEST (Invitrogen) for an LR reaction to generate pLenti6/U6-ADAM10^{shRNA}.

Lentivirus was produced in 293FT cells by cotransfecting pLenti6/U6-ADAM10^{shRNA} (*pLenti6/V5-DEST^{ADAM10}*, *pLenti6/V5-DEST^{GFP-L1}*, or *pLenti6/V5-DEST^{GFP-CD44}*) with the ViraPower Packaging Mix according to the manufacturer's instructions (Invitrogen). The media was changed 14 h later and the cells continued to incubate for 2 days. The media were collected and filtered through 0.45 µm sterile filters and directly added to the target cells.

Lentiviruses (1 ml) were added to 35 mm dishes seeded with AtT-20 cells; after 12 h, the media were replaced with another 1 ml of fresh lentiviruses, and the cells were incubated for another 24 h for further experiments.

HA coating and cell detachment assay

Streptococcal hyaluronan (5 mg/ml; Sigma–Aldrich) was added in a volume of 500 µl to 35 mm dishes for 36 h at 4 °C, then 500 µl of 2% BSA was added, and incubated for 12 h. The dishes were washed with F-12K medium, and AtT-20 cells were immediately seeded onto these dishes. Twenty-four hours later, shRNA transfection was performed and the cells were cultured for ~3 days. The cells were then treated with 5 µM ionomycin in a serum-free medium for 2 h. The suspended and adherent cells were collected, and the cell numbers were counted.

Migration assay

The filter inserts (6.5 mm-diameter polycarbonate filters of 8 µm pore size, Corning) were coated with 1 mg/ml hyaluronan (Sigma–Aldrich) or 20 µg/ml fibronectin (Sigma–Aldrich) overnight at 4 °C. The inserts were air-dried before the cells were added. Three days after infection, AtT-20 cells were digested and washed with FBS-free medium and then 2×10^4 cells were added to the top of the insert (in 100 µl of serum-free medium). F-12k medium (2.5% FBS and 7.5% horse serum) was added in a volume of 1 ml to the lower chamber, the filter was placed into the lower chamber, and the cells were incubated at 37 °C. After 24 h, inserts

with the cells on the topside of the filter were removed and the cells on the lower surface were stained with Diff-Quik solution. The cells were washed twice, and the cell numbers in the lower compartments of the chambers were counted using a light microscope in five defined high-power fields.

Statistical analysis

Each experiment was repeated a minimum of three times, and the results were expressed as the mean $\pm$ s.d. Statistical differences were determined using the *t*-test for the cell detachment and migration assays. The Pearson's χ^2 test was used to assess the relationship between *ADAM10*, *p-418Src*, and *p-ERK1/2* expression and each clinicopathological factor and the correlation between *p-418Src* and *p-ERK1/2* protein expression. Computations were performed using the SPSS 10.0 software (Aspire Software International, Leesburg, VA, USA). A *P* value of <0.05 was considered significant.

Results

Patient and tumor characteristics

Of the 90 patients, 64 were females and 26 were males. The tumor size was ≤ 1 cm in 29 patients, 1–3 cm in 22 patients, and ≥ 3 cm in 39 patients. Fifty-one patients showed cavernous sinus invasion, while 39 patients had noninvasive tumors. Based on the secreting group classification, 60 patients were functional and 30 were nonfunctional adenomas (Table 1). Among 60 patients with functional adenomas, there are 38 cases of GH-secreting tumors and 22 cases of PRL-secreting tumors (Table 1).

