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Research Article **Oncology**

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Epithelial-mesenchymal transition (EMT) is a tightly regulated process that is critical for embryogenesis but is abnormally activated during cancer metastasis and recurrence. Here we show that a switch in CD44 alternative splicing is required for EMT. Using both in vitro and in vivo systems, we have demonstrated a shift in CD44 expression from variant isoforms (CD44v) to the standard isoform (CD44s) during EMT. This isoform switch to CD44s was essential for cells to undergo EMT and was required for the formation of breast tumors that display EMT characteristics in mice. Mechanistically, the splicing factor epithelial splicing regulatory protein 1 (ESRP1) controlled the CD44 isoform switch and was critical for regulating the EMT phenotype. Additionally, the CD44s isoform activated Akt signaling, providing a mechanistic link to a key pathway that drives EMT. Finally, CD44s expression was upregulated in high-grade human breast tumors and was correlated with the level of the mesenchymal marker N-cadherin in these tumors. Together, our data suggest that regulation of CD44 alternative splicing causally contributes to EMT and breast cancer progression.

Introduction

Tumor recurrence and metastasis represent the two major obstacles in the successful treatment of cancer. Emerging lines of evidence suggest that the aggressive phenotype of this disease is associated with epithelial-mesenchymal transition (EMT), a developmental process in which epithelial cells lose polarity and change to a mesenchymal phenotype (1–4). EMT plays a fundamental role in developmental processes including mesoderm and neural tube formation. Key characteristics of EMT include a morphological change from a cobblestone-like epithelial appearance to an elongated, spindle-like fibroblastic shape, cytoskeletal reorganization, cadherin switching that involves downregulation of epithelial E-cadherin and upregulation of mesenchymal N-cadherin, enhanced resistance to cell death, and acquisition of a migratory phenotype. Among these characteristics, cell death resistance may well explain the importance of EMT in tumor recurrence, in which malignant cells survive chemotherapy or radiation treatment, and in metastasis, in which tumor cells avoid apoptosis when disseminating to distal organs. Therefore, it is of critical importance to understand the mechanisms by which EMT is regulated in order to develop effective therapeutic strategies for the treatment of recurrent and metastatic cancer.

Previous studies have revealed that EMT can be transcriptionally regulated by a family of transcription repressors, including Snail, Twist, Slug, and Zeb1/2, that suppress E-cadherin expression (3, 5–7). EMT is also regulated by microRNAs such as miR-200, miR-155, and miR-9 that target key proteins involved in EMT (8–11). However, the potential role of alternative splicing, which represents another important mechanism of gene regulation, in EMT and the aggressive cellular behavior that contributes to cancer progression remains unclear.

Alternative RNA splicing is a process by which cells generate multiple protein products from a single gene, thereby contributing to the complexity of mammalian genomes. It is estimated that nearly all mammalian genes undergo alternative splicing (12, 13), and observational studies have also indicated that aberrant alternative splicing frequently occurs in cancer (14–17). These findings suggest a role for alternative splicing in cancer progression, but a direct link has not yet been established. In this study, we specifically addressed this issue by studying the *CD44* gene. CD44 is a cell surface protein that modulates cellular signaling by forming coreceptor complexes with various receptor tyrosine kinases (18–21). Through alternative splicing, cells produce a family of CD44 protein isoforms that are involved in multiple distinct cellular functions, including proliferation, adhesion, and migration (22). Our results reveal that CD44 alternative splicing is differentially regulated during EMT, resulting in a switch in expression from the variable exon–containing CD44v isoforms to the standard isoform, CD44s, which is devoid of all CD44 variable exons. We also establish that the switch in expression to CD44s, mediated by changes in alternative splicing, accelerates both EMT and breast cancer progression. Finally, we demonstrate that the mesenchymal CD44s isoform is upregulated in advanced human breast tumors. Given the prevalence of alternative splicing in humans, these data thus suggest that regulation at the level of alternative splicing constitutes a critical mechanism in controlling EMT and cancer progression.

Results

A switch in CD44 isoform expression occurs during EMT. To assess the regulation of CD44 alternative splicing during EMT, we used an inducible EMT system in which immortalized human mammary

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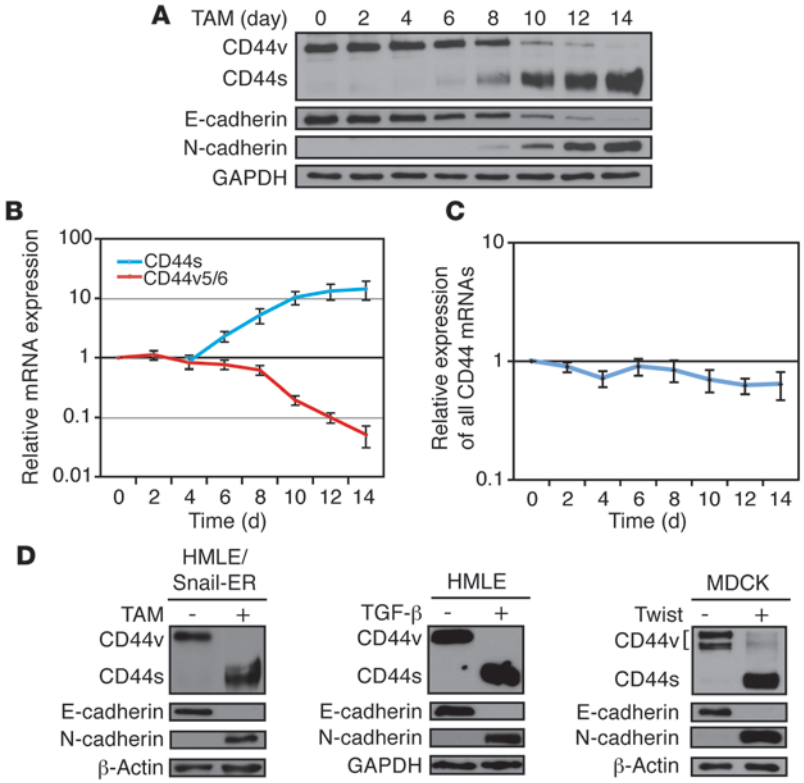

