ADAM10 Is the Constitutive Functional Sheddase of CD44 in Human Melanoma Cells

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CD44 proteins are cell surface receptors for hyaluronic acid (HA), a component of the extracellular matrix that has multiple effects on cell behavior. CD44 can be shed from the cell surface by proteolytic cleavage. The resulting soluble form can interfere with the interaction between HA and membrane-bound CD44. Soluble CD44 can abolish the cell proliferation-promoting effect of HA on melanoma cell lines, suggesting that a better understanding of the shedding process might identify ways of blocking tumor cell proliferation. ADAM10, ADAM17, and MMP14 have previously been implicated in the shedding of CD44 from various tumor cells. Using immunohistochemistry we demonstrate that ADAM10 and ADAM17 but not MMP14 are significantly expressed on melanoma cells in histological sections. In human melanoma cell lines expression of these proteases could be blocked by transfection with appropriate siRNAs. However, only blocking of ADAM10 expression led to decreased shedding of CD44. In parallel, cell proliferation was promoted. Confocal microscopy demonstrated that ADAM10 and CD44 colocalize on the cell surface. We conclude that ADAM10 is the predominant protease involved in the constitutive shedding of endogenous CD44 from melanoma cells, and that enhancement of ADAM10 activity could be an approach to decrease the proliferation of melanoma cells.

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INTRODUCTION

CD44 constitutes a family of transmembrane glycoproteins that are generated by alternative splicing and glycosylation, resulting in a variety of related proteins that range in size from 85 to 200 kDa and that regulate a number of aspects of cell behavior (Naor *et al.*, 1997). This regulatory activity is mediated in part through the ability of CD44 proteins to interact with hyaluronic acid (HA; Aruffo *et al.*, 1990), a major component of the extracellular matrix. The interaction between HA and CD44 has multiple effects on cell proliferation, cell motility, and cell survival for both malignant and nonmalignant cells (Goebeler *et al.*, 1996; Naor *et al.*, 1997; Morrison *et al.*, 2001; Ahrens *et al.*, 2001a, b).

CD44s, the standard CD44 isoform that lacks epitopes encoded by the variant exons, is 85–90 kDa in size and binds strongly to HA (Stamenkovic *et al.*, 1991). It is widely expressed in different tissues and cell types, and furthermore its expression is enhanced in a variety of malignant tumors (Dietrich *et al.*, 1997; Naor *et al.*, 1997). Previously we have shown that CD44s is the principal HA receptor expressed on the surface of human melanoma cells (MM; Ahrens *et al.*, 2001a, b). Moreover, we could demonstrate that HA promotes the proliferation of MM cells and that this is fully dependent on the interaction between CD44 and HA (Ahrens *et al.*, 2001a).

In addition to binding to HA, CD44 can also serve as an organizing platform for other proteins. For example, it directs proteases such as MMP9 (Yu and Stamenkovic, 1999), MMP7 (Yu *et al.*, 2002), and MMP14 (Mori *et al.*, 2002) to the leading edge of migrating cells. CD44 also functionally interacts with growth factors (Bennett *et al.*, 1995) and growth factor receptors such as EGFR (Wobus *et al.*, 2002) and PDGFβR (Li *et al.*, 2006), and thereby regulates cell proliferation. On the cytoplasmic side, the tightly regulated intracellular interaction between CD44 and Ezrin or NF2 proteins can be pivotal in determining whether cells proliferate or apoptose (Morrison *et al.*, 2001).

CD44 can be shed from the cell surface by proteolytic cleavage. Increased plasma levels of soluble CD44 (solCD44) have been observed in different malignancies (Sheen-Chen *et al.*, 1999; Yamane *et al.*, 1999; Stickeler *et al.*, 2000; Yasasever *et al.*, 2000; Molica *et al.*, 2001; Niitsu and Iijima, 2002). Furthermore, studies with melanoma cell lines have demonstrated that the cell proliferation-promoting effect of

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Abbreviations: ADAM, a disintegrin and metalloproteinase domain; ERM, Ezrin-Radixin-Moesin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, hyaluronic acid; MM, melanoma cell; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline; solCD44, soluble CD44

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HA can be abolished *in vitro* and *in vivo* by solCD44 (Ahrens *et al.*, 2001b). This blocking of HA-induced proliferation was strongly dependent on the ability of solCD44 to bind to HA (Ahrens *et al.*, 2001b), suggesting that solCD44 competes with cell surface-bound CD44 for HA binding. Other groups have found that shedding of the ectodomain of CD44 plays a critical role in tumor cell migration (Goebeler *et al.*, 1996; Nagano *et al.*, 2004). In addition, migrating tumor cells traversing the extracellular matrix have been shown to release membrane vesicles containing integrins, CD44, and cytoplasma (Mayer *et al.*, 2004), possibly part of the detachment strategy that increases mobility of the tumor cells. External cleavage of the CD44 ectodomain can be followed by a second intramembraneous cleavage by a γ-secretase that releases the cytoplasmic domain of CD44 from the membrane into the cytoplasm (Nakamura *et al.*, 2004). This step perturbs the interaction between CD44 and attached protein complexes, proteins of the cytoskeleton and growth factor signaling cascades. Additionally, the cytoplasmic domain can enter the nucleus and act as a transcription factor, changing the gene expression pattern of the cells (Nagano and Saya, 2004; Nakamura *et al.*, 2004). CD44 shedding may thus have important effects on tumor cell behavior.

Several proteases have been implicated in the cleavage of CD44 from the surface of tumor cells, the most important being MMP14, ADAM-10, and ADAM-17. The involvement of MMP14 in the shedding of the CD44 ectodomain has been demonstrated in the fibrosarcoma HT1080 and gastric carcinoma MKN-28 cell lines (Ueda *et al.*, 2003). In the context of MM, transfection of the melanoma cell line A375 with CD44 and MMP14 allowed the ability of MMP14 to shed CD44 from the cell membrane to be demonstrated (Nakamura *et al.*, 2004), but the relevance of these findings to the constitutive cleavage of endogenous CD44 in MM remains unclear. Furthermore, inhibitor studies indicate that CD44 shedding is not completely sensitive to matrix metalloproteinase (MMP) inhibitors, indicating that another type of protease must contribute to CD44 cleavage from the membrane (Okamoto *et al.*, 1999; Gasbarri *et al.*, 2003). A number of observations suggest that members of the ADAMs (a disintegrin and metalloproteinase domain) family of metalloproteinases that have important functions in extracellular matrix processing, organogenesis, and hemostasis (Reiss *et al.*, 2006) are involved. ADAMs have been suggested to be involved in the release of CD44 from the cell surface of fibroblasts (Shi *et al.*, 2001) and tumor cells (Nakamura *et al.*, 2004). In human glioma cells there is evidence that ADAM10 and ADAM17 mediate the effects of agents that stimulate the shedding of CD44, but are not involved in constitutive CD44 shedding (Nagano *et al.*, 2004).

Here we report expression of ADAM10 and ADAM17 and to a lesser extent of MMP14 in human melanoma biopsies, as well as in cultured CD44-positive human melanoma cells. Functional studies employing specific inhibitors and siRNA techniques revealed the involvement of ADAM10, but not of MMP14 or ADAM17, in the constitutive shedding of native CD44 from human melanoma cells. Confocal microscopy revealed a colocalization of ADAM-10 and CD44 on the surface of melanoma cells. Moreover, ADAM10 knockdown augmented HA-induced proliferation of the cells in a CD44-dependent manner. These data are consistent with the notion that ADAM10 is critically involved in the constitutive shedding of the CD44 ectodomain from human melanoma cells to generate solCD44, a process that has impact on melanoma cell proliferation.