ADAM10 expression increased in high-grade pituitary adenomas and was associated with high levels of p-418Src and p-ERK1/2

Substantial previous research has indicated that the expression level of *ADAM10* increases in many cancers. We evaluated *ADAM10* expression in pituitary adenomas by immunohistochemistry and found that *ADAM10* was expressed at high levels in high-grade pituitary adenomas compared with low-grade pituitary adenomas. Because *p-418Src* and *p-ERK1/2* are involved in the cleavage of cell adhesion molecules, such as L1 (which might be driven by *ADAM10*), we also examined their expression levels in tumors from pituitary adenoma patients. High levels of expression of *p-418Src* and *p-ERK1/2* were also observed in high-grade pituitary adenomas compared with low-grade pituitary adenomas (Fig. 1). Western blotting analysis of

Figure 1 High levels of expression of *ADAM10*, *p-418Src*, and *p-ERK1/2* are present in high-grade pituitary adenomas. *ADAM10*, *p-418Src*, and *p-ERK1/2* are detected in low- and high-grade pituitary adenomas by immunohistochemistry.

the pituitary adenomas samples further confirmed the results (Fig. 2A and E).

To determine whether *ADAM10* expression correlates with tumor invasion, we analyzed the relationship between *ADAM10*, *p-418Src*, *p-ERK1/2*, and clinicopathological features in 90 pituitary adenoma patients. We subdivided the adenomas into 'high'- or 'low'-expressing *ADAM10*, full-length *CD44*, *CD44EXT*, *p-418src*, or *p-ERK1/2* according to Kirkegaard's valuation standard (Kirkegaard *et al.* 2006), and tumors were subdivided into those with high (above the median) or low (below or equal to the median) expression. We found that *ADAM10* expression correlates with the invasiveness of the tumor but has no relationship with the age or gender of the patient or the tumor size and secreting character of the tumor. The expression of *p-418Src*, but not *p-ERK1/2*, showed a similar relationship (Table 1). We also detected *ADAM10*, *p-Src*, and *p-ERK1/2* expression levels in the two subtypes of the functional pituitary adenomas respectively. The results revealed no obvious changes in the expression of three proteins between these two subtypes. We next analyzed the relationship between *Src* or *ERK1/2* activation and the protein expression of *ADAM10* in patient tumors. Our data indicated that the activation of *Src* and *ERK1/2* correlated with *ADAM10* expression (Table 2). These data suggest that *ADAM10*, together with *p-418Src* and *p-ERK1/2*, plays a role in high-grade pituitary adenomas.

Cleavage of CD44 was increased in high-grade pituitary adenomas and was associated with increased *ADAM10* expression, which might be mediated by CaM and Src signaling

To determine whether *ADAM10* expression levels were correlated with tumor invasiveness, we examined expression levels of *CD44* in normal pituitary and in low- and high-grade pituitary adenomas, which is one of the important substrates of *ADAM10* and plays the critical role in cell adhesion and migration (Gunthert *et al.* 1996, Schachner 1997, Brummendorf *et al.* 1998, Hortsch 2000, Murai *et al.* 2004). For the experiments, we chose 29 low-grade tumors with no cavernous sinus invasions and diameters below 1 cm and 39 high-grade tumors above 3 cm in diameter and with invasion in the cavernous sinus and surrounding the unilateral carotid artery.

Our data showed that Pro-*ADAM10* expression was increased in high-grade pituitary adenomas, as was the cleavage of *CD44* (Fig. 2A). However, no obvious changes were observed in normal pituitary and low-grade pituitary adenomas (Fig. 2B). And the increased cleavage of *CD44* correlated with high expression of Pro-*ADAM10* (Table 3). These data suggested that *ADAM10* exerts its function in high-grade pituitary adenomas, probably through *CD44*.