Figure 1
Switch in CD44 isoform expression during EMT. (**A**) Immunoblot analysis of CD44 isoforms during tamoxifen-induced (TAM-induced) EMT in HMLE cells expressing Twist-ER (HMLE/Twist-ER). The CD44 antibody recognizes CD44v and CD44s isoforms, although with conceivably higher affinity for CD44s. Immunoblots of EMT markers E-cadherin and N-cadherin confirm that these cells undergo EMT. (**B**) qRT-PCR analysis of levels of CD44 isoforms using primers that specifically detect either CD44s or CD44v containing variable exons v5 and v6 (v5/6). Relative expression levels of CD44s or CD44v5/6 in TAM-treated cells were normalized to untreated cells at each time point, and the results are shown relative to day 0. Error bars indicate SD; $n = 4$. (**C**) Relative mRNA levels of all CD44 isoforms in TAM-treated cells were normalized to untreated cells at each time point, and results are shown relative to day 0. Error bars indicate SD; $n = 4$. (**D**) Immunoblot analysis of CD44 isoforms during EMT induced by tamoxifen-mediated Snail-ER translocation to the nucleus in HMLE/Snail-ER cells (left), TGF-β (5 ng/ml) treatment in HMLE cells (middle), or Twist expression in MDCK cells (right). Immunoblots of EMT markers E-cadherin and N-cadherin confirm that these cells undergo EMT.

epithelial cells stably express Twist-ER (HMLE/Twist-ER). In this system, tamoxifen treatment leads to nuclear translocation of Twist-ER, causing the cells to undergo EMT in 12–14 days (23). Completion of EMT is indicated by loss of the epithelial marker E-cadherin, acquisition of the mesenchymal marker N-cadherin, and transition to a fibroblastic cellular morphology (ref. 23; Figure 1A shows the cadherin switch from E-cadherin expression in epithelial cells to N-cadherin expression in mesenchymal cells, and Supplemental Figure 1A [supplemental material available online with this article; doi:10.1172/JCI44540DS1] indicates morphological changes during EMT).

A striking observation is that CD44 isoform expression undergoes a switch during EMT (Figure 1). Using a CD44 antibody that recognizes all CD44 isoforms, we found that the epithelial cells predominantly expressed the alternatively spliced CD44v. Upon tamoxifen-induced Twist-ER translocation, CD44v was gradually converted to CD44s, with the initial appearance of CD44s at day 6. When EMT was complete at day 14, CD44s was almost exclusively expressed (Figure 1A). Quantitative RT-PCR (qRT-PCR) analysis of *CD44* mRNA using isoform-specific primers (Supplemental Figure 1B) showed that expression of CD44v containing exons v5/v6 was decreased 13-fold, while expression of CD44s was increased 14-fold during EMT (Figure 1B). The switch in CD44 isoform expression was also evidenced by the ratio of CD44s to CD44v, which increased approximately 3-fold by day 6, rising further to 280-fold at day 14 (Supplemental Figure 1C). Importantly, total levels of CD44 transcription varied by less than 2-fold throughout the EMT time course. These data indicate that the switch in CD44 isoform expression represents a change in CD44 alternative splicing, rather than an alteration in baseline CD44 transcription (Figure 1C). The shift in CD44 isoform expression was specifically associated with EMT, as culturing the HMLE/Twist-ER cells in the absence of tamoxifen or the parental HMLE cells in the presence of tamoxifen for 14 days did not cause a change in CD44 isoform expression (Supplemental Figure 2).

To establish that CD44 isoform switching during EMT is a general phenomenon and not unique to Twist-induced EMT in HMLE cells, we analyzed the levels of CD44 isoforms during EMT using different EMT inducers (Twist, Snail, or TGF-β) and epithelial cell lines (HMLE or Madin-Darby canine kidney [MDCK]). We found that a switch in CD44 isoform expression from CD44v to CD44s was indeed a common feature that occurred during EMT (Figure 1D). These results indicate that, rather than changing overall CD44 transcription, cells primarily employ alternative splicing regulation to produce the CD44s isoform during EMT.

Knockdown of CD44 inhibits EMT. We hypothesized that the switch in expression to the CD44s isoform is a critical process required for cells to undergo EMT. To test this idea, we assessed whether eliminating CD44 impairs EMT induction in the HMLE/Twist-ER cells. We first knocked down CD44 expression in these epithelial cells using an shRNA that targets all forms of CD44 (24). CD44 depletion alone in the epithelial cells did not cause a detectable effect on the levels of epithelial markers, suggesting that loss of CD44v in these cells does not induce EMT (Figure 2A). Upon treatment with tamoxifen for 14 days, control luciferase shRNA-expressing cells underwent EMT, as evidenced by decreased levels of epithelial markers E-cadherin, γ-catenin, and occludin, increased levels of mesenchymal markers N-cadherin and vimentin (Figure 2A, compare control shRNA lanes before and after tamoxifen treatment), and the acquisition of a mesenchymal spindle-like morphology (Figure 2B). In contrast, cells with diminished CD44 expression were resistant to EMT induction by tamoxifen treatment. The

