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CD44S-hyaluronan interactions protect cells resulting from EMT against anoikis

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Abstract

The detachment of normal epithelial cells from matrix triggers an apoptotic response known as anoikis, during homeostatic turnover. Metastatic tumor cells evade anoikis, by mechanisms that are only partly characterized. In particular, the epithelial–mesenchymal transition (EMT) in a subset of invasive tumor cells confers anoikis-resistance. In some cases, EMT up-regulates the cancer stem cell marker CD44S and the enzyme hyaluronic acid synthase-2 (HAS2). CD44S is the major receptor for hyaluronan in the extracellular matrix. Herein, we demonstrate that CD44S, unlike the CD44E isoform expressed in normal epithelial cells, contributes to the protection against anoikis. This protection requires the interaction of CD44S with hyaluronan (HA). CD44S–HA interaction is proposed to play an important role in tumor metastasis through enhanced cell survival under detached conditions.

Keywords

CD44; Hyaluronan; Anoikis; Apoptosis; Epithelial-mesenchymal transition

Introduction

Normal epithelial cells respond to detachment from their extracellular matrix by undergoing apoptosis, through a process known as anoikis (ancient Greek, meaning homelessness) [1]. A subset of breast cancer cells occurring at the invasive tumor–stromal interface display an altered gene expression program in which epithelial genes are down-regulated and mesenchymal genes are up-regulated, a process known as Epithelial–Mesenchymal Transition (EMT) [2]. In addition to invasiveness, EMT also confers chemo-resistance, pre-

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Declarations of interest

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disposes tumors to late recurrence, and, in some contexts, favors the generation/stabilization of tumor-initiating cells [2–5].

Resistance to anoikis prominently accompanies EMT. The molecular mechanisms coupling these processes are understood incompletely. They include cytoskeletal changes that alter transcription factor localization/activity, activation of pro-survival gene expression by EMT-transcription factors, and the down-regulation of pro-apoptotic gene expression due to the loss of epithelial transcription factors [6].

The cell adhesion receptor CD44 is a lymphocyte homing receptor for the ligand hyaluronan. Although expressed ubiquitously, multiple isoforms arise from complex differential splicing, and individual isoforms tend to be expressed in specific tissues or cell types within a tissue [7]. Individual isoforms may function differently, due to variations of the extracellular domain in the context of a uniform intracellular domain [7,8]. For example, the CD44S (standard) isoform has higher affinity for the ligand, HA than does CD44E (epithelial) isoform; CD44E contains three additional exons (exons 8–10) that extend the extracellular domain, generating novel glycosylation sites that interfere with HA binding [9,10].

The CD44 gene is highly regulated, both transcriptionally and by alternative splicing mechanisms. Transcriptionally, the gene promoter is positively regulated by the p63 protein and by Wnt signaling through TCF4-related factors [11,12]. P53 represses the promoter by preventing the recruitment of p63 [12]. Epithelial cells generally express the sequence specific splicing factors ESRP1/2, promoting the inclusion of exons 8–10 and causing CD44E to predominate over CD44S. ESRP1/2 are down-regulated by EMT, permitting the accumulation of CD44S [13].

Significant evidence links high CD44 expression with metastasis and disease progression in several cancer types [14–16]. For example, CD44 blocking antibodies suppress both metastasis and disease recurrence following chemotherapy in human × mouse xenografts [17]. Hyaluronan (HA)-blocking peptides restrict tumor progression in mouse models as well [18]. CD44 up-regulation also correlates with mammary tumor aggressiveness [19]. Mechanistically, this may reflect, in part, the co-receptor function that CD44 isoforms provide for c-met, EGFR and perhaps other receptors [8,14]. In addition, CD44–HA interaction stimulates migration and invasion through Ezrin/Radixin/Moesin proteins, ankyrin-G, and rhoA [20]. Interestingly, CD44 appears to be a major antagonist of the pro-apoptotic functions of p53, by promoting the survival of p53-null cell lines with respect to DNA damaging agents, in vivo and in vitro [12].

In HMLE (*H*uman *M*ammary *E*pithelial cells immortalized with telomerase and SV40 early region *L*arge T) cells, a well characterized cell culture model for mammary epithelial cell EMT, the induction of EMT with Twist, Snail, E-cadherin depletion or TGF-β induces a $CD44^{high}CD44^{low}$ phenotype with extraordinary tumor-initiating potential, indicative of cancer stem cells [21]. Conversely, subpopulations of HMLE cells that are flow-sorted for this marker set show a gene expression profile indicating EMT. These results indicate that, at least in this particular cell line, EMT generates a cancer stem cell-like phenotype, and, in

fact, this marker set is diagnostic of cancer stem cells in a subset of human mammary tumors.

The functional significance of CD44 in CSCs and metastasis is understood incompletely. In this paper, we demonstrate that CD44S partially protects mammary epithelial cells against anoikis, through interaction with the HA ligand.

Results

Depletion of CD44S sensitizes cells to anoikis

We hypothesized that CD44S expression accompanying EMT contributes to anoikis-resistance. To test this hypothesis, we first characterized CD44 isoform expression before EMT or following EMT that was induced in HMLE cells by the stable knockdown of E-cadherin or the expression of a Twist-estrogen receptor (Twist-ER) fusion protein after induction with 4-hydroxytamoxifen. A substantial shift from CD44E expression in parental cells to CD44S expression in EMT-derived cells was seen, and the total CD44 was significantly increased in the latter, reflecting both promoter up-regulation as well as differential splicing (Fig. 1A), consistent with previous reports [21].