The calcium-dependent molecule CaM has been reported to interact with *ADAM10* and suppress the

Figure 2 ADAM10/CaM interaction is reduced, and Src/Shc interaction is increased in high-grade pituitary adenomas expressing high level of ADAM10, along with increased cleavage of CD44. (A) Typical examples of western blots for Pro-ADAM10, full-length CD44, and CD44EXT in pituitary adenomas. Tubulin is used as the internal control. Histogram of band densities comparing the amounts of the three proteins in the two different grade tumors, calculated from western blot experiments of 29 low-grade tumors and 39 high-grade tumors. (B) The protein expression levels of CD44EXT and full-length CD44 in normal pituitary tissues and pituitary adenomas. Tubulin is used as the internal control. (C) Pro-ADAM10 lost the interaction with CaM in high-grade pituitary adenomas, along with increased CD44EXT. Whole-tissue lysates were immunoprecipitated with anti-ADAM10 antibody and analyzed by immunoblotting with anti-CaM antibody and anti-ADAM10 antibody. Separate aliquots of the lysates were immunoblotted with the indicated antibodies. The histogram shows the relative amounts of CaM immunoprecipitated with Pro-ADAM10 in high-grade pituitary adenomas and low-grade pituitary adenomas. (D) Increased Shc interaction with Src in high-grade pituitary adenomas. Whole-tissue lysates were immunoprecipitated with anti-Src antibody and analyzed by immunoblotting with anti-Shc antibody and anti-Src antibody. Separate aliquots of the lysates were immunoblotted with the indicated antibodies. The histogram shows the relative amounts of Shc immunoprecipitated with Src in high-grade pituitary adenomas and low-grade pituitary adenomas. (E) The protein expression levels of shed form L1, p-ERK1/2, and p-418Src in high-grade pituitary adenomas and low-grade pituitary adenomas. Tubulin is used as the internal control. Histogram of band densities comparing the amounts of the three proteins in the two different grades of tumors.

ADAM10 cleavage function (Nagano *et al.* 2004). Therefore, we next examined the endogenous protein interaction between Pro-ADAM10 and CaM in low- and high-grade pituitary adenomas. A co-immunoprecipitation assay indicated that the interaction between Pro-ADAM10 and CaM is almost abolished in high-grade pituitary adenomas, which is associated with increased cleavage of CD44 (Fig. 2C). However, several groups have demonstrated that the interaction between Src and Shc can promote the activation of ERK1/2 (Barberis *et al.* 2000, Bulayeva & Watson 2004, Kleiner *et al.* 2007), which has been reported to be a regulator of the ADAM10-mediated cleavage of L1 (Gutwein *et al.* 2000). Then, we next examined the interaction between Src and Shc in low- and high-grade pituitary adenomas using co-immunoprecipitation and found that the interaction between Src and Shc was increased in high-grade pituitary adenomas, which indirectly implied the increased L1 cleavage (Fig. 2D). The western blotting experiment result confirmed that cleavage of L1 was increased in high-grade pituitary adenomas (Fig. 2E). These data suggested that Pro-ADAM10 expression was increased in high-grade pituitary adenomas with increased cleavage of ADAM10 and CD44 and increased interaction between Src and Shc.

ADAM10-induced L1 cleavage is mediated by Src/ERK1/2 signaling

L1 has important functions in cell adhesion and migration and can enhance invasiveness and metastasis (Schachner 1997, Brummendorf *et al.* 1998, Hortsch 2000). The overexpression of L1 has been observed in many tumor types (Ohnishi *et al.* 1998, Meckersheimer *et al.* 2001, Thies *et al.* 2002). We also found that cleavage of L1 was increased in high-grade pituitary adenomas (Fig. 2E). To investigate the details of how ADAM10 regulates L1, we took advantage of the pituitary adenoma cell line AtT-20 to detect L1 cleavage. PMA, which is well known to induce L1 cleavage, was introduced into our assay. Our data showed that the

Table 3 The relationship between CD44 or CD44 cleavage and the protein expression of ADAM10 in 39 high-grade tumor specimens and 29 low-grade tumor specimens