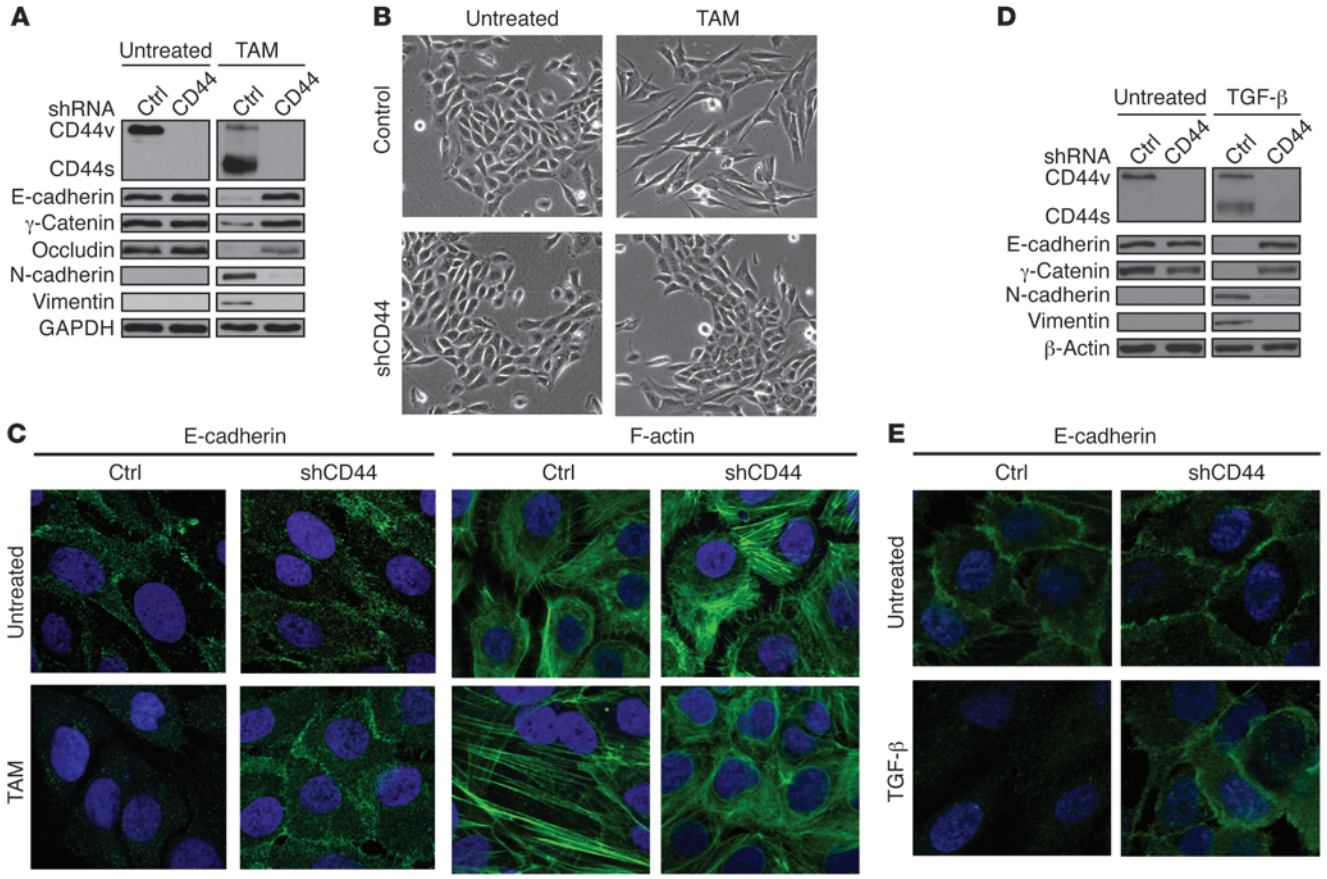

Figure 2

Depletion of CD44 inhibits EMT. (**A**) Immunoblot analysis of EMT markers in HMLE/Twist-ER cells expressing luciferase (control) or CD44 shRNA targeting all CD44 isoforms before (untreated) and after 14 days of tamoxifen treatment. Upon TAM treatment, CD44 shRNA–expressing cells show impaired downregulation of epithelial markers and upregulation of mesenchymal markers. (**B**) Phase contrast images (×10) illustrating impaired morphological changes in cells expressing CD44 shRNA (shCD44) after 14 days of TAM treatment compared with control cells. (**C**) Immunofluorescence images (original magnification, ×63) indicating that, unlike control cells, shCD44 cells maintain E-cadherin localization at cell junctions and do not undergo cytoskeletal reorganization after 12 days of TAM treatment. Green staining indicates E-cadherin or F-actin (left or right panels, respectively). DAPI staining (blue) indicates nuclei. (**D**) Immunoblot analysis of EMT markers in HMLE cells expressing control luciferase shRNA (Ctrl) or CD44 shRNA before (untreated) and after 18 days of 5 ng/ml TGF-β treatment. Upon TGF-β treatment, CD44 shRNA–expressing cells show impaired downregulation of epithelial markers and upregulation of mesenchymal markers. (**E**) Immunofluorescence images (original magnification, ×60) demonstrating that E-cadherin localization is preserved at cell junctions in CD44 shRNA–expressing cells after 18 days of TGF-β treatment. Green staining indicates E-cadherin, and blue staining indicates nuclei.

CD44 shRNA–expressing cells showed impaired downregulation of epithelial markers and upregulation of mesenchymal markers and maintained a cobblestone-like epithelial appearance (Figure 2, A and B). Furthermore, immunofluorescence analysis of tamoxifen-treated control cells showed a loss of E-cadherin localization at cell-cell junctions as well as a reorganized cytoskeletal structure, as indicated by F-actin staining. However, the CD44-silenced cells preserved high expression of E-cadherin at cell-cell junctions and exhibited an unchanged cytoskeletal structure (Figure 2C). These morphological and molecular results demonstrate that silencing CD44, thus preventing the upregulation of CD44s, inhibits EMT.

The critical role of CD44 in promoting EMT was also evident when HMLE cells were treated with the cytokine TGF-β to induce EMT. As anticipated, exposure of control HMLE cells to TGF-β caused them to undergo EMT in 18 days (Figure 2D). Interestingly, cells that expressed CD44 shRNA showed impaired transition to a mesenchymal phenotype upon TGF-β treatment. These cells maintained a cobblestone-like morphology indicative of epithelial cells, expressed high levels of epithelial markers, preserved E-cadherin at cell-cell junctions, and showed impaired upregulation of mesenchymal markers (Figure 2, D and E, and Supplemental Figure 3). These data, again, emphasize that CD44 knockdown inhibits EMT.