RESULTS

The candidate CD44 sheddases ADAM-10, ADAM-17, and MMP-14 are expressed in malignant melanoma cells *in vivo* and *in vitro*

MMP14, ADAM-10, and ADAM-17 have all been implicated in the shedding of the CD44 ectodomain, yet their relative expression in human MM remains to be determined. We therefore investigated the expression of these proteases in healthy human skin and in a panel of malignant melanomas of various types (superficial spreading melanoma $n=10$, nodular melanoma $n=8$, skin metastases $n=5$) using established antibodies. Both ADAM-10 and ADAM-17 were expressed by the tumor cells in 20/23 samples (Figure 1a and b). MMP14 was also expressed on melanoma cells in 20/23 samples, albeit at a weaker staining intensity (Figure 1c). Note that CD44 is also prominently expressed by the melanoma cells (Figure 1d). In normal skin, all three proteases were detected in the epidermis and to a lesser extent in dermal fibroblasts (Figure 1e–g). Based on these *in vivo* expression data it appears that all of the three proteases implicated in the shedding of CD44 are expressed on tumor cells in tissue samples of melanoma.

To be able to perform functional experiments to address the relative roles of MMP14, ADAM-10, and ADAM-17 in the shedding of CD44 from MM, we next examined the level of CD44 shedding in a panel of six MM cell lines. Of these, Bro and HT144 showed the highest levels of constitutive endogenous CD44 shedding (data not shown) and were therefore selected for further studies. Both cell lines expressed ADAM10, ADAM17, and MMP14 mRNAs, with ADAM10 expression being highest when normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in qPCR experiments (Figure 2a). In Western blot analysis, both the inactive 90/125 kDa pro-forms and the 75/100 kDa mature forms of ADAM 10 and ADAM 17 could be detected. MMP14 was detectable by western blot analysis as a single band of about 65 kDa (Figure 2b). MMP14 activity was detectable in both cell lines using a MMP14 activity ELISA (Figure 2c).

CD44 shedding from melanoma cells is sensitive to ADAM inhibitors

To investigate the possible contribution of ADAM10 and ADAM17 to the shedding of CD44 in MM cells, we first employed two previously described ADAM-specific protease inhibitors. One inhibitor, namely GI254023X, preferentially blocks ADAM10 with a 100-fold higher specificity toward ADAM10 than to other proteases, whereas the other, designated GW280264X, blocks both ADAM10 and

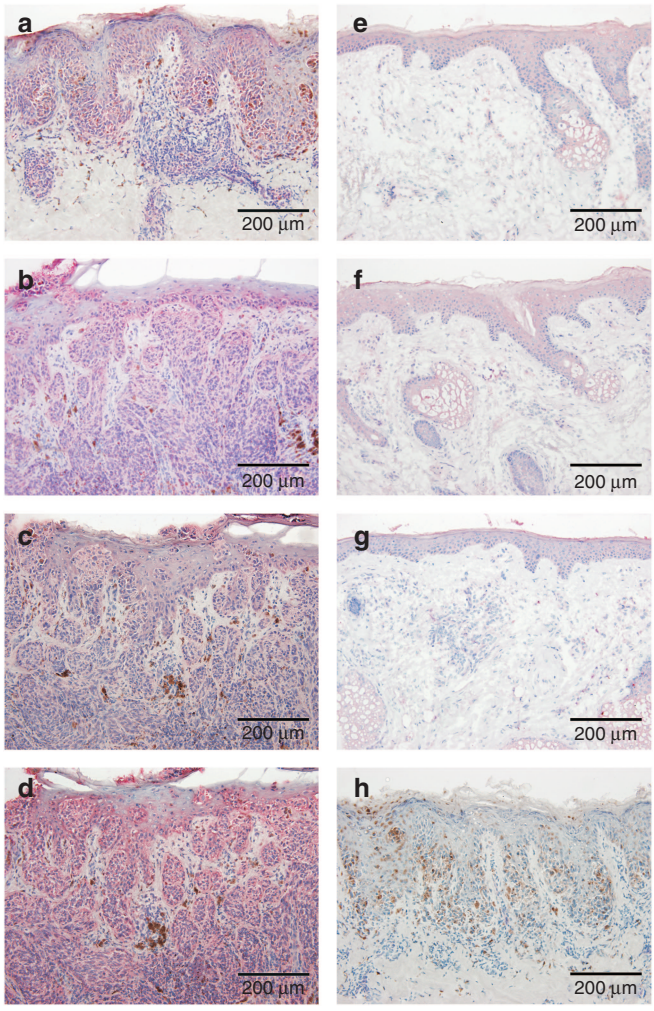

Figure 1. Candidate sheddases as well as CD44 are expressed in malignant melanoma. A representative example of a superficially spreading melanoma (out of $n = 10$) is shown here, with prominent ADAM10 expression in tumor cells (**a**). ADAM17 (**b**) shows a similar expression pattern but MMP14 (**c**) shows a different signal distribution (red color). CD44 (**d**) is expressed on the tumor cells as well. In normal skin ADAM10 (**e**), ADAM17 (**f**) and MMP14 (**g**) are expressed at lower intensity and prevail in the epidermis. Specificity of the staining was checked using an isotype control antibody (**h**). Scale bar = 200 μm.

ADAM17 with comparable IC_{50} values (Hundhausen *et al.*, 2003; Ludwig *et al.*, 2005). In cleavage assays we found by ELISA that both inhibitors reduced the shedding of CD44 in the MM cell lines (Figure 3) without exerting any toxic effects, as assessed by trypan blue exclusion (data not shown). To our knowledge this is the first evidence that that ADAM10 rather than ADAM17 is involved in the constitutive shedding of CD44 in MM cells.

Knockdown of ADAM10 but not of ADAM17 effectively reduces CD44 shedding in melanoma cells

To confirm the findings obtained with the inhibitors that suggested that ADAM10 rather than ADAM17 might be the relevant CD44 sheddase in MM, we used a RNA knockdown approach. The melanoma cell lines Bro and HT144 were transfected with stealth siRNAs (Invitrogen, Karlsruhe, Germany) specific for either ADAM10 or ADAM17 and subsequently mRNA and protein levels for ADAM10 and ADAM17 were determined. Specificity of the knockdown was confirmed using scrambled control siRNAs and cross detection of ADAM10 mRNA expression in ADAM17 silenced cells and vice versa (data not shown). All six different siRNAs specific for ADAM10 or for ADAM17 specifically reduced the respective mRNA levels over a time period from 24 to 96 hours after transfection. Likewise, ADAM10 and ADAM17 protein levels were effectively decreased from 48 until 96 hours after transfection by the respective siRNAs (Figures 4a, b, 5a and b) compared to transfection of MM cells with scrambled control siRNAs.

We then assessed the effect of knockdown of ADAM 10 and ADAM17 on the shedding of CD44 by measuring the concentrations of soluble CD44 in the culture supernatants of siRNA-transfected melanoma cells. Importantly, knockdown of ADAM10 (Figure 4c) but not of ADAM17 (Figure 5c) effectively blocked the shedding CD44 into the culture supernatant. However, decreased shedding was not accompanied by changed expression of CD44 on the cell surface as assayed by flow cytometry (Figure S1) and Western blot (data not shown). These data, together with the inhibitor studies shown in Figure 3, provide strong evidence that ADAM10 is crucially involved in the CD44 shedding process in human melanoma cells.

MMP14 is not involved in constitutive CD44 shedding

To date, only indirect evidence has suggested a role for MMP14 in the shedding of CD44 in MM cells (Nakamura *et al.*, 2004). We found little MMP14 expression in human melanomas using immunohistochemistry (Figure 1). Furthermore, although MMP14 mRNA and protein were detected in Bro and HT144 cell lines, MMP14 was expressed at substantially lower amounts compared to ADAM10 in Bro and HT144 cells, consistent with the finding of low MMP14 activity in melanoma cells using functional assays (Figure 2). To test the relative importance of MMP14 in CD44 shedding from melanoma cells, we again chose the siRNA approach.