	Full-length CD44		P value	CD44EXT		P value
	High	Low		High	Low	
Protein expression ADAM10						
High	13	22	0.476	25	10	0.602
Low	19	14		15	18	

phosphorylation of 418Src increases significantly in PMA-induced samples. This phenomenon is enhanced when ADAM10 is overexpressed. Moreover, when we pretreated the cells with the Src inhibitor PP2, the cleavage of L1 induced by PMA and the overexpression of ADAM10 were abolished (Fig. 3A). The opposite result was observed upon knockdown of the endogenous *Adam10* expression by RNA interference (Fig. 3B). Similar results were also observed with the MEK inhibitor PD98059 (Fig. 3D). These data indicated that the cleavage of L1 induced by PMA is regulated by ADAM10. Src and ERK1/2 signaling participates in this process. Moreover, upon PMA stimulation, increased interaction between Src and Shc was observed (Fig. 3E). These results are consistent with those found in high-grade pituitary adenoma patients (Figs 1 and 2D). We also performed immunocytochemistry to detect the influence of ADAM10 on L1 cleavage. We found that L1 localizes on the cell membrane of unstimulated cells, and membrane L1 decreases with PMA treatment. However, upon *Adam10* knockdown, the membrane L1 did not decrease with PMA treatment, indicating that ADAM10 mediates L1 cleavage induced by PMA (Fig. 3F). Taken together, these data indicated that ADAM10 promotes L1 cleavage induced by PMA, and this process is at least partially mediated by the Src and ERK1/2 pathways.

Calcium signals enhance ADAM10-induced CD44 cleavage

CD44, another substrate of ADAM10, is a cell surface adhesion molecule that plays an important role in tumor invasion and metastasis (Gunthert *et al.* 1996, Murai *et al.* 2004) through the cleavage of its ectodomain. Calcium signals have been reported to activate the ADAM10-mediated cleavage of CD44 (Nagano *et al.* 2004). Our data also showed that ADAM10 activation is related to CaM, a calcium regulator protein. Here, we treated the cells by mechanical scraping, which can lead to the activation

Table 2 The relationship between Src or ERK activation and the protein expression of ADAM10 in tumor specimens from 90 patients

	p-418Src		P value	p-ERK		P value
	High	Low		High	Low	
Protein expression ADAM10						
High	30	16	0.036	28	18	0.035
Low	19	25		17	27

Figure 3 Src and ERK1/2 are involved in ADAM10-mediated L1 cleavage in PMA-treated AtT-20 cells. (A) PP2, a Src inhibitor, blocks the cleavage of L1 induced by PMA in ADAM10 overexpressing AtT-20 cells. AtT-20 cells were transfected with ADAM10 and treated as indicated earlier. Protein levels in the whole-cell extract were analyzed by western blotting. (B) The absence of ADAM10 blocks the cleavage of L1 induced by PMA in AtT-20 cells. AtT-20 cells were transfected with shADAM10 and treated as indicated earlier. (C) shADAM10 effectively reduces the expression of ADAM10. After transfection with shADAM10, the total cell lysate of AtT-20 cells was analyzed. (D) PD98059, an MEK inhibitor, further reduced the cleavage of L1 induced by PMA in shADAM10-treated AtT-20 cells. AtT-20 cells were transfected with *ADAM10* expression plasmid or shADAM10 and treated as indicated earlier. (E) Interaction between Shc and Src was increased in PMA-treated AtT-20 cells. Whole-tissue lysates were immunoprecipitated with anti-Src antibody and analyzed by immunoblotting with the indicated antibodies. (F) Subcellular location of L1 and ADAM10 in AtT-20 cells after PMA treatment. After transfection with the indicated plasmids and PMA treatment, the cells were observed by confocal microscopy.