The CD44s isoform is essential for EMT. We next sought to address the isoform specificity of CD44 in promoting EMT. Strikingly, we found that the impaired EMT phenotype observed in the CD44-silenced cells could be rescued by reexpressing the mesenchymal CD44s isoform, but not the epithelial CD44v isoform. As shown in Figure 3A, we introduced, separately, shRNA-refractory CD44v or CD44s cDNA into the CD44-knockdown HMLE/Twist-ER cells, thereby restoring CD44v expression to a level comparable to that observed in the control luciferase shRNA–expressing epithelial cells, or CD44s expression to a level similar to that seen in the control mesenchymal cells. Reexpression of CD44s alone was

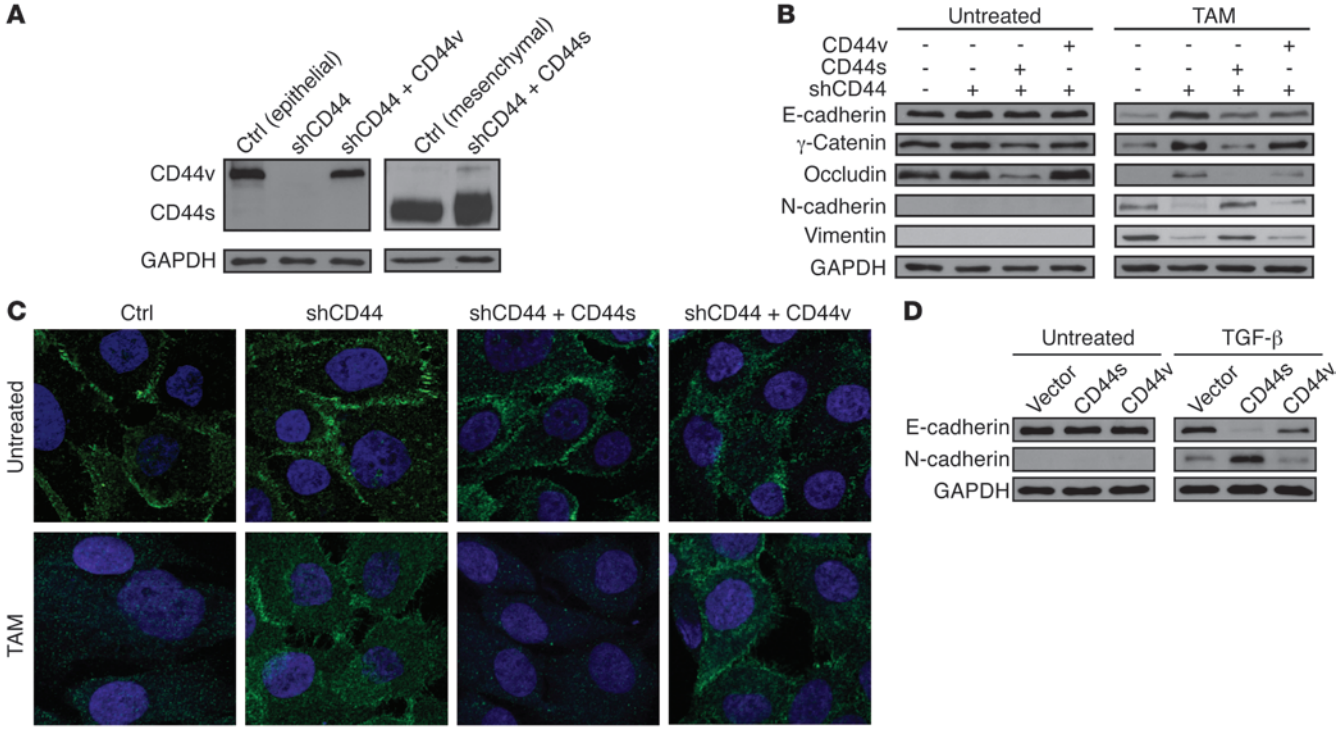

Figure 3

The specific CD44s isoform is essential for EMT. (**A**) Immunoblot analysis of CD44 in HMLE/Twist-ER cells expressing CD44 shRNA (shCD44) and reconstituted with CD44s or CD44v. (**B**) Immunoblot analysis of EMT markers in cells reconstituted with CD44s or CD44v before (untreated) and after 12 days of TAM treatment, indicating that reconstituting CD44s, but not CD44v, rescues the impaired EMT phenotype in shCD44 cells. (**C**) Immunofluorescence images (original magnification, ×63) of cells expressing control, shCD44, or shCD44 reconstituted with CD44s or CD44v, before and after 12 days of TAM treatment. Green staining indicates E-cadherin. DAPI staining (blue) indicates nuclei. (**D**) Immunoblot analysis of EMT markers in MCF10A cells expressing CD44s or CD44v before (untreated) and after 20 days of TGF-β (1 ng/ml) treatment, indicating that CD44s expression accelerates TGF-β–induced EMT.

sufficient to cause a decrease in the level of the epithelial marker occludin in the untreated epithelial cells (Figure 3B). Furthermore, CD44s reexpression fully rescued the impaired EMT observed in the CD44-knockdown cells, evidenced by decreased expression of epithelial markers E-cadherin, γ-catenin, and occludin and increased expression of mesenchymal markers N-cadherin and vimentin following tamoxifen treatment (Figure 3B). In contrast, expression of CD44v was not able to rescue the impaired EMT phenotype. Reconstitution of the CD44s isoform also led to loss of E-cadherin at cell-cell junctions following tamoxifen treatment, as shown in Figure 3C. However, cells in which CD44v had been reexpressed maintained E-cadherin at cell-cell junctions, emphasizing the importance of the CD44s isoform in promoting EMT.