Sequence specific blocking of mRNA transcription led to decreased MMP14 mRNA and protein expression levels in the transfected cells (Figure 6a and b). Importantly, efficient blocking of MMP14 expression did not change the release of CD44 from the transfected cell lines (Figure 6c). Parallel transfection of the cells with ADAM10-specific siRNA, and cotransfection of an MMP14-siRNA and the ADAM10-specific siRNA proved the specificity of the RNA knockdown and furthermore demonstrated that only ADAM10-specific siRNA treatment blocks CD44 shedding in the MM cells (Figure 6a and c). Importantly, cotransfection of ADAM10- and MMP14-specific siRNAs did not further decrease CD44 shedding compared to transfection with ADAM10-specific siRNAs alone (Figure 6c).

Together, these data demonstrate that ADAM10, but not ADAM17 or MMP 14, is the relevant protease involved in the endogenous shedding of CD44 from human melanoma cells.

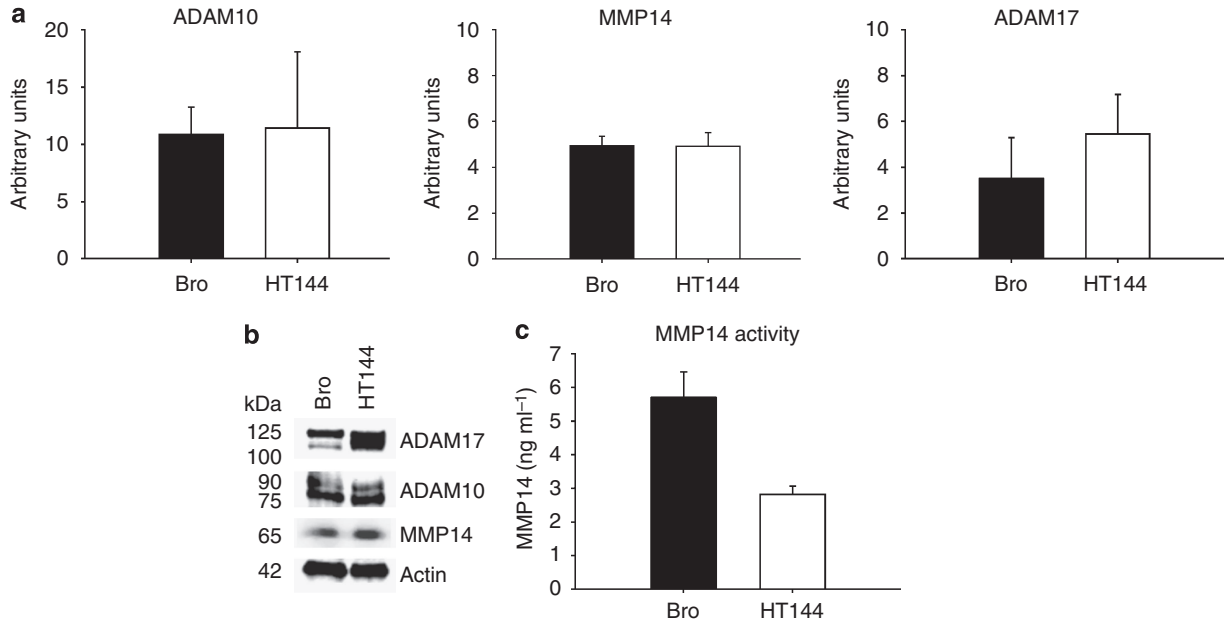

Figure 2. MM cell lines express ADAM10, ADAM17 and MMP14. Subconfluent cultures were analyzed by quantitative RT-PCR (**a**), and immunoblotting (**b**). MMP14 activity was determined by ELISA (GE Healthcare, Munich, Germany) (**c**). A representative Western blot is shown in (**b**). The quantitative RT-PCR expression data in (**a**) were obtained from three independent experiments.

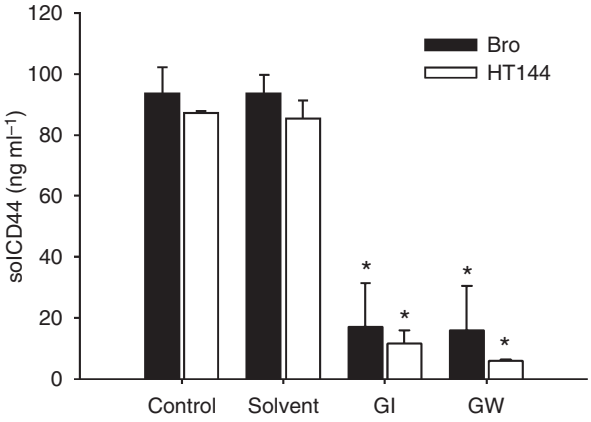

Figure 3. CD44 shedding is sensitive to ADAM-specific inhibitors. Bro and HT144 cells were seeded into 6-well plates at 2×10^5 cells per well. To synchronize the cells they were incubated overnight with medium containing 1% FCS. The cells were incubated with complete medium or with medium containing GI254023X (GI, 5 μM) or GW280264X (GW, 5 μM) for 24 hours. After removal of the supernatant the cells were detached and counted. No toxic effect of the inhibitors on the cells was observed, as assessed by trypan blue exclusion. The cell-free supernatant was assessed for solCD44 levels using an ELISA. The CD44 content (ng ml^{-1}) was normalized to 5×10^5 cells. The mean and standard error from 3 independent experiments is presented. *$P<0.005$.

ADAM10 and CD44 colocalize, whereas ADAM17 and CD44 do not colocalize in Bro MM cells

To further substantiate the notion that ADAM10 expression is involved in the shedding of CD44 from MM cells, we used immunofluorescense techniques to determine whether CD44 and ADAM10 or ADAM17 colocalize on the cell membrane of MM cells. Confocal laser scanning microscopy revealed that CD44 and ADAM10 proteins could be found at the cell membrane, and furthermore indicated that these two proteins colocalize within the cell membrane (Figure 7a). These data therefore indicate that ADAM10 and CD44 can be found together in the same cellular compartment, providing the necessary spatial proximity of the proteins for ADAM10 to be directly involved in CD44 cleavage. Conversely, no colocalization of ADAM17 and CD44 could be detected (Figure 7b) indicating that there is no constitutive interaction between CD44 and ADAM17. These data could be also confirmed for the melanoma cell line HT144 (data not shown; see Figure S3).

ADAM10 knockdown stimulates melanoma cell proliferation in a CD44-dependent manner

In a first attempt to investigate the functional relevance of constitutive ADAM10 expression in melanoma cells we examined its effect on tumor cell proliferation. As we had previously shown that solCD44 reduces the proliferative capacity of melanoma cells both *in vitro* and *in vivo* (Ahrens *et al.*, 2001b), we examined whether the blocking of CD44 shedding by ADAM10-knockdown changes the proliferation rate of melanoma cells. For this purpose MM cells were transfected with ADAM10-specific siRNA. In addition, we silenced CD44 expression in combination with ADAM10, which effectively abolished CD44 mRNA levels and CD44 shedding as shown by qRT-PCR (data not shown) or solCD44 ELISA (Figure 8b). First we show that ADAM10 silencing significantly enhanced melanoma cell proliferation (Figure 8a) in the presence of coated or soluble HA. Importantly, simultaneous knockdown of CD44 abolished the increased proliferative response induced by ADAM10 silencing (Figure 8a). Further corroborative evidence for the notion that silencing of ADAM10 stimulates cell proliferation in a