of the cellular calcium signal (Nagano *et al.* 2004). After treatment, CD44 cleavage increased, while the expression of full-length CD44 decreased. This phenomenon could be blocked by the knockdown of *Adam10* expression (Fig. 4A). Similar results were also observed with ionomycin treatment (Fig. 4B). Immunocytochemistry was also performed to detect the influence of ADAM10 on CD44 cleavage. With ionomycin treatment, less CD44 localized to the cell membrane compared with normal cells. Upon *Adam10* knockdown, the membrane CD44 showed no difference compared with normal cells (Fig. 4D). These results suggested that ADAM10 regulates CD44 cleavage in calcium flux-stimulated pituitary adenoma cells. We then pretreated the cells with EGTA to chelate the calcium before ionomycin was added. We found that ADAM10 activation was blocked by EGTA (Fig. 4C), which suggests that calcium flux regulates ADAM10

activation. To further investigate how calcium flux regulates ADAM10 activation, we examined the interaction between Pro-ADAM10 and CaM. We observed that the interaction between Pro-ADAM10 and CaM decreases in high-grade pituitary adenomas, which is associated with increased CD44 cleavage (Fig. 2C). In the pituitary adenoma cell line treated by mechanical scraping or treated with the CaM inhibitor TFP, the interaction between CaM and ADAM10 was decreased significantly (Fig. 5A). CaM has been reported to suppress ADAM10 activation by protein interactions. Our results suggested that extracellular calcium flux decreases the interaction between Pro-ADAM10 and CaM. Taken together, our data indicated that ADAM10-mediated cleavage of CD44 depends on the cellular calcium signal and is at least partially mediated by the release of the CaM-mediated suppression of ADAM10.

Figure 4 Calcium upregulates the active form of ADAM10, which increases the number of detached cells and causes CD44 cleavage. (A) The absence of ADAM10 blocks the cleavage of CD44 induced by mechanical scraping in AtT-20 cells. AtT-20 cells were transfected with the indicated shRNA and treated as indicated earlier. Protein levels in whole-cell extract were analyzed by western blotting. (B) The absence of ADAM10 blocks the cleavage of CD44 induced by ionomycin in AtT-20 cells. AtT-20 cells transfected with the indicated plasmids were treated with or without 5 μ M ionomycin for 1 h. Protein levels in whole-cell extract were analyzed by western blotting. (C) EGTA prevents Pro-ADAM10 from becoming active ADAM10 in ionomycin-treated AtT-20 cells. AtT-20 cells were treated with or without 5 μ M ionomycin for 1 h in the absence or presence of 10 mM EGTA. Protein levels in whole-cell extract were analyzed by western blotting. (D) Subcellular localization of CD44 and ADAM10 in AtT-20 cells after ionomycin treatment. AtT-20 cells transfected with the indicated plasmids were treated with or without 5 μ M ionomycin for 1 h. The cells were then examined by confocal microscopy. (E) The absence of ADAM10 reduces the detachment of AtT-20 cells. * $P < 0.05$.

ADAM10 plays a role in regulating cell adhesion and migration, which is mediated by CD44

We also examined whether ADAM10 is involved in the modulation of cell adhesion and migration. We used shRNA to knockdown *Adam10* expression in pituitary adenoma cells and then compared the percentage of detached cells. The results indicated that cell adhesion increases significantly when *ADAM10* expression is impaired (Fig. 4E). Next, we wanted to investigate whether the ADAM10-mediated regulation of cell adhesion and migration is dependent on cleavage of CD44. As CD44 can interact with hyaluronic acid, but not with fibronectin, we examined the cell migration ability under both hyaluronic acid- and fibronectin-coated conditions upon *Adam10* knockdown. We found that knockdown of *Adam10* greatly repressed cell migration with hyaluronan but had no effect on cell migration with fibronectin (Fig. 5B and C). Importantly, overexpression of CD44EXT reversed the effect of *Adam10* knockdown on cell migration. These results

suggested that CD44 mediates the function of ADAM10 in the regulation of pituitary adenoma cell adhesion and migration.