To further demonstrate isoform specificity of CD44s in accelerating EMT, we used TGF-β–induced EMT in normal mammary MCF10A cells as an additional model. In MCF10A cells, the endogenous level of CD44 was relatively low. However, when the MCF10A cells were exposed to TGF-β, we observed elevated expression of CD44s (Supplemental Figure 4A). Interestingly, we found that ectopic expression of CD44s (Supplemental Figure 4B) enhanced TGF-β–induced EMT in MCF10A cells. As shown in Figure 3D, after 20 days of TGF-β treatment, CD44s-expressing MCF10A cells showed an approximately 4-fold increase in N-cadherin expression and undetectable E-cadherin expression compared with control cells. In contrast, expression of CD44v failed to accelerate EMT. In addition to the cadherin switch observed during EMT, another key functional characteristic of EMT is increased cell motility. We therefore overexpressed CD44s in MDCK cells, a cell line commonly utilized for migration assays, to determine the effect of CD44s on motility. Indeed, ectopic expression of CD44s in MDCK cells resulted in enhanced cell migration in Transwell assays (Supplemental Figure 5). Together, these results demonstrate that the CD44s isoform is critical for EMT.

The splicing factor ESRP1 regulates CD44 isoform switching during EMT. The above findings led us to investigate the mechanisms controlling the switch in CD44 isoform expression during EMT. Splicing factors are known to play a critical role in regulating alternative splicing by directly modulating splice exon usage. Given that the splicing factor ESRP1 (epithelial splicing regulatory protein 1) has been implicated in promoting CD44 alternative splicing (25), we tested whether ESRP1 affects CD44 alternative splicing in the HMLE/Twist-ER inducible EMT system. In agreement with previous results indicating that ESRP1 is an epithelium-specific splicing factor (25), we found that *ESRP1* was highly expressed in untreated epithelial HMLE/Twist-ER cells. Additionally, we demonstrated that treating these cells with tamoxifen caused a gradual decrease in the level of *ESRP1*, culminating in a 31-fold downregulation by day 14, when EMT was complete (Figure 4A). Interestingly, this decline in *ESRP1* expression coincided with the switch in expression from CD44v to CD44s (Figure 1, A and B). These