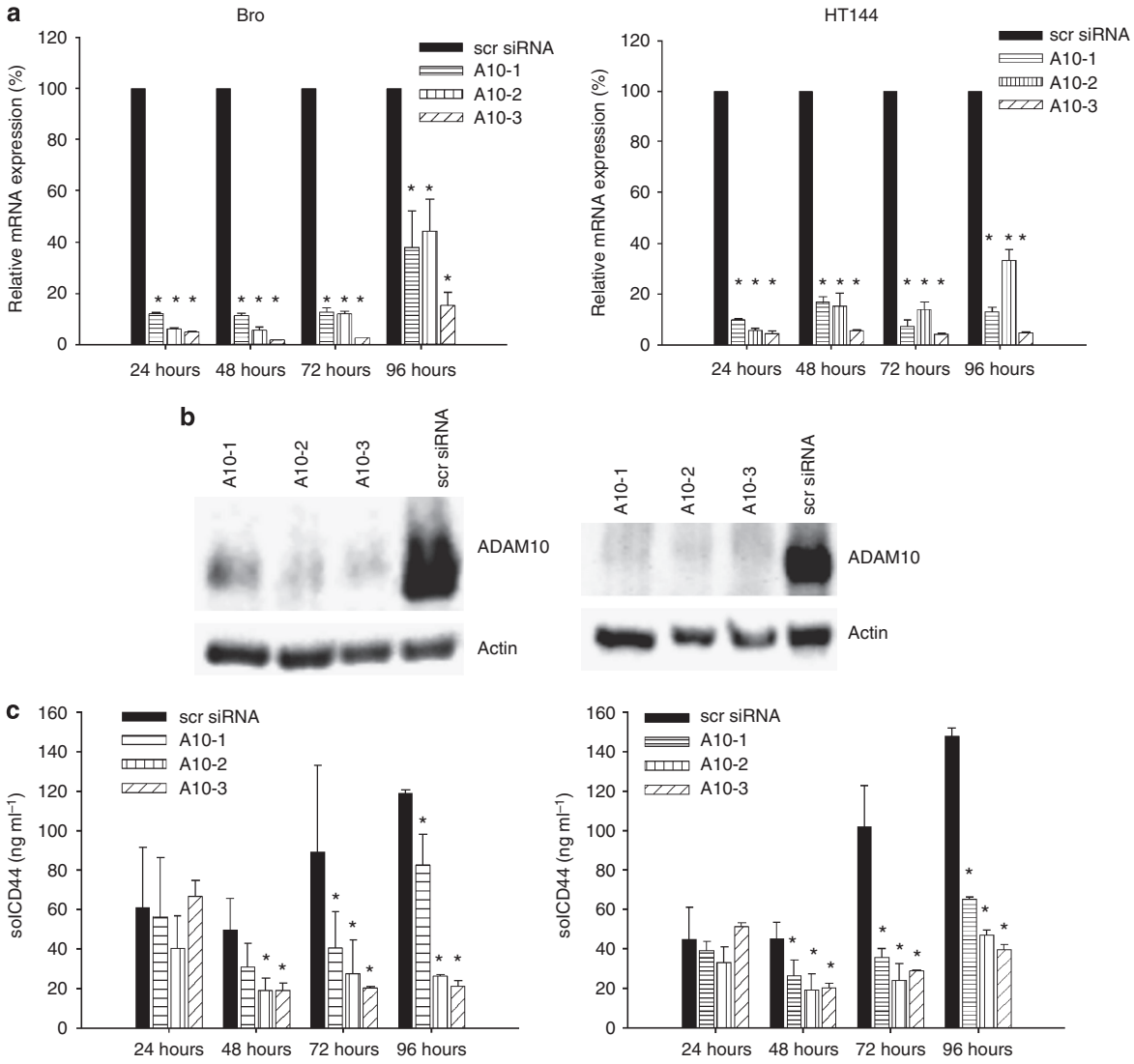

Figure 4. ADAM10 siRNA decreases ADAM10 mRNA and protein levels and efficiently reduces CD44 shedding. Growing cultures of MM cells were transfected with specific and scrambled siRNAs. After various time points the ADAM10 mRNA expression was determined by real-time PCR (**a**). Parallel cells were analyzed by immunoblotting for ADAM10 protein expression (**b**). Quantitation of solCD44 in the culture supernatants from the cells shows that all three ADAM10-specific siRNAs but not the scrambled controls significantly block CD44 shedding (**c**). The mean and standard error from 5 independent experiments is presented. *$P<0.005$.

CD44-dependent manner was also obtained by comparing the effect of ADAM10 silencing in three melanoma cell lines differing in CD44 expression. The cell lines Bro and HT144 express CD44 and shed significant amounts of CD44. The human melanoma cell line RPM-MC (Hoashi *et al.*, 2001; Morrison *et al.*, 2001) expresses equivalent levels of ADAM10 as Bro and HT144 cells, but it neither expresses CD44 nor releases solCD44. The results of these experiments showed that ADAM10 knockdown significantly induced cell proliferation compared to scrambled siRNA treatment in both of the CD44 expressing Bro and HT144 cell lines (Figure 8c). Importantly, silencing of ADAM10 did not affect cell proliferation in RPM-MC cells which do not express CD44. Together with the data obtained using ADAM10 and CD44 cosilencing, these data indicate that ADAM10 mediates shedding of the CD44 ectodomain that in turn strongly affects the proliferation of MM cells.

DISCUSSION

Human melanoma cells are capable of shedding constitutively the ectodomain of CD44, and our previous work has shown that the resulting solCD44 abolishes proliferation of MM *in vitro* and *in vivo* (Ahrens *et al.*, 2001b). Until now, the protease(s) responsible for this shedding has remained obscure. The ADAMs, a family of zinc-dependent membrane-associated proteases that cleave a variety of substrates including growth factors, extracellular matrix proteins, and transmembrane receptors, are prominently expressed in many cancers such as in oral cavity, stomach, ovary, uterine, colon, leukemia, prostate (reviewed in (Mochizuki and