Previously, we showed that, accompanying this shift, EMT conferred resistance to anoikis in this cell line [27]. To test the role of CD44 in anoikis, a doxycycline-inducible CD44 shRNA lentivirus was infected into HMLE + Twist-ER cells, which are HMLE cells that stably express Twist-ER fusion protein and had been induced with 4-hydroxytamoxifen (4-OHT) to generate irreversible EMT, characterized previously [22,21]; the efficiency of the knockdown was confirmed by Western blotting (Fig. 1B). A significant sensitization (~2.5-fold) was observed in response to the knockdown of CD44S, essentially the only isoform expressed in this post-EMT cell line (Fig. 1B). To confirm that this effect could be observed using a different knockdown targeting site and cell line, MCF10a cells were transiently transfected with an siRNA pool. A similar anoikis--sensitization effect was observed, ruling out off-target effects of the shRNA (Fig. 1C), although this basal cell line expresses a low level of CD44E relative to a larger amount of CD44S, confounding specific attribution of the knockdown effect to the latter isoform only.

The expression of Twist protein confers upon HMLE cells the ability to generate mammospheres in suspension culture [21], a CSC trait that requires anoikis-resistance. The expression of CD44 shRNA decreased both the number and size of mammo-spheres in HMLE + Twist-ER cells (Fig. 1D).

These data revealed for the first time that CD44S protected EMT-like tumor cells against anoikis.

CD44S, but not CD44E, protects against anoikis

In light of the isoform switch from CD44E to CD44S accompanying EMT, we next compared the capacity of these isoforms to affect anoikis. As shown above (Fig. 1C), a pan-CD44 shRNA knockdown exacerbated anoikis. By contrast, an siRNA sequence specific for

the exons uniquely found in the CD44E isoform protected MCF10a cells partially against anoikis, indicating that CD44E was pro-apoptotic with regard to anoikis (Fig. 2A).

To confirm this conclusion, we knocked down both isoforms in MCF10a cells using an shRNA that targeted the 3′UTR of CD44, and then infected these cells with a CD44E expression vector (that lacks the CD44 3′UTR). The resulting cells, expressing only the CD44E isoform, were more sensitive to anoikis than were the pan-CD44 knockdown cell line by about 1.8-fold (Fig. 2B). Furthermore, CD44E, ectopically expressed in HMLE–Twist-ER cells that had undergone EMT and lack endogenous CD44E, sensitized these cells to anoikis (Fig. 2C).

These observations indicated that, while CD44S protected against anoikis in both epithelial and EMT-derived cells, CD44E actually promoted anoikis in normal epithelial cells.

HA–CD44 interaction is critical for anoikis protection

The opposite effects of CD44S vs. CD44E in anoikis protection motivated us to compare the ability of cells expressing these isoforms to interact stably with the ligand hyaluronan (HA). First, we compared HMLE + Twist-ER cells before vs. after EMT, which had undergone the switch from CD44S to CD44E expression, for their ability to bind an HA-coated plastic surface. The post-EMT (4-OHT-treated) cells showed dramatically higher efficiency of binding on this matrix component (Fig. 3A). In addition, CD44S or CD44E were ectopically overexpressed in parental HMLE–Twist-ER cells that were not induced with 4-OHT (and therefore had endogenous expression of CD44E only). CD44S, but not CD44E (expressed at similar levels), promoted the binding of these cells to HA coated surfaces (Fig. 3B), consistent with previous observations in other cell lines [10]. These results suggested that the CD44 interaction with HA correlated with anoikis protection.

To further test the role of CD44–HA in anoikis, we constructed a point mutation in CD44S that abolishes its interaction with HA, which was previously characterized [28]. An HMLE–Twist-ER cell line that had undergone EMT but expressed very low levels of CD44 (due to stable shRNA knockdown) were rescued with wild-type or R41A mutant forms of CD44S and assayed for anoikis. While CD44-WT protected against anoikis, confirming our previous results above, the HA non-binding mutant failed to protect (Fig. 3C). This result indicated that CD44–HA interaction plays a critical role in anoikis resistance.

To explore the role of HA in the anoikis-protective effect of CD44 further, we compared HA Synthase-2 (HAS2) mRNA levels and HA levels in HMLE–Twist-ER cells before vs. after induction of EMT with 4-OHT. HAS2 protein levels could not be determined directly to a paucity of specific antibodies. HAS2 mRNA increased in response to EMT, as did the production of endogenous HA, as determined by specific labeling with biotin-labeled HA-binding protein (HABP-bio; Fig. 4A).

As a further test of the role of HA in anoikis-protection, post-EMT cells (4-OHT-treated HMLE + Twist-ER, or sh-Ecadherin) cells were treated with the HA synthase inhibitor, 4-methylumbelliferone (4MU) for four days and assayed for anoikis. In both cell lines, 4MU

sensitized dramatically (5–8-fold) to anoikis (Fig. 4B). These results supported the important role of HA in protecting against anoikis.

Finally, we tested the role of CD44-ligand interaction in anoikis using the CD44-agonistic antibody Hermes-3, which induces downstream signaling matching that of HA as the ligand [29]. MCF10a cells were grown as three-dimensional acinar cultures in a model where anoikis occurs in order to generate luminal clearance. Growth of these acini in the presence of Hermes-3 decreased the number of apoptotic cells in the lumens of these structures (Fig. 4C).