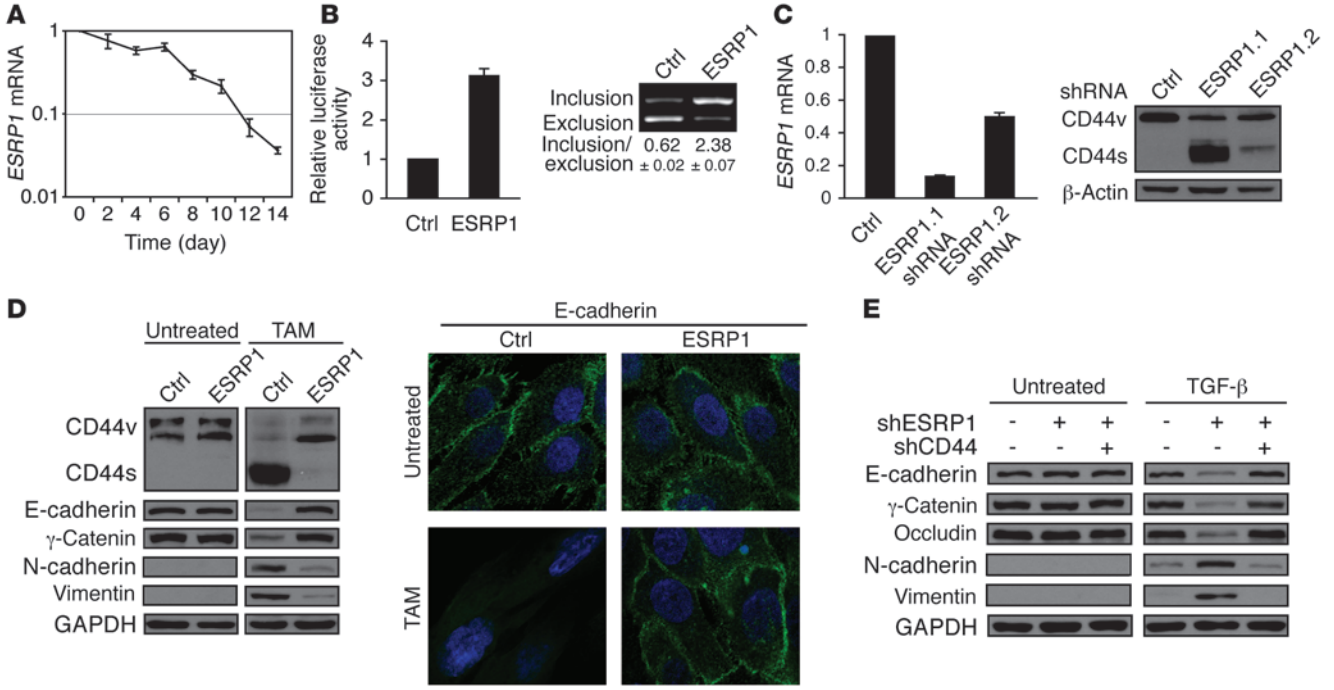

Figure 4

The splicing factor ESRP1 regulates CD44 isoform switching during EMT. (**A**) qRT-PCR analysis of *ESRP1* levels in HMLE/Twist-ER cells during TAM-induced EMT. (**B**) Relative luciferase activity (left) and semiquantitative RT-PCR analysis (right) in 293 cells cotransfected with ESRP1 and a CD44v5 luciferase reporter construct, indicating that ESRP1 promotes inclusion of CD44 variable exons. (**C**) Left: qRT-PCR analysis showing knockdown efficiency of ESRP1 in HMLE cells using two different shRNAs. Right: Immunoblot analysis showing decreased expression of CD44v and increased expression of CD44s in HMLE cells in which ESRP1 has been silenced. (**D**) Immunoblot analysis of CD44 and EMT markers in control and ESRP1-overexpressing HMLE/Twist-ER cells before (untreated) and after 14 days of TAM treatment (left), demonstrating that ESRP1 overexpression inhibits both the CD44 isoform switch and EMT. Immunofluorescence imaging (right) shows that overexpression of ESRP1 results in maintenance of E-cadherin at cell junctions during TAM-induced EMT in HMLE/Twist-ER cells, as compared with control cells. (**E**) Immunoblot analysis of EMT markers in HMLE cells expressing ESRP1 shRNA alone or in combination with CD44 shRNA before (untreated) and after 14 days of TGF-β treatment. Silencing ESRP1 promotes EMT, and loss of CD44 in the ESRP1-silenced cells leads to preservation of expression of epithelial markers E-cadherin, γ-catenin, and occludin and impaired upregulation of mesenchymal markers N-cadherin and vimentin following TGF-β treatment.

results raised the possibility that ESRP1 promotes the production of CD44v through regulation of alternative splicing, such that the decrease in expression of ESRP1 during EMT favors the downregulation of CD44v and the switch in expression to CD44s.

To confirm that CD44 alternative splicing is stimulated by ESRP1, we used a CD44v5 reporter minigene construct that contains the CD44v5 exon and its flanking introns located upstream of a luciferase gene (Supplemental Figure 6A). Inclusion of the CD44v5 exon results in luciferase expression; therefore, luciferase activity provides a direct readout of the level of CD44 alternative splicing. When cotransfecting 293 cells with ESRP1 and the CD44v5 minigene, we observed a 3.2-fold increase in luciferase activity compared with control (Figure 4B and Supplemental Figure 6B). To more directly assess CD44v5 inclusion, we also used semiquantitative RT-PCR to analyze the levels of v5 inclusion and exclusion in mRNA isolated from the transfected 293 cells. Analysis of the ratios of v5 inclusion to exclusion showed that ESRP1 overexpression caused a 3.8-fold increase in v5 inclusion compared with control (Figure 4B). We next examined the effects of ESRP1 on the endogenous levels of CD44 isoforms in HMLE cells. Knockdown of ESRP1 by two different shRNAs in these cells resulted in a shift in expression from CD44v to CD44s, while the transcription level of CD44 varied only slightly (Figure 4C and Supplemental Figure 6C). Together, these results indicate that ESRP1 stimulates CD44 alternative splicing, thus promoting the production of CD44v. These data also suggest that ESRP1 may act to maintain an epithelial cell state by preventing the switch from CD44v to CD44s.

To test whether overexpression of ESRP1 impairs EMT by inhibiting the isoform switch from CD44v to CD44s, we ectopically expressed ESRP1 in the HMLE/Twist-ER cells (Supplemental Figure 7A) and exposed them to tamoxifen to induce EMT. In contrast to control cells, which displayed a shift in expression from CD44v to CD44s during tamoxifen-induced EMT, ESRP1-overexpressing cells did not exhibit this switch in isoform expression. Even after 14 days of tamoxifen treatment, these cells maintained high levels of CD44v expression, while control cells predominantly expressed CD44s (Figure 4D). Furthermore, ESRP1 overexpression prevented cells from undergoing EMT, as indicated by the preservation of high levels of the epithelial markers E-cadherin and γ-catenin, impaired upregulation of the mesenchymal markers N-cadherin and vimentin, and the preservation of E-cadherin at cell junctions and a cobblestone-like epithelial morphology after tamoxifen treatment (Figure 4D and Supplemental Figure 7B). These results suggest that ESRP1

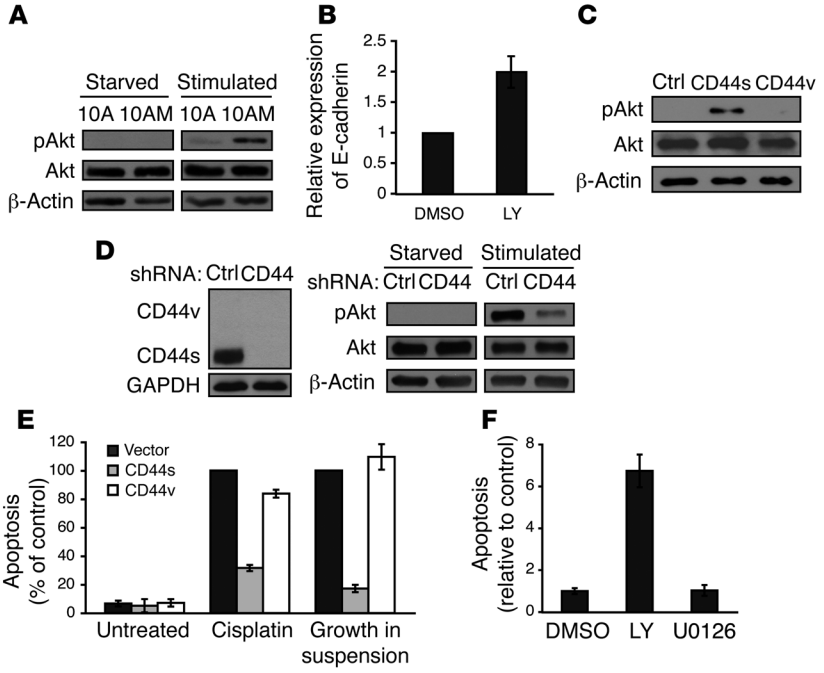

Figure 5

CD44s potentiates Akt activation and promotes cell survival in TGF-β–induced mesenchymal MCF10A cells. (**A**) Immunoblot analysis of activated Akt (pAkt) in epithelial MCF10A cells (10A) and TGF-β–induced mesenchymal MCF10A cells (10AM), indicating an increased level of Akt phosphorylation in mesenchymal MCF10A cells. Cells were starved for 24 hours and then stimulated with 2 μg/ml insulin. (**B**) qRT-PCR analysis of E-cadherin levels in TGF-β–treated mesenchymal MCF10A cells treated with DMSO (control) or the PI3K inhibitor LY-294002 (LY; 50 μM) for 20 hours. Error bars indicate SD; $n = 3$. (**C**) Immunoblot analysis of pAkt in TGF-β–treated MCF10A cells expressing vector (Ctrl), CD44s, or CD44v. (**D**) Left: Knockdown efficiency of CD44 shRNA in TGF-β–treated MCF10A cells that predominantly express CD44s. Right: Levels of pAkt after serum starvation for 24 hours, followed by insulin (1 μg/ml) stimulation for 30 minutes, showing that silencing CD44s impairs insulin-stimulated Akt activation. (**E**) Results of apoptosis assays showing that ectopic expression of CD44s, but not CD44v, inhibits apoptosis in TGF-β–induced mesenchymal MCF10A cells when treated with cisplatin (100 μM) or cultured in suspension. Error bars indicate SEM; $n = 3$. (**F**) Results of apoptosis assays showing that treatment with LY-294002, but not the MEK inhibitor U0126, causes an increase in apoptosis in CD44s-expressing TGF-β–induced mesenchymal MCF10A cells. Error bars indicate SEM; $n = 4$.

overexpression inhibits EMT by stimulating CD44 alternative splicing, which promotes high levels of CD44v and prevents the isoform switch from CD44v to CD44s.

To test whether ESRP1 acts directly through regulation of CD44 alternative splicing to inhibit EMT, we examined the effects of ESRP1 knockdown on TGF-β–induced EMT in HMLE cells. We found that after only 14 days of TGF-β treatment, at which time control cells were still epithelial in nature, the majority of the ESRP1-knockdown cells had already undergone EMT, evidenced by a decrease in levels of the epithelial markers E-cadherin, γ-catenin, and occludin, an increase in the levels of the mesenchymal markers N-cadherin and vimentin, and loss of E-cadherin expression at cell junctions (Figure 4E and Supplemental Figure 7). These results indicate that silencing ESRP1 accelerates EMT. We hypothesized that if CD44 is the major downstream splice target of ESRP1 affecting EMT, depleting CD44 in the ESRP1-knockdown cells (Supplemental Figure 7, C and D) would offset the accelerated EMT observed in the ESRP1-silenced cells. Indeed, as shown in Figure 4E and Supplemental Figure 7E, shRNA knockdown of CD44 in the ESRP1-silenced HMLE cells prevented these cells from undergoing an accelerated EMT. These results indicate that ESRP1 acts primarily on CD44 alternative splicing to regulate EMT. Collectively, our findings reveal that ESRP1 negatively regulates EMT by preventing the isoform switch from CD44v to CD44s. Thus, the decrease in expression of ESRP1 during EMT facilitates the CD44 isoform switching that is required for the transition to the mesenchymal state.

CD44s mediates activation of Akt during EMT. Our next objective was to identify downstream effectors of CD44s that are able to drive EMT. Because CD44 acts on the cell surface and is known to affect several signaling cascades (22), we wished to determine whether CD44s alters signaling nodes involved in promoting EMT. Using a phosphoarray assay that simultaneously detects a panel of activated receptor tyrosine kinases and key downstream effectors, we first identified signaling molecules whose activities were enhanced upon TGF-β–induced EMT in MCF10A cells. Interestingly, one of the major changes detected in this screen was elevated Akt activation in the TGF-β–treated cells (Figure 5A and Supplemental Figure 8), consistent with previous findings demonstrating that the PI3K/Akt signaling cascade is a central pathway that promotes EMT (26–32). Previous work has shown that overexpressing a constitutively activated viral Akt promotes EMT, whereas expression of a dominant negative form of Akt inhibits EMT (26, 28). Indeed, when treating TGF-β–induced mesenchymal MCF10A cells with the PI3K inhibitor LY-294002, we observed a 2-fold upregulation of the epithelial marker E-cadherin (Figure 5B and Supplemental Figure 9A), supporting the importance of Akt signaling in potentiating EMT. To determine whether CD44s promotes Akt activation, we compared the level of Akt phosphorylation in TGF-β–treated mesenchymal MCF10A cells expressing a control vector, CD44s, or CD44v. As shown in Figure 5C, under normal growth conditions, the level of activated Akt was very low in control TGF-β–treated mesenchymal MCF10A cells. Ectopic expression of CD44s augmented the activity of Akt in these cells. However, ectopic expression of CD44v was not able to increase the level of phosphorylated Akt under these conditions, suggesting that only the CD44s isoform promotes Akt activation in TGF-β–treated mesenchymal MCF10A cells. This observation was further strengthened by analysis of Akt activation in the CD44s-depleted mesenchymal MCF10A cells. Treatment of control mesenchymal MCF10A cells with insulin resulted in an increase in Akt phosphorylation. However, this phosphorylation was greatly impaired in cells in which CD44s had been silenced (Figure 5D). Collectively, these data indicate that CD44s plays a critical role in Akt activation during TGF-β–induced EMT in MCF10A cells.

Given that cell death resistance is a key feature of EMT and that Akt activation promotes cell survival, we next examined whether CD44s affects cell death sensitivity through Akt activation. As

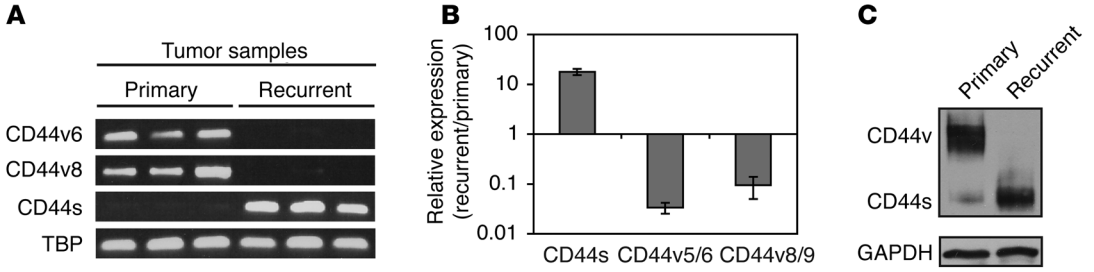

Figure 6
A change in expression of CD44 isoforms occurs during murine breast tumor progression. (**A**) Semiquantitative RT-PCR analysis of CD44 isoforms indicating differential CD44 isoform expression during progression from HER2/*Neu*-dependent epithelial primary tumors to HER2/*Neu*-independent mesenchymal recurrent tumors. (**B**) qRT-PCR analysis of CD44 isoforms in primary and recurrent tumors. Results are shown as fold change in mRNA level in recurrent tumors relative to that of the primary tumors. Error bars represent SEM; $n = 6$. (**C**) Immunoblot analysis of CD44 isoforms in HER2/*Neu* primary and recurrent tumor cell lines.

shown in Figure 5E, subjecting TGF-β–induced mesenchymal MCF10A cells to the chemotherapy drug cisplatin or growth in suspension, which mimics loss of matrix attachment, induced cell death. Expression of CD44s in these cells resulted in a 3- or 6-fold inhibition of apoptosis caused by cisplatin or growth in suspension, respectively. In contrast, ectopic expression of CD44v failed to inhibit apoptosis (Figure 5E). These findings indicate that only CD44s promotes cell survival. To confirm that the observed apoptotic resistance was dependent on Akt signaling, we treated the CD44s-expressing mesenchymal MCF10A cells with the PI3K inhibitor LY-294002 and subsequently analyzed cisplatin-induced apoptosis. Cells incubated with LY-294002 showed an approximately 7-fold increase in apoptosis compared with control, indicating that Akt inhibition greatly abolishes the antiapoptotic effect conferred by CD44s (Figure 5F and Supplemental Figure 9B). These results suggest that Akt acts downstream of CD44s to promote cell survival. In contrast, treatment of cells with the MEK inhibitor U0126 did not significantly increase apoptotic sensitivity to cisplatin, supporting the notion that CD44s potentiates cell survival mainly via Akt signaling (Figure 5F and Supplemental Figure 9C). Furthermore, we found that the level of the antiapoptotic protein Bcl-2, a downstream target of Akt, was increased in the CD44s-expressing TGF-β–treated MCF10A cells (Supplemental Figure 10). Together, these data suggest that CD44s augments Akt signaling and upregulates Bcl-2, leading to apoptosis resistance, a key characteristic of EMT. These results also indicate isoform specificity of CD44s in EMT-associated cell death resistance.

A change in CD44 isoform expression occurs during EMT-associated breast cancer progression. The above data demonstrate that CD44 isoform switching occurs during EMT and that the shift in expression to CD44s accelerates EMT. We next sought to determine whether the change in CD44 isoform expression observed in cellular model systems could be recapitulated in vivo. We analyzed the expression of CD44 isoforms in tumors from a conditional HER2/*Neu*-dependent breast cancer mouse model (1, 33). In this model, doxycycline-induced expression of oncogenic HER2/*Neu* in the mammary epithelium results in the development of breast tumors. Upon doxycycline withdrawal, these primary tumors fully regress, indicating a dependence on HER2/*Neu* signaling. However, recurrent tumors develop at the primary site up to a year after regression of the HER2/*Neu*-induced primary tumors. These recurrent tumors are HER2/*Neu* independent, show upregulated expression of Snail, and acquire mesenchymal characteristics in a manner resembling EMT (1). In accordance with our in vitro observations, the primary tumors from these mice, which are epithelial in nature, predominantly expressed CD44v, as indicated by the levels of exon v6– or v8–containing CD44v isoforms (Figure 6A). In contrast, the recurrent breast tumors, which are mesenchymal in nature, primarily expressed the CD44s isoform (Figure 6A). Quantification of the change in CD44 isoform expression indicated 30- and 11-fold decreases in CD44v5/6 and CD44v8/9 expression, respectively, and an 18-fold increase in CD44s in recurrent tumors (Figure 6B). A change in CD44 isoform expression was also apparent in tumor cell lines that had been derived from these primary and recurrent tumors (Figure 6C). Together, these results indicate that CD44 alternative splicing is differentially regulated in an in vivo model of breast cancer progression, resulting in a shift in expression from CD44v to CD44s during the development of recurrent mesenchymal breast tumors.

CD44s is necessary for the formation of tumors that display EMT characteristics in vivo. To determine the significance of CD44s expression in the formation of recurrent breast tumors with mesenchymal characteristics, we silenced CD44s expression with shRNA in the recurrent tumor–derived cell line (Figure 7A). We then injected 5×10^5 control or CD44 shRNA–expressing cells into mouse mammary fat pads and compared the incidence of tumor formation. Loss of CD44s in the recurrent tumor cells resulted in a significant decrease in both tumor incidence and tumor burden (Figure 7, B and C). We also analyzed the potency of tumor formation by inoculating limited numbers of tumor cells in mouse mammary fat pads. Interestingly, injection of only 500 control recurrent tumor cells was sufficient to produce palpable tumors in 5 weeks in 79% (11 of 14) of the animals. In contrast, only 21% (3 of 14) of mice injected with CD44-silenced cells formed tumors (Figure 7D). Notably, all tumors derived from CD44 shRNA–expressing cells reexpressed the CD44s isoform, while CD44v levels remained undetectable, further emphasizing the necessity of CD44s, but not CD44v, for formation of these tumors that exhibit EMT properties (Supplemental Figure 11).

One of the key features of recurrent tumor cells is resistance to cell death, which allows tumor cells to survive despite chemotherapy or radiation treatment. Given our observation that CD44s promotes cell death resistance during EMT, we speculated that reduced cell survival may have contributed to the decreased tumor incidence upon depletion of CD44s. Therefore,