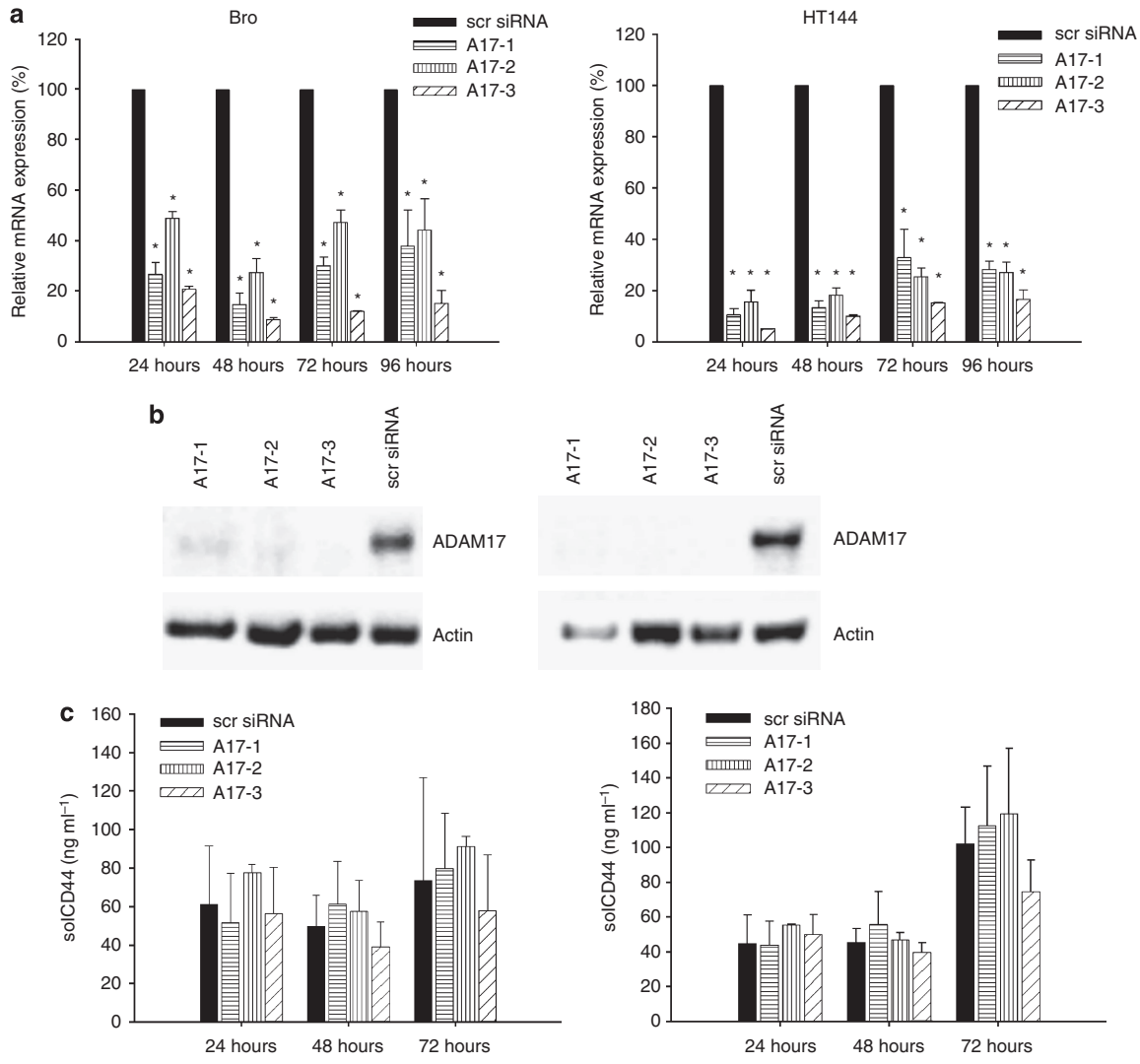

Figure 5. ADAM17 siRNA decreases mRNA and protein levels but does not impair CD44 shedding. Growing cultures of MM cells were transfected with specific and scrambled siRNAs. After various time points the ADAM17 mRNA expression was determined by real-time PCR (**a**). Parallel cells were analyzed by immunoblotting for ADAM17 protein expression (**b**). Quantitation of solCD44 in the culture supernatants from the cells shows that neither ADAM17-specific siRNAs nor the scrambled controls impair CD44 shedding (**c**). The mean and standard error from 5 independent experiments is presented. *$P<0.005$.

Okada, 2007)). Here we show that ADAM10 and ADAM17 are constitutively expressed in human melanoma cells. More importantly, we demonstrate that blockade of ADAM10 activity or its expression has a potent inhibitory effect on CD44 shedding, and also strongly stimulates MM cell proliferation in a CD44-dependent manner. Together, these observations are consistent with the notion that ADAM10 plays a major role in regulating melanoma cell proliferation, at least in part by controlling the shedding of the ectodomain of CD44 from MM cells.

Is ADAM10 the only protease involved in constitutive shedding of CD44 from MM cells? In addition to ADAM10, ADAM17 and MMP14 have been suggested previously to play a role in CD44 shedding. Using the well established inhibitors GW280264X and GI254023X that have been shown to inhibit ADAM10 and ADAM17 (Hundhausen *et al.*, 2003; Ludwig *et al.*, 2005) together with siRNA techniques, we clearly demonstrated that ADAM10 but not ADAM17 is involved in the constitutive shedding of native CD44 from MM cells. This is consistent with previous reports that found that CD44 shedding was not sensitive to ADAM17 depletion in mouse monocytes and fibroblasts (Shi *et al.*, 2001). A possible involvement of MMP14 in CD44 shedding has been suggested by cotransfection studies of MMP14, CD44, and TIMPs in breast carcinoma, osteosarcoma (Kajita *et al.*, 2001), and melanoma cells (Nakamura *et al.*, 2004). However, it is important to note that cotransfection generates dramatically changed molecular ratios between the putative sheddases, their inhibitor proteins and CD44 compared to the steady state levels of natively expressed proteins in untreated cells. Indeed, we found little expression or activity of MMP14 in MM. Further, blocking its expression had no effect on the endogenous constitutive cleavage of CD44 from MM cells, ruling out a role for MMP14 in cleavage of CD44 in this

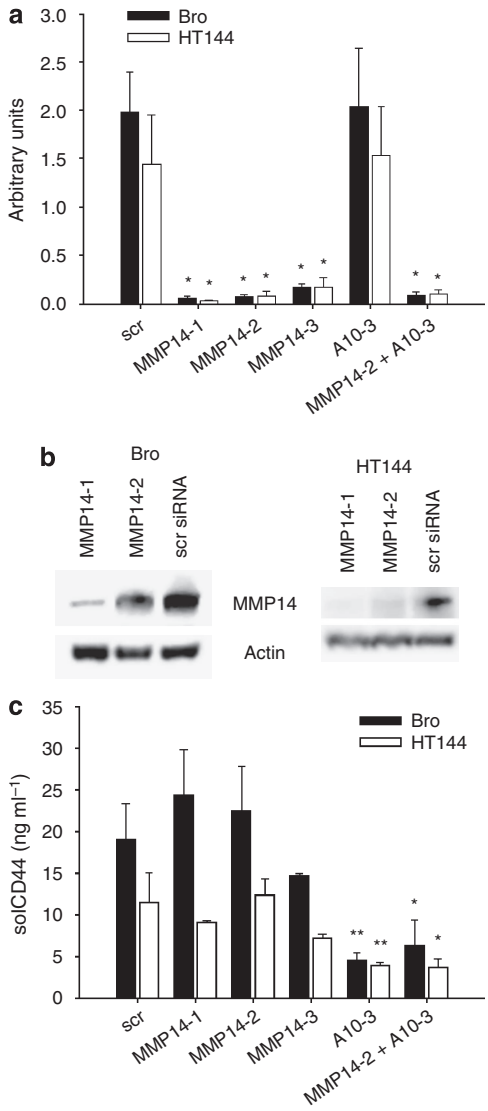

Figure 6. MMP14 knockdown does not affect CD44 shedding in melanoma cells. Growing cultures of MM cells were transfected with siRNAs against MMP14, ADAM-10, a combination of both, or with scrambled siRNAs. After 48 hours the mRNA expression was determined by real-time PCR (**a**). After 72 hours cells were analyzed by immunoblotting for protein expression (**b**). Quantitation of solCD44 in the culture supernatants from the cells by ELISA after 72 hours culture shows that neither MMP14-specific siRNAs nor the scrambled controls impair CD44 shedding (**c**). Cotransfection of ADAM10-specific siRNA with MMP14-specific siRNA did not enhance the blocking effect on CD44 shedding mediated by ADAM10-specific siRNA alone. The mean and standard error from 3 independent experiments is presented. *$P<0.05$; **$P<0.005$.

context. Although other proteases such as ADAM9 have been reported to be expressed in human malignant melanoma (Zigrino *et al.*, 2005), there is no evidence that they might be involved in CD44 cleavage. Most importantly, our data show that inhibition of ADAM10 alone has a dramatic effect on the shedding of CD44 from MM cells, with functional consequences for cell proliferation. Cell adhesion to HA (Figure S1) and cell motility on HA (assayed by time lapse videomicroscopy), however, were not changed significantly by ADAM10 silencing (data not shown). This might be due to the fact that the amount of membrane-bound CD44 on the MM cells was not affected by ADAM10 knockdown (Figure S2). These data strongly support the notion that ADAM10 plays at least the major role in regulating shedding of CD44 from MM cells.

The mechanism of CD44 cleavage by ADAM10 in MM cells remains to be determined, and whereas it is likely that CD44 is a direct target of ADAM10, we cannot currently rule out the possibility that ADAM10 acts indirectly, for example by activating another protease. ADAM10 has previously been implicated in the cleavage of CD44 from glioblastoma cells, where ADAM10-dependent shedding can be induced by Ca2 + influx (Nagano and Saya, 2004). In these cells it has also been demonstrated that EGF can induce ADAM10-mediated cleavage of CD44 in a manner that is dependent on Rac1 and mitogen-activated protein kinase activation (Murai *et al.*, 2004). Recent investigations with ovarian carcinoma cell lines have also demonstrated that ADAM10 can initiate cleavage of CD44 and CD171 in the endosomal compartment, with the fragments subsequently being released in the form of exosomes, within which cleavage continues (Stoeck *et al.*, 2006). Regarding a possible ADAM10 substrate sequence on CD44, cleavage of the Axl receptor tyrosine kinase by ADAM10 is critically dependent on membrane-proximal sequences of the receptor (Budagian *et al.*, 2005), and the same may hold true for CD44. ADAM10 also contains a sorting signal within its cytoplasmic domain that can target it to adherens junctions, and this targeting is required for ADAM10-mediated cleavage of E-cadherin (Maretzky *et al.*, 2005; Wild-Bode *et al.*, 2006). In this context it is interesting to note that CD44 can also localize to adherens junctions (Kooy *et al.*, 1999).