EMT induces CD44S, but not CD44E, to interact with cytoskeleton

CD44 interacts with the cytoskeleton via ezrin, ankyrin-G and perhaps other molecules, generating signaling complexes affecting diverse cellular behaviors [30]. We hypothesized that CD44S-cytoskeletal interaction might be induced by HA, which accumulates during EMT, through the up-regulation of HAS2. HMLE + Twist-ER cells that stably expressed ectopic CD44S or CD44E were generated and assayed for CD44-cytoskeletal interaction before EMT (without 4-OHT) or after EMT (with 4-OHT) (Fig. 5). Interestingly, before EMT, both isoforms were essentially fully extractable with Triton X-100, indicative of free, non-cytoskeletally anchored receptors. By contrast, the CD44S—but not CD44E—ectopically expressed in post-EMT cells was anchored to cytoskeleton. This was consistent with the notion that HA, present in higher concentrations following EMT, induced cytoskeletal CD44 complexes involved in downstream signaling (see Discussion). These complexes are known to be involved in cell migration; an additional role in anoikis regulation is suggested.

Discussion

Our data have revealed that CD44S attenuated, while CD44E promoted anoikis. The switch from CD44E to CD44S expression accompanying EMT is functionally significant, in that it partially protects cells against anoikis. The protection by CD44S may contribute significantly to the dissemination of tumor cells throughout metastasis, complementing its established effects on migration, invasion and chemoresistance [8,12,14–20]. Accompanying EMT, HAS-2, the major enzyme that synthesizes the CD44 ligand hyaluronan, is up-regulated as well [31]. The results reported herein demonstrate that the resulting increase in CD44S and HA partially protects EMT-like cells from anoikis. The hyaluronan interaction was evidently critical for the protective effect, as only the high-affinity receptor CD44S but not the low affinity receptor CD44E conferred it. Moreover, a point mutation of the CD44S that is known to decrease HA binding affinity abrogated the protection, as did an inhibitor of HA synthase.

It should be noted that this monotypic cell culture model, while informative, suggests that other cell types may participate to promote anoikis-resistance in vivo. In particular, HA tends to accumulate at inflammatory sites [32]. In principle, the interactions of these HA-rich matrices with CD44S on tumor cells may program the latter to become better able to survive under detached conditions. This may provide a novel mechanism by which inflammation could promote tumor progression.

A plethora of interactions with CD44 endow it with multiple tumor-promoting functions. The intracellular domain of CD44 has been proposed to interact with Ezrin/Radixin/Moesin (ERM) proteins, ankyrin, and regulators of Rho/Rac/cdc42 signaling [14]. Generally, however, these interactions have not been documented in endogenous co-immunoprecipitation experiments, so their utility as readouts of HA–CD44 interaction is limited. In addition, HA-stimulated CD44 enhances the activities of several receptor tyrosine kinases (RTK), through both CD44-ligand and CD44-receptor interactions [14]. Thus, activation of PI3Kinase, src and Ras downstream of CD44–HA have been observed, although it is difficult to unravel whether these signals emanate from CD44 itself vs. RTKs that use CD44 as a co-receptor. HA has been proposed to induce the internalization of CD44, which binds to STAT3 and p300 and activates the transcriptional activity of the STAT3 in the nucleus, inducing cyclin D expression [33]. Relatedly, gamma-secretase mediated cleavage of the intracellular domain (ICD) of CD44 enhances the transforming activity of c-Ret in a cell culture based assay [34]. The implications of these mechanisms for the modulation of anoikis remain to be explored.

Superimposed upon the well established up-regulation of CD44S and HA during EMT, there is some support for a functional role of CD44S in promoting EMT itself. For example, Tumor Necrosis Factor induces CD44S expression and moesin phosphor-ylation, generating a CD44–moesin–actin complex that activates Smad2/3 and downstream induction of TGF-b target genes that program EMT [35]. A mechanistically undefined role for CD44S in Twist-induced EMT of HMLE cells has also been proposed [36]. Nevertheless, abundant evidence indicates an important role of CD44–HA interaction in tumor cell migration and invasion [37], biologically consistent with the anoikis-resistance function that we demonstrate here.

While the mechanistic basis for the anoikis-protective effect of CD44S–HA remains to be elucidated, it has potential translational value. In particular, antagonistic CD44-blocking antibodies, stimulation of HA turnover by hyaluronidases, and competition by small HA oligos may provide tools for restoring the anoikis response in tumor cells, thereby inhibiting tumor progression.

Conclusions

In this study, we have found that CD44S, the isoform that is up-regulated by EMT, partially protected mammary epithelial cells against anoikis. This effect involved the interaction of CD44S with the ligand, hyaluronan. The normal epithelial cell isoform CD44E interacted less efficiently with hyaluronan and, surprisingly, it promoted anoikis. The results indicate that the CD44 isoform switch associated with EMT serves the function of protecting cells against anoikis, an important feature of the meta-static phenotype.