Discussion

ADAM10 is a membrane-anchored member of the ADAM family and is ubiquitously expressed throughout most tissues, including neurons, the vasculature, leukocytes, and tumor cells (Moss *et al.* 2008). ADAM10 has been reported to be crucial for developmental processes, based on the data from mice with impaired *Adam10* expression (Hartmann *et al.* 2002). However, the most important function of ADAM10 is still considered to be its proteinase activity. A consensus sequence of HEXGHXXGXXHD is located within the active sites in the metalloproteinase domain of the proteinase-type ADAM molecules. This sequence is also present in the catalytic metalloproteinase domain of

Figure 5 ADAM10 mediates AtT-20 cell migration in a CD44-dependent manner. (A) The interaction of CaM with Pro-ADAM10 decreases after mechanical scraping or TFP treatment. (B) CD44EXT overexpression reversed the effects of *Adam10* knockdown on migration of AtT-20 cells under the hyaluronic acid-coated condition. (C) Quantitative measurement of the migration of AtT-20 cells. Bars show mean $\pm$ s.d. * $P < 0.01$. All experiments were repeated three times.

MMP family members (Mochizuki & Okada 2007). Through its proteinase activity, ADAM10 can process several critical signaling molecules associated with cancer development and tumor progression, including Her2, CD44, and L1 (Moss *et al.* 2008). These molecules play important roles in cell adhesion and migration, which are widely known to be the key steps in tumor invasion. Overexpression of ADAM10 has been found in oral cavity (Ko *et al.* 2007), stomach (Yoshimura *et al.* 2002), ovary, uterine (Fogel *et al.* 2003), colon (Gavert *et al.* 2005), leukemia (Wu *et al.* 1997), and prostate (McCulloch *et al.* 2004) carcinoma patients. ADAM10 has been reported to participate in the proliferation of some tumor cells; overexpression of *ADAM10* can promote cell proliferation, and blocking *ADAM10* expression reduces cell growth (Carl-McGrath *et al.* 2005, Ko *et al.* 2007). Some studies have indicated that the stimulation of proliferation mediated by ADAMs is involved in early tumorigenesis, possibly by releasing growth factors or allowing the cells to escape immune surveillance (Sanderson *et al.* 2006, Moss *et al.* 2008). However, more details regarding the functions of ADAM10 in cancers remain to be elucidated.

In this study, we first identified that *ADAM10* expression is higher in high-grade pituitary adenoma

patients and is associated with the cleavage of L1 and CD44. In addition, we found that two important signaling molecules, CaM and Src, present abnormal interactions with ADAM10 and Shc respectively. In high-grade pituitary adenomas or a stimulated pituitary adenoma cell line, further investigation indicated that L1 and CD44 are shed by ADAM10 through different signals; CaM releases its suppression of ADAM10 to promote CD44 cleavage, whereas increased Src/ERK1/2 signal leads to increased L1 shedding. These results demonstrate that adhesion molecules are involved in promoting tumor invasiveness through ADAM10.

L1 can be cleaved constitutively at the cell surface, and this process is enhanced by stimulation of PMA. Overexpression of L1 has been found in many types of tumors and is associated with enhanced invasiveness and metastasis (Ohnishi *et al.* 1998, Mechtersheimer *et al.* 2001, Thies *et al.* 2002). Interestingly, ADAM10 is overexpressed at the invasive edge of many cancers, where L1 overexpression is also observed (Fogel *et al.* 2003, Gavert *et al.* 2005). Soluble L1 has been shown to enhance cell migration *in vitro*, which might be mediated by binding to integrins (Mechtersheimer *et al.* 2001). Metalloprotease inhibitors can block cell

migration mediated by L1. In neuronal cells, L1-dependent adhesion and haptotactic migration are blocked when L1 shedding is inhibited (Maretzky *et al.* 2005). Some ADAMs contain SH3-binding sites in their cytoplasmic tail. Therefore, a series of SH3-domain-containing signaling factors might be activated by ADAMs, such as Src and its downstream MAPK pathway (Barberis *et al.* 2000, Seals & Courtneidge 2003, Kleiner *et al.* 2007). Our data show that the activation of Src and ERK1/2 correlates with *ADAM10* expression in high-grade pituitary adenoma patients. ADAM10-mediated L1 cleavage is blocked by the inhibitors of Src and MEK. Therefore, one might speculate that these signaling molecules are activated in high-grade pituitary adenoma patients and modify the intracytoplasmic tail domains of ADAMs, which leads to conformational changes and enables them to interact with specific substrates (Mochizuki & Okada 2007). However, these possibilities remain to be elucidated by further studies.