We observed a clear colocalization between ADAM10 and CD44, but not between ADAM17 and CD44. In this regard it is worth noting that CD44 can interact with a variety of other cell surface proteins, including integrins and various growth factor receptors (reviewed in Ponta *et al.*, 2003). The colocalization between ADAM10 and CD44 also suggests that the molecules are in the appropriate spatial proximity for ADAM10-mediated cleavage of CD44 to function in *cis*. However, we cannot rule out the possibility that ADAM10-mediated cleavage of CD44 functions in *trans*. Indeed, cleavage of ephrin-A5 by ADAM10 occurs in *trans*, with ADAM10 and its substrate being on the membranes of opposing cells (Janes *et al.*, 2005).

We have previously reported that soluble CD44 inhibits HA-stimulated proliferation of melanoma cells *in vitro* and *in vivo* (Ahrens *et al.*, 2001b). In this study we could demonstrate that the silencing of ADAM10 suppresses constitutive CD44 shedding and also promotes melanoma cell proliferation. The effect of inhibiting ADAM10 expression/activity on MM cell proliferation was dependent on their CD44 expression. This observation can be explained on a number of levels: Reduced constitutive shedding of CD44 as a consequence of ADAM10 knockdown could also possibly increase the density of transmembrane CD44 on the cell surface, leading to increased proliferation as a consequence

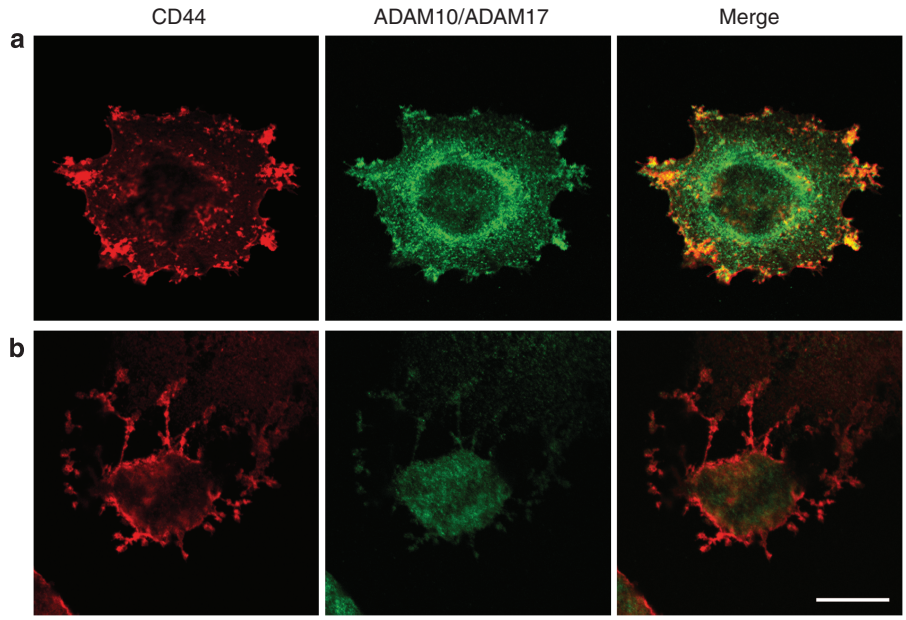

Figure 7. ADAM10 and CD44 colocalize on the cell membrane of MM cell line Bro, whereas ADAM17 does not co-localize with CD44 in resting MM cells. Cells were cultured on glass cover slips, fixed and stained with anti ADAM10/goat anti rabbit-CY2 (green) and biotinylated antiCD44/Streptavidin-Alexa-Fluor-555 (red) (**a**, upper line) or with ADAM17/goat anti rabbit-CY2 (green) and biotinylated antiCD44/Streptavidin-Alexa-Fluor-555 (red) (**b**, lower line). Inspection of the stained cells with a confocal laser scanning microscope revealed ADAM10 expression in the cytoplasm and at the cell membrane, whereas CD44 was expressed predominantly on the cell membrane. In the merged picture the yellow spots indicate colocalization of both proteins in the cell membrane. Scale bar = 20 μm.

of enhanced ligation of HA by the MM cells, which we have previously shown promotes their proliferation (Ahrens *et al.*, 2001b). However, this is unlikely as we did not observe changes in steady state cell surface levels of CD44 in response to blockade of ADAM10 expression/activity as assessed by FACS analysis (see Figure S1) and Western blotting (data not shown). This stable CD44 surface expression could also explain unchanged cell functions that rely on HA–CD44 interaction such as cell attachment (see Figure S2) and motility on HA-coated surfaces. Another possibility is that reduced levels of solCD44 as a result of blocking ADAM10 expression/activity could decrease the interference by solCD44 in the interaction between HA and membrane-bound CD44, which would be consistent with our previous findings that soluble CD44 can inhibit the HA-stimulated proliferation of melanoma cells (Ahrens *et al.*, 2001b). Blocking of CD44 shedding might also change intracellular signaling events. Proliferation of nonconfluent, growing cell cultures depends on the interaction of the CD44 cytoplasmic domain with Ezrin-Radixin-Moesin (ERM) proteins (Morrison *et al.*, 2001). On the other hand, dissociation of this CD44–ERM complex results in NF2-mediated growth arrest (Morrison *et al.*, 2001). Shedding of the CD44 ectodomain is followed by the sequential cleavage of the CD44 cytoplasmic domain by a γ-secretase (Nagano and Saya, 2004) in many cells. It may be possible that following ADAM10-mediated shedding of the CD44 ectodomain the CD44 cytoplasmic domain is released from internal complexes with ERM proteins, which then results in an antiproliferative signal. Thus, inhibition of CD44 shedding by ADAM10 siRNA could result in persistence of CD44–ERM–NF2 complexes, resulting in a more efficient proliferation. Future work will focus on deciphering which of these various possibilities accounts for the effect of ADAM10 on MM cell proliferation.

How might the activity of ADAM10 be increased in MM cells? Recent work suggests that ADAM10 activity is upregulated in the context of squamous skin tumors by kallikrein 6 (Klucky *et al.*, 2007). Consistently, the postulated cleavage site in the latent ADAM10 protein whose proteolysis activates ADAM10 enzymatic activity shares similarities with amino-acid sequences that are efficiently cleaved by kallikrein 6 (Angelo *et al.*, 2006). To the best of our knowledge no studies have examined the expression of kallikrein 6 in the context of melanoma, and it will therefore be interesting to examine whether kallikrein 6 might be responsible for the ADAM10 expression and/or activity we observed in human MM tumors and cell lines.

In conclusion, our data indicate that ADAM10 is the major protease responsible for constitutive CD44 cleavage from MM cells, and that its expression can impair tumor cell proliferation in MM. Upregulation of ADAM10 expression or activity, thus stimulating the production of solCD44 and at the same time inhibiting MM cell proliferation, might therefore represent a therapeutic approach for MM.