Experimental procedures

Cell lines

HMLE + Twist-ER (described above, contributed by Robert Weinberg, MIT) and MCF10a (a well characterized mammary basal epithelial cell line, obtained from Karmanos Cancer Center) were cultured as described previously [22]. Briefly, HMLE medium consisted of

DMEM/F12 with 5% horse serum, 1× penicillin–streptomycin–glutamine, 10 μg/ml insulin, 10 ng/ml Epidermal Growth Factor, and 0.5 μg/ml hydrocortisone; MCF10a cells were grown in the same medium with 0.1 μg/ml cholera toxin (EMD Biosciences). Stable post-EMT populations were generated from the HMLE + Twist-ER cells by culturing the cells in the presence of 4-hydroxytamoxifen (4-OHT) for two weeks and characterizing them for EMT as described [22]. The shRNA sequence used for stable CD44 knockdown was AATGGTGTAGGTGTT ACAC, expressed in the lentiviral vector pTRIPZ (Open Biosystems, clone RHS4696-99703186), packaged and infected as described previously [22]. Knockdown cells were selected in puromycin-containing medium followed by flow sorting of RFP-positive cells resulting after 24 h of induction with doxycycline (1 μg/ml); knockdown was verified by Western blotting. For transient pan-CD44 knockdown, using distinct target sequences, Dharmacon Smart-Pool was used in conjunction with a non-targeting control siRNA, via Lipofectamine RNAi-Max, using manufacturer's protocol (Invitrogen). For isoform-specific knockdown of CD44E, three double stranded RNA oligonucleotides corresponding to the target sequences: (siRNA1): GGAAGAAGATAAAGAC-CAT, (siRNA2): TGACAACGCAGCAGAGTAA and (siRNA3): ACACAGGTTTGGTGGAAGA were synthesized with dTdT 3′ overhangs (Sigma). The three siRNAs were pooled for siRNA transfection. For ectopic expression of CD44, CD44S and E were first cloned by reverse-transcription from HMLE + Twist-ER cells with or without 4-OHT treatment (respectively) and completely sequenced. These cDNAs were re-amplified and cloned into the retroviral vectors pMIG or MSCV-IRES-puro (Addgene). To inhibit HA synthesis, cells were treated with 1 μM 4-methylumbelliferone (Sigma) for 48 h prior to the assay.

Anoikis and mammosphere assays

For anoikis assays, cells were dissociated using Tryple Express, counted and suspended at 100,000 cells per well of a 6 well Ultra Low Cluster dish (Corning) in growth media containing 0.5% methyl-cellulose. Cells were collected by diluting 4–8× with complete growth media, pelleted, and lysed in PBS + 0.5% Triton X-100 + 10 mM EDTA. Apoptosis was then quantified using either DNA Fragmentation ELISA (Roche) or a caspase activity assay [22]. Mammosphere assays were carried out as described in [23] and modified in [24]; quantitation was at 14 days post-plating.

Western blots

Protein lysates prepared in standard 1× SDS sample buffer were electrophoresed on 4–20%SDS-PAGE Tris–Glycine gels (Invitrogen). Proteins were transferred electrophoretically onto PVDF membranes (Immobilon-FL) at 50 V overnight in Tris–glycine transfer buffer containing 5% methanol. Membranes were blocked in phosphate-buffered saline containing 5% nonfat milk, and 0.1% Tween-20. Primary antibodies were diluted in blocking buffer as recommended by the manufacturer. Following incubation for 2 h (room temperature) or overnight (4°), secondary antibodies (anti-mouse or anti-rabbit, conjugated to horseradish peroxidase, Bio-Rad, diluted 1:3000 in blocking buffer) were incubated for 1 h. Chemiluminescent signal was detected using ECL-West Pico, Pierce).

Antibodies were from the following sources: CD44: Santa Cruz Biotechnology (anti-HCAM), Alpha-tubulin: Cell Signaling (clone 11H10), caspase-8: Cell Signaling (clone 1C12), RIP1: Becton-Dickinson (#610458).

RT-PCR

Total RNA was prepared from cell lines using the RNeasy Plus kit (Qiagen) and converted to cDNA using the Invitrogen Superscript III kit. qRT-PCR was conducted in 20 μl reactions, using Invitrogen Sybr-Green master mix (#4309155) and an Applied Biosystems 7500 instrument. Primers that flanked the CD44 variable exons were designed as described [25]: F: CCAAGATGATCAGCCATTCTGG and R: ATGCAA ACTGCAAGAATC. The two sets of primers used for HAS2 detection (with similar results) were: F1: GCCTC ATCTGTGGAGATGGT and R1: TCCCAGAGGTCCA CTAATGC; F2: GATGACAGGCATCTCACGAA and R2: CAGCCATTCTCGGAAGTAGG.

Detection of endogenous HA

Endogenous HA was detected using the biotinylated HA-Binding Protein (HABP) method [26]. Briefly, cells were fixed with 2% PFA in PBS, washed 2× with PBS and permeabilized with 0.3% Triton X-100 + 5% BSA for 5 min, followed by 100 mM Glycine wash. Blocking was in PBS + 5% BSA for 30 min. 3 μg/ml biotinylated-HABP (US Biological) was incubated in blocking solution overnight at 4°. 2 μg/ml Texas-Red-streptavidin (Molecular Probes) in blocking solution was incubated for 1 h at room temperature. Slides were then washed, mounted and imaged with a Zeiss Axiovert microscope.

Cell-substratum binding assay

6 well plates were coated with 50 μg/ml HA for 1 h, then washed 3 times with PBS and blocked in 2% BSA in PBS for 1 h. 50,000 cells per well were allowed to bind for 5 min and then washed 3 times with media. Bound cells were incubated with Presto-Blue viability reagent (Invitrogen) and quantified using a fluorescent plate reader (Molecular Devices).