CD44 also mediates tumor cell migration and invasion, and its function might be regulated by ADAM10 on several different levels (Nagano *et al.* 2004). ADAM10 contributes to the production of soluble CD44 by ectodomain cleavage. This process is enhanced by calcium ionophores. Soluble CD44 has been reported in various tumor cells, and in many cases this phenomenon correlates with decreased cell adhesion and increased detachment of the cells from the ECM (Nagano *et al.* 2004, Moss *et al.* 2008). An explanation for this correlation is that the shedding of CD44 reduces the density of the surface molecule and soluble CD44 acts as an antagonist of transmembrane CD44 by binding to extracellular hyaluronan and reducing cell adhesion (Okamoto *et al.* 2002, Murakami *et al.* 2003). Another consequence of ADAM10-dependent CD44 shedding is the release of a cytosolic fragment, which regulates gene expression, including the expression of CD44 itself (Nagano *et al.* 2004). Thus, this type of feedback for CD44 expression may further amplify CD44 activity in cancer cells.

Calcium is considered to contribute to increased cell mobility through blocking cell–matrix adhesion. Recently, many studies have indicated that extracellular Ca^{2+} influx promotes the shedding of many proteins, including CD44 and E-cadherin (Ito *et al.* 1999). Ca^{2+} -influx-induced ectodomain cleavage is mediated by ADAM10 metalloproteinases. CaM, which suppresses ADAM10 activity through protein interaction (Nagano *et al.* 2004), dissociates from ADAM10 under calcium overload and subsequently enhances CD44 shedding and CD44-dependent cell migration.

Our study demonstrates the molecular mechanism of how ADAM10 participates in the invasiveness of pituitary adenomas through the regulation of adhesion factors. However, distinct mechanisms are involved in this process. The changes in some proinflammatory

factors, such as tumor necrosis factor- α , epidermal growth factor, Fas ligand, cytokines, and chemokines, require further investigation. While features, such as necrosis, mitosis, and hemorrhage, are unreliable markers of the invasiveness of these tumors, ADAM10 may be a useful molecule for determining invasiveness. Notably, an ADAM10 inhibitor could potentially represent a beneficial method to treat cancers. Recently, more attention has been focused on ADAM10 inhibitors because of their ability to inhibit ErbB signaling (Moss *et al.* 2008). Therefore, the development of specific ADAM10 inhibitors that target pathogenic shedding events will be a promising strategy in cancer therapy.

MMPs are important in digestion of the ECM during tumor invasion and metastasis. Many researches have illustrated that MMP2 (Liu *et al.* 2005) and MMP9 (Gong *et al.* 2008) play important roles in pituitary tumor invasion. Both MMP2 and MMP9 are type IV collagenases, which can break down basement membrane, in particular, cleaving type IV collagen (Kawamoto *et al.* 1996). Like MMP2 and MMP9, ADAM10 is also a type IV collagenase (Millichip *et al.* 1998). As we mentioned earlier, *ADAM10* expression increased in various types of cancer (Wu *et al.* 1997, Yoshimura *et al.* 2002, Fogel *et al.* 2003, McCulloch *et al.* 2004, Gavert *et al.* 2005, Ko *et al.* 2007). Here, we found that *ADAM10* expression is enhanced in pituitary adenomas and associated with the invasiveness of pituitary adenomas, and further investigation revealed that ADAM10 facilitated cell migration through modulation of CD44 and L1 cleavage. These results discovered a new mechanism of pituitary adenoma invasiveness and indicated new potential therapeutic targets for pituitary adenomas.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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