MATERIALS AND METHODS

Antibodies and inhibitors

Rabbit anti-human ADAM10 polyclonal antibodies, rabbit anti-human ADAM17 polyclonal antibodies, and rabbit anti-human MMP14 polyclonal purified IgG antibodies were purchased from Chemicon Int. (Billerica,MA). Anti-CD44 antibody was purchased from Bender MedSystems (Vienna, Austria). For Western blot

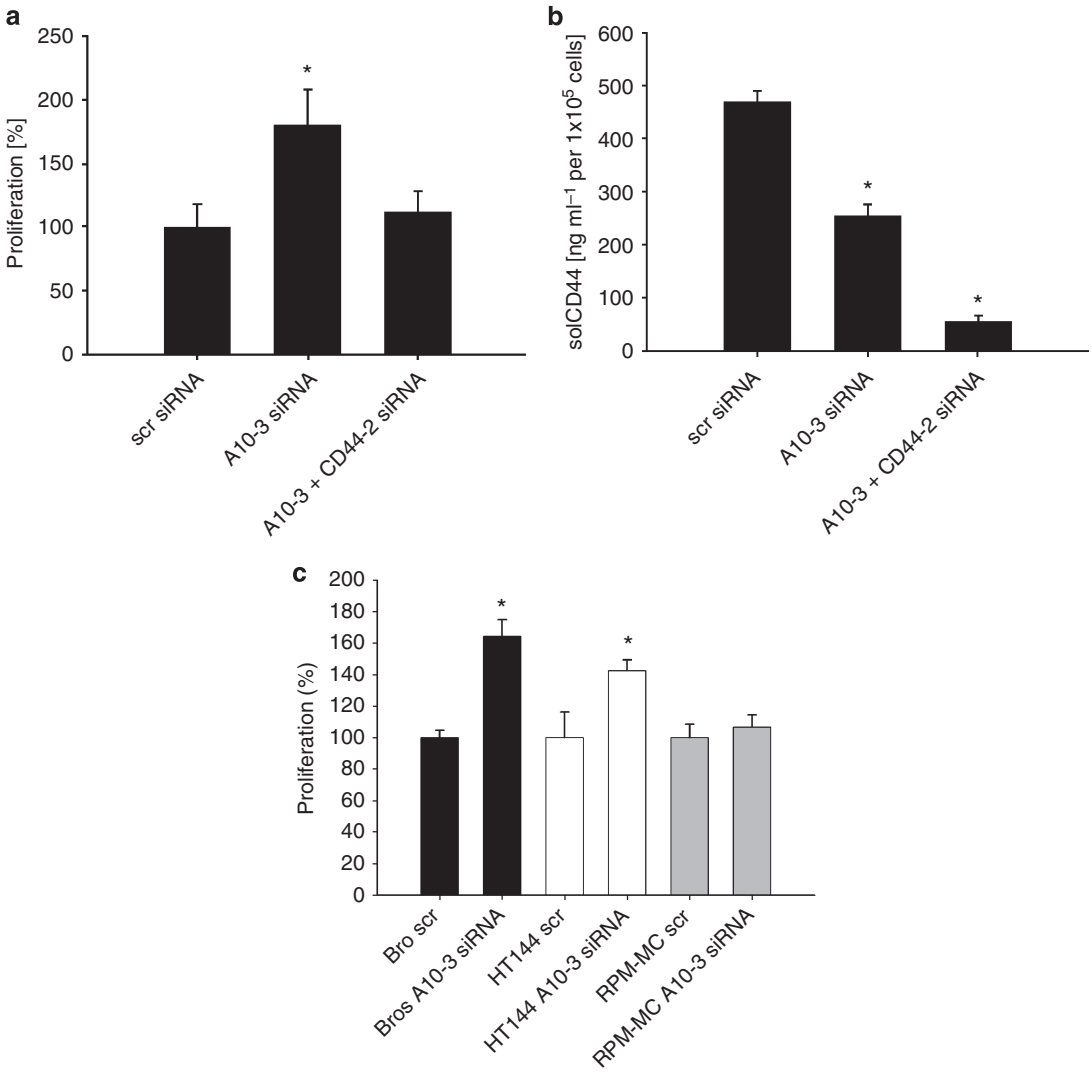

Figure 8. ADAM10 silencing enhances cell proliferation in a CD44-dependent manner. MM cells (1×10^4 cells per well) were seeded into a 24 well plate and transfected with siRNAs as described. Proliferation was assayed by the wst-Proliferation test 48 hours after transfection of the cells. The proliferation in wells of scrRNA-transfected cells was set to 100%. (**a**) Cell proliferation of Bro cells was determined after silencing of ADAM10 (A10-3), and after cosilencing of ADAM10 (A10-3) and CD44 (CD44-2) simultaneously. (**b**) The effectiveness of gene silencing on the CD44 shedding was analyzed by CD44 ELISA. (**c**) ADAM10 silencing promotes cell proliferation in CD44 expressing cell lines Bro and HT144 whereas it has no impact on cell proliferation in the CD44 negative cell line RPM-MC. The mean and standard error from 3 independent experiments is presented. *$P<0.05$.

development, anti-rabbit F(ab)-fragments coupled to horseradish peroxidase (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were used. Equal loading and transfer of proteins was ensured using a rabbit anti-human β-actin polyclonal antibody (Abcam, Cambridge, UK). The metalloproteinase inhibitors GW280264X ((2R,3S)-3-(formyl-hydroxyamino)-2-(2-methyl-1-propyl) hexanoic acid ((1S)-5-benzyloxycarbamoylamino-1-(1,3-thiazol-2-ylcarbamoyl)-1-pentyl) amide) and GI254023X ((2R,3S)-3-(formyl-hydroxyamino)-2-(3-phenyl-1-propyl) butanoic acid ((1S)-2,2-dimethyl-1-methylcarbamoyl-1-propyl) amide) were synthesized as described in US Patents US 6 172 064, US 6 191 150, and US 6 329 400. The compounds were assayed for inhibition of recombinant human ADAM17 and ADAM10 ectodomains as described with final concentrations of 5 μM (Hundhausen *et al.*, 2003; Ludwig *et al.*, 2005). The inhibitors were dissolved in DMSO at 5 mM and the control experiments were performed by adding 1/1,000 volume DMSO.

Cell culture

The melanoma cell lines Bro (kindly provided by Dr J Eberle Berlin (Lockshin *et al.*, 1985)), and HT144 (kindly provided by Dr van Muijen (van Muijen *et al.*, 1995)) were cultured in RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 10 or 1% fetal bovine serum (PAA, Pasching, Austria) and maintained at 37 °C in a humidified 5% CO2 atmosphere. Cultures were passaged by detaching the cells with trypsin/EDTA solution (Biochrom). Experiments were started after 12 hours of serum starvation to synchronize the cell cycles. For transfection, melanoma cells were grown in six-well plates until they reached approximately 50% confluence.

RNA preparation and real-time PCR

Total RNA was prepared using the innuPREP RNA Mini Kit (Analytikjena, Jena, Germany). The quantity and quality of the