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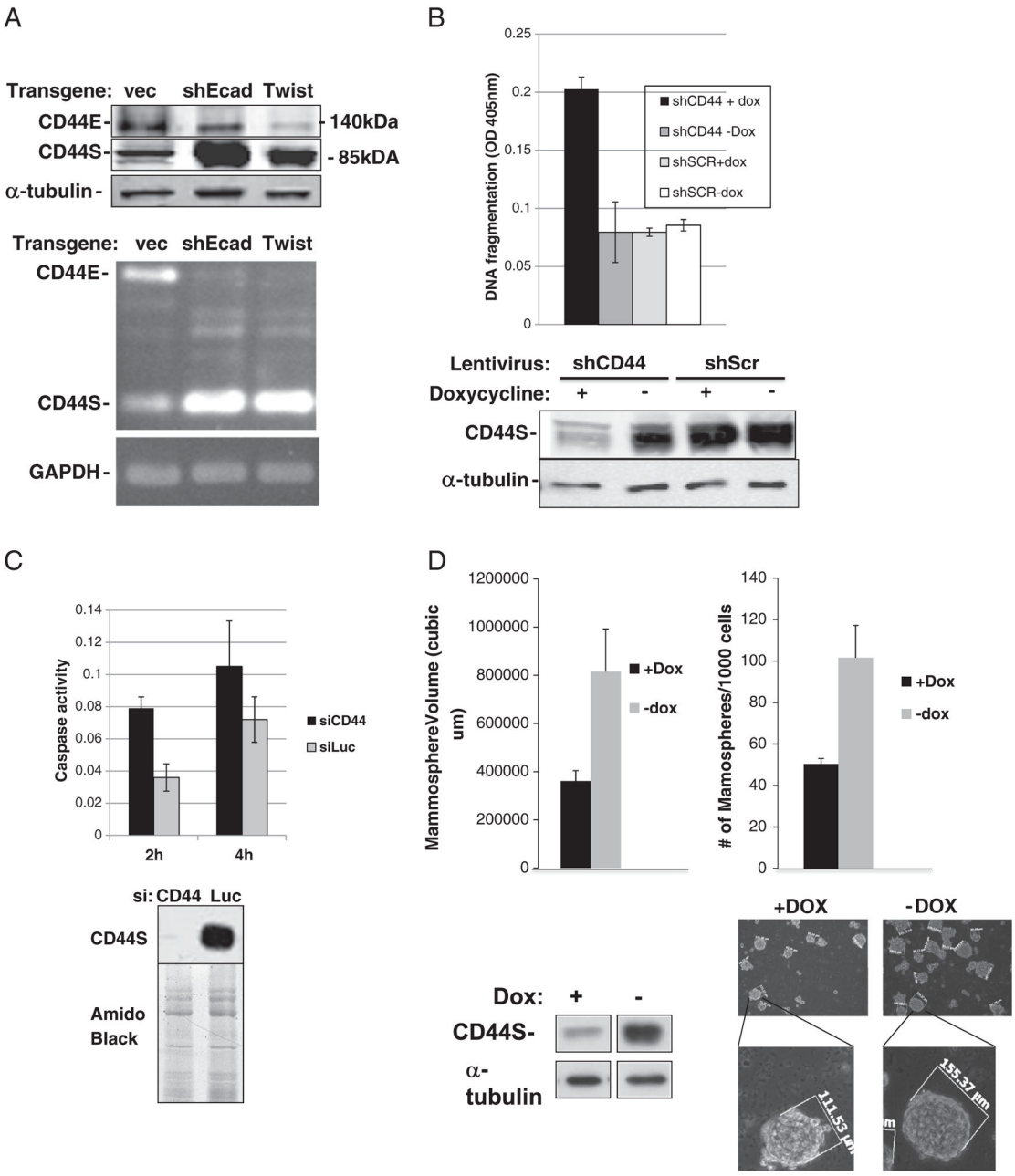

Fig. 1.

CD44S expression is induced by EMT and protects cells against anoikis. (A) EMT causes a switch from CD44E to CD44S expression. HMLE cells expressing control vector or HMLE cells that were induced to undergo EMT via shRNA for E-cadherin or by induction of retrovirally transduced Twist-ER protein with 4-hydroxytamoxifen (4-OHT). Cells were then assayed for CD44 expression via Western blotting (upper panels) or RT-PCR (lower panel). (B) Depletion of CD44 enhances anoikis. HMLE + Twist-ER cells that had been induced with 4-OHT were transduced with a doxycycline-inducible shRNA targeting CD44 (all isoforms). Anoikis was assayed in doxycycline-induced (shRNA expressing) or non-

induced cells at 24 h of suspension (upper panels). Confirmation of the knockdown is shown in the lower panel. shSCR is scrambled shRNA control; DOX is doxycycline-treated to induce shRNA expression. (C) Confirmation of specificity of knockdown effect using different CD44 targeting sequences. MCF10a cells were transfected with CD44 siRNA (Dharmacon SmartPool) followed by assaying for anoikis. siLUC is luciferase siRNA control. Anoikis assays in a–c were conducted in biological duplicates and represent one of two independent experiments. (D) Depletion of CD44 attenuates mammosphere forming capacity. HMLE + Twist-ER cells with doxycycline induction of CD44 shRNA or without doxycycline induction were assayed for average mammosphere number and mammosphere volume in triplicate; graphical result represents one of two independent experiments and N > 26 mammospheres per replicate; triplicate samples were scored (N > 78 per experimental group).

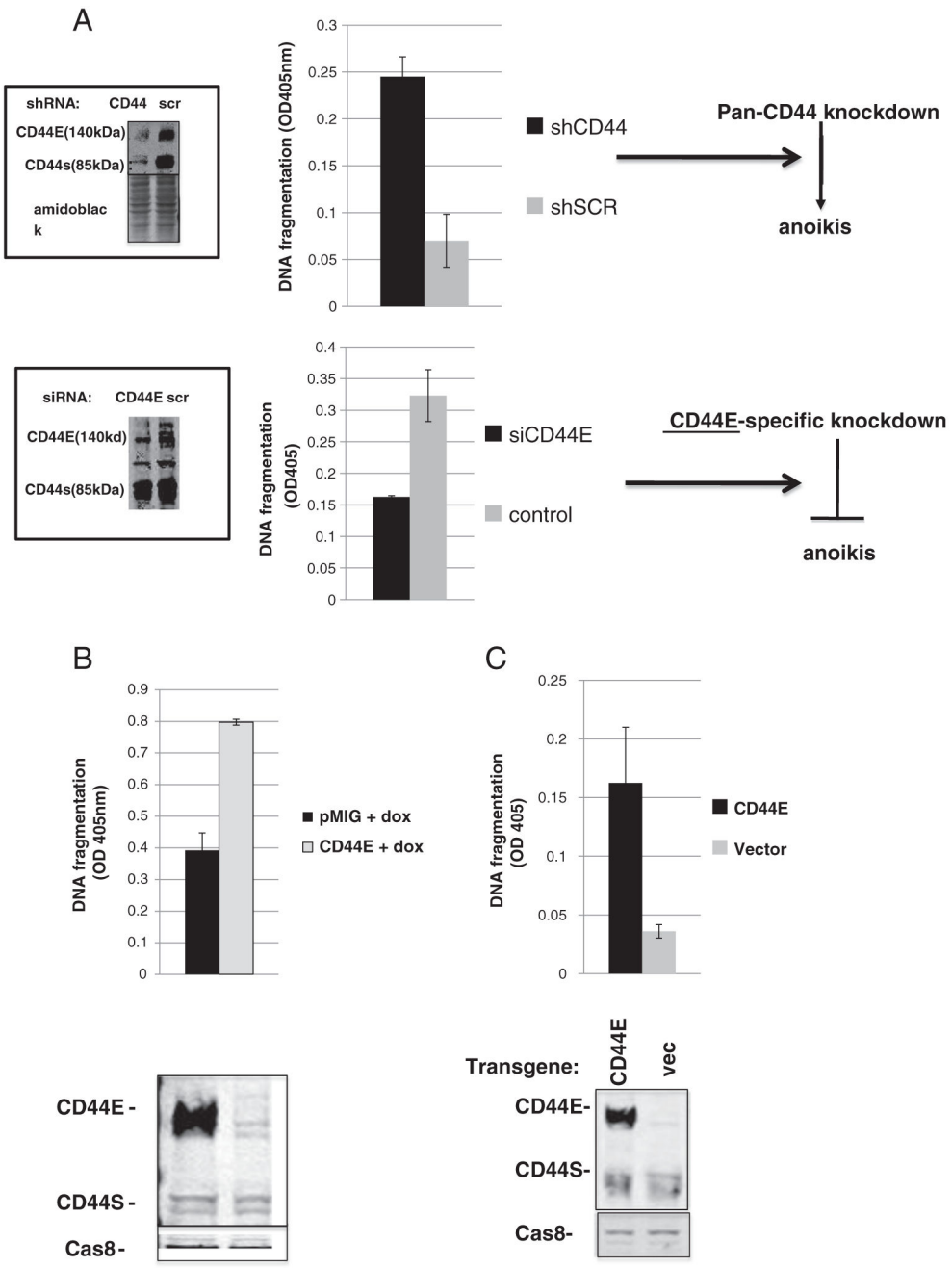

Fig. 2.

Protection from anoikis is CD44 isoform-specific. (A) Pan-CD44 knockdown sensitizes MCF10a cells to anoikis. MCF10a cells expressing doxycycline-inducible CD44 shRNA (targeting the 3′UTR) were induced with doxycycline or untreated, followed by an anoikis assay conducted at 8 h of suspension (upper panels). MCF10a cells transfected with a CD44E-specific siRNA were assayed for anoikis under the same conditions (lower panels). Confirmation of the knockdowns is shown to the left of the corresponding anoikis assays. (B, C) CD44E was ectopically expressed using a retrovirus containing the coding sequence only, without the 5′UTR or 3′UTR, in MCF10a (B) or HMLE + Twist-ER (4-OHT-treated)

cells (C) that stably expressed a doxycycline-inducible CD44 shRNA that targeted only endogenous CD44 but not ectopic CD44E (because this shRNA targeted the 3′UTR of CD44 mRNA). Cells with or without CD44E expression were then assayed for anoikis. Experiments were conducted in biological duplicate, and the results presented here are representative of two duplicate, independent experiments.

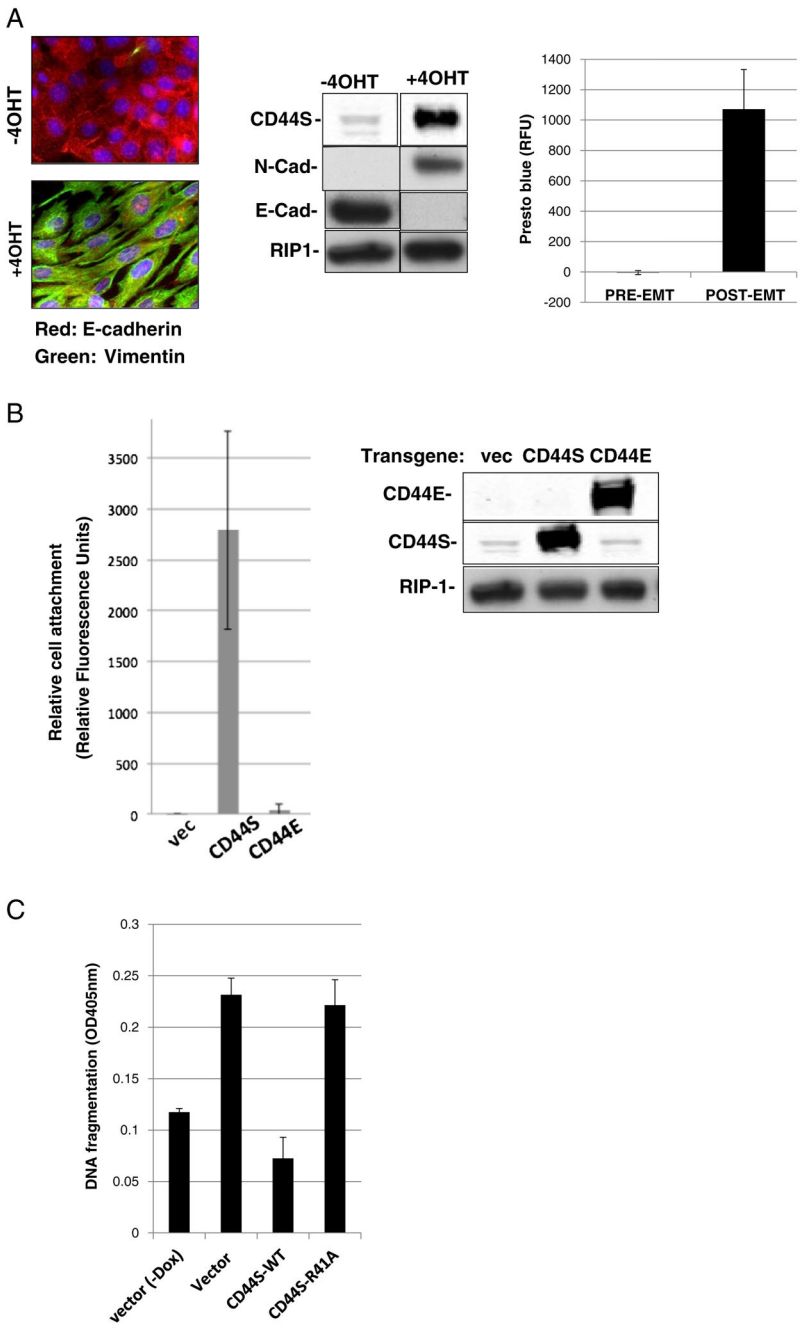

Fig. 3.

The ability of CD44 to bind HA is important for anoikis-resistance. (A) Comparison of HMLE + Twist-ER cells (containing empty retroviral vector) without 4-OHT pre-treatment (pre-EMT) or with 4-OHT pre-treatment (post-EMT) for ability to stably bind to an HA-coated surface (graph). Evidence for EMT is shown by E-cadherin and vimentin immunofluorescence (left panel) or Western blotting for EMT markers (middle panel); (B) Comparison of HMLE cells with ectopically expressed CD44S vs. CD44E for the cell binding to an HA-coated surface (note: the empty vector data point in b and a represent the

same data point, replicated for clarity of presentation.) (C) Effect of mutating the HA-binding domain of CD44S on anoikis-protection. HMLE cells with ectopic expression of CD44S (wild-type) or CD44S (R41A) mutant were assayed for anoikis. The results presented are the average of biological duplicates, and representative of two duplicate experiments.

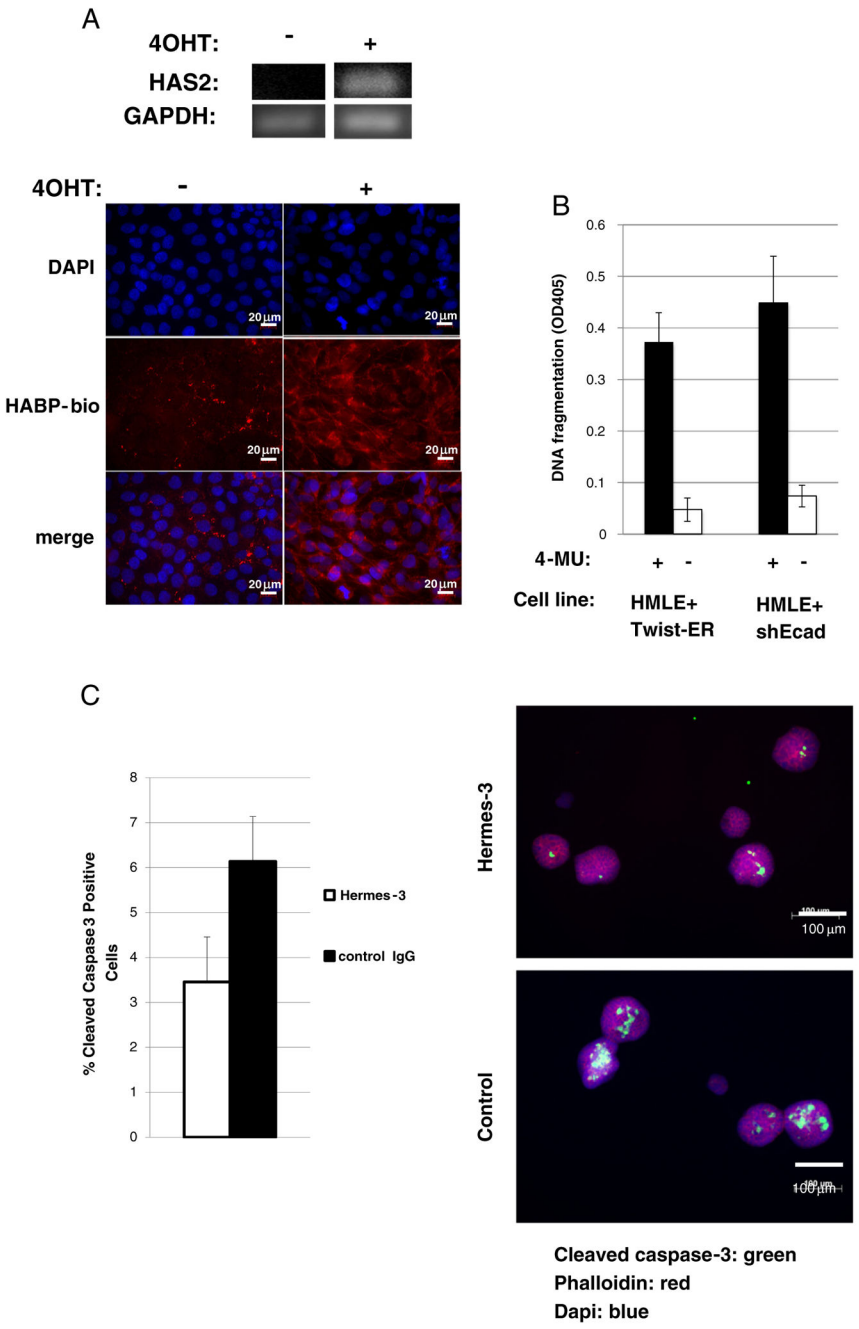

Fig. 4.

HA is important for anoikis-resistance. (A) EMT was induced in HMLE + Twist-ER cells by treatment with 4-OHT. The effect on HAS2 mRNA expression (RT-PCR, upper panel) or HA accumulation in the matrix, assayed by fluorescent HABP detection (lower panels) are shown. (B) HMLE cells that had undergone EMT due to shEcad or Twist-ER expression were treated with the HAS inhibitor 4-MU (1 µM) prior to assaying for anoikis. Time zero values were subtracted from the 24-h values. Biological replicates were performed on two independent cell lines. (C) An agonistic antibody, Hermes-3, was used to stimulate CD44

signaling in MCF10a cells, which were then assayed for anoikis using an acinar morphogenesis/ cleaved caspase-3 assay. Assessed 32 acini per group each for % cleaved caspase 3 positive cells. Each acinus consisted of an average of 309 cells for control and 403 cells for experimental, counted via DAPI stain.

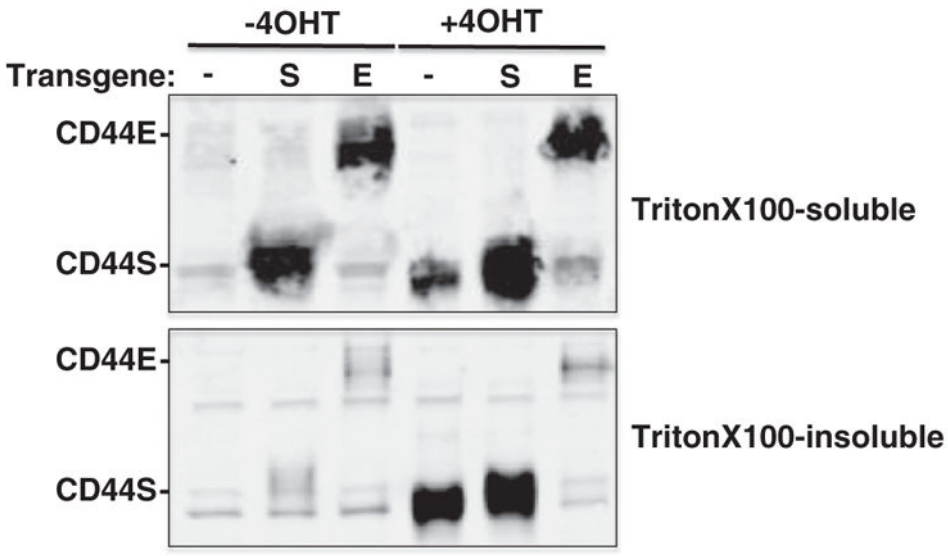

Fig. 5.

EMT induces cytoskeletal association of CD44S. HMLE–Twist-ER cells that were induced to undergo EMT (with 4-OHT) or untreated were transduced with CD44S or CD44E expression vectors. Cells were then fractionated as described in Experimental Procedures and assayed for free vs. cytoskeleton-associated CD44 by Western blotting.