RNA was determined by spectrophotometry (ND-1000; NanoDrop Technologies Inc., Wilmington, DE). Total RNA (1 μg) from each sample was used for first strand cDNA synthesis using M-MLV reverse transcriptase (Promega, Madison, WI) following the manufacturer's instructions. Real-time PCR was monitored by EvaGreen fluorescence (Biotium Inc., Hayward, CA) using GenTherm DNA-Polymerase (Rapidozym, Berlin, Germany) with 2.5 mM $MgCl_2$, 0.5 mM Primer, 250 mM dNTPs, and 0.5 U polymerase per reaction (20 μl) under primer-specific conditions. The following experimental protocol for PCR reaction (40 cycles) was performed on a Rotor-Gene 3000 cycler (Corbett Research, Sydney, Australia): denaturation for 5 minutes at 95 °C, followed by 40 amplification cycles at 95 °C (10 seconds), annealing under primer-specific conditions (20 seconds), and extension for 30 seconds at 72 °C. Fluorescence was measured for 15 seconds at 80–85 °C depending on the melting temperature of the specific PCR product. Primers with the following sequences were chosen: GAPDH (GenBank NM_002046): forward: 5′-CTTCACCACCATGGAGAAGGC-3′, reverse: 5′-CCAGTGAGCTTCCCGTTCAGC-3′, annealing temperature 58 °C. ADAM-10 (GenBank NM_001110): forward: 5′-CAAAGTCTGAGAAGTGTCGGG-3′, reverse: 5′-CTGCACATTGCCCATTAATG-3′, annealing temperature 60 °C. ADAM-17 (GenBank NM_003183): forward: 5′-ACCTGAAGAGCTTGTTCATCGAG -3′, reverse: 5′-CCATGAAGTGTTCCGATAGATGTC-3′, annealing temperature 60 °C. MMP-14 (GenBank NM_004995): forward: 5′-ACATTGGAGGAGACACCCAC-3′, reverse: 5′-TAGGCAGTGTTGATGGACGC-3′; 2′-5′ oligoadenylate synthetase (GenBank: NM_016816): forward: AGAAAGAGGGCGAGTTCTCC, reverse: 5′-GCATAGACCGTCAGGAGCTC-3′; annealing temperature 60 °C. All Primers were obtained from Metabion (Martinsried, Germany). To confirm the specificity of the amplified DNA, a melting curve was determined at the end of each run. The reaction efficiency was determined with a dilution series of plasmids containing the PCR products. Genes were normalized to the unregulated housekeeping gene GAPDH and the results were expressed as ratio of target gene and GAPDH expression (arbitrary units). Control experiments were also performed to ensure that GAPDH expression was not differentially regulated under the experimental conditions employed, and expression of additional house keeping genes was also analyzed to verify the reliability of normalization relative to GAPDH.

CD44 ELISA

Soluble CD44 protein in culture supernatants was quantified using an ELISA kit from Bender MedSystems according to the manufacturer's instructions. All samples were assayed in triplicate.

Immunohistochemistry

Cryostat sections of superficial spreading melanoma were incubated with the anti-ADAM10, ADAM-17, anti-MMP14, or anti-CD44 antibodies for 1 hour, followed by avidin–biotin complex technique according to the manufacturer's recommendation (supersensitive multilink alkaline phosphatase ready-to-use detection system; Biogenix, San Ramon, CA). Bound proteins were detected colorimetrically using the New Fuchsin substrate system (DAKO, Hamburg, Germany). As a negative control, the same procedure was performed with a rabbit IgG control antibody. The study was conducted according to Declaration of Helsinki Principles.

Immunofluorescense staining

Bro and HT144 cells were cultured on cover slips for 24 hours in complete medium. The medium was removed and the cells were washed with warm phosphate-buffered saline (PBS). Next the cells were fixed with cold paraformaldehyde (4% in PBS) for 15 minutes. After rinsing with PBST (PBS + 0.1% Tween20) the fixed cells were pre-blocked with PBST containing 5% goat serum for 30 minutes. Cells were stained with anti-ADAM10 or anti-ADAM17 for 45 minutes at room temperature and after several washes with PBST a CY2-labeled goat-anti-rabbit antibody was added. In a second round, a biotinylated antiCD44-antibody was used as the primary antibody that was secondarily detected with Stretavidin-Alexa555. Normal rabbit and mouse IgG were used as controls.

Finally the sections were extensively washed with PBST, briefly placed in distilled water, mounted on fluorescence-free slides, air dried, and cover slipped with Entelan (dissolved in toluene; Merck, Darmstadt, Germany). The objects were viewed using a Zeiss confocal laser scanning microscope (LSM 510). Confocal images of Cy2 fluorescence were obtained with the argon laser (488 nm) and emission filter BP 505–530. The HeNe 1 laser (543 nm) and the emission filter BP 560–615 were used to detect Cy3 fluorescence.

Transfection of melanoma cells with siRNA

siRNA (Stealth Select RNAi) specific for ADAM10 (Stealth Select RNAi HSS100165-67), ADAM17 (Stealth Select RNAi HSS110434-36), and MMP14 (Stealth Select RNAi HSS106639-41) as well as scrambled siRNA (Stealth RNAi Negative Control (High and Medium GC)) were purchased from Invitrogen.

MM cells with a confluence of 30–50% were transfected with either 40 pmol ml^{-1} of gene-specific siRNA or 40 pmol ml^{-1} of scrambled control siRNA using Lipofectamine RNAiMax Transfection Reagent (Invitrogen) according to the manufacturer's instructions. After various times of incubation, transcript levels of the gene of interest were detected by quantitative RT-PCR and protein expression was analyzed by Western blotting. The influence of the treatment on CD44 shedding was monitored by ELISA. To control for transfection efficiency, FITC-labeled control siRNA (Santa Cruz, Heidelberg, Germany) was transfected into the cells in parallel experiments. This control indicated a transfection efficiency of between 60 and 85% 48 hours after transfection as measured by flow cytometry (data not shown). Off target effects were evaluated by analysis of ADAM10 mRNA expression in ADAM17-silenced cells and vice versa. Unspecific knockdown could be excluded by this method. Silencing of MM cells was achieved without eliciting critical stress responses confirmed by qRT-PCR of the stress gene 2′-5′ oligoadenylate synthetase (Sledz *et al.*, 2003; Scacheri *et al.*, 2004).

Western blot analysis

Western blot analysis of endogenous ADAM10 and ADAM17 was performed as described previously (Hundhausen *et al.*, 2003; Ludwig *et al.*, 2005). In brief, the cells were washed with PBS and directly lysed in Triton buffer (20 mM Tris-HCl, pH 7.6; 0.5 M NaCl, 0.5% Triton X-100). The protein concentration in the lysates was determined using the Advanced Protein Assay Reagent from Cytoskeleton (Denver, CO), and equivalent amounts of protein were subjected to SDS-PAGE under reducing conditions using 8–16% Tris-Glycine gels (Lonza, Bruxelles, Belgium). Proteins were transferred

onto PVDF membranes (Hybond-P; Amersham, Freiburg, Germany). Blocking was performed for 1 hour with 5% non-fat dried milk powder in PBS. The membranes were incubated with dilutions of rabbit antiserum against ADAM10 (1 $\mu g\,ml^{-1}$ in PBS containing 0.01% Tween 20 and 5% BSA) or rabbit antiserum against ADAM17 (1 $\mu g\,ml^{-1}$) combined with a rabbit-anti β-actin antibody (0.5 $\mu g\,ml^{-1}$) for a loading control. Bound Ig was detected with HRP-coupled goat anti-rabbit Ig (40 $ng\,ml^{-1}$ in PBS containing 0.01% Tween 20 and 5% non-fat dried milk powder), followed by incubation with chemiluminescence substrate (ECL; Pierce, Rockford, IL). Chemiluminescent signals were recorded on a luminescent image analyzer (MultiImage Light Cabinet; Alpha Innotech, San Leandro, CA).

Proliferation assay

Twenty-four-well flat bottom plates (Nunc Maxisorb) were coated overnight at 4 °C with 300 µl HA (1.0 $mg\,ml^{-1}$ in 50 mM $NaHCO_3$ buffer, pH 9.6) that had been heat inactivated for 20 minutes at 100 °C. 3×10^4 cells per well were seeded and cultured overnight. Cells were transfected with the siRNAs (40 $pmol\,ml^{-1}$) and cultured for further 48 hours. To measure cell proliferation, the wst-proliferation assay (Roche Applied Science, Mannheim, Germany) was performed according to manufacturer's protocol. In some experiments soluble HA (0.5 $mg\,ml^{-1}$ in complete culture medium) was added 6 hours after transfection. Statistical analysis of the data was performed using the Student's *t*-test. All probes were performed as quintuplicate samples.

Statistical analysis

All data are expressed as the mean ± SEM. Statistical analysis was performed with the SigmaPlot9 software (Systat, Erkrath, Germany) using the Student's *t*-test.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIAL

Figure S1. ADAM10 silencing does not change CD44 surface expression.

Figure S2. ADAM10 silencing does not change significantly cell attachment to HA-coated surfaces.

Figure S3. ADAM10 and CD44 colocalize on the cell membrane of MM cell line HT144, whereas ADAM17 does not localize with CD44 in resting MM cells.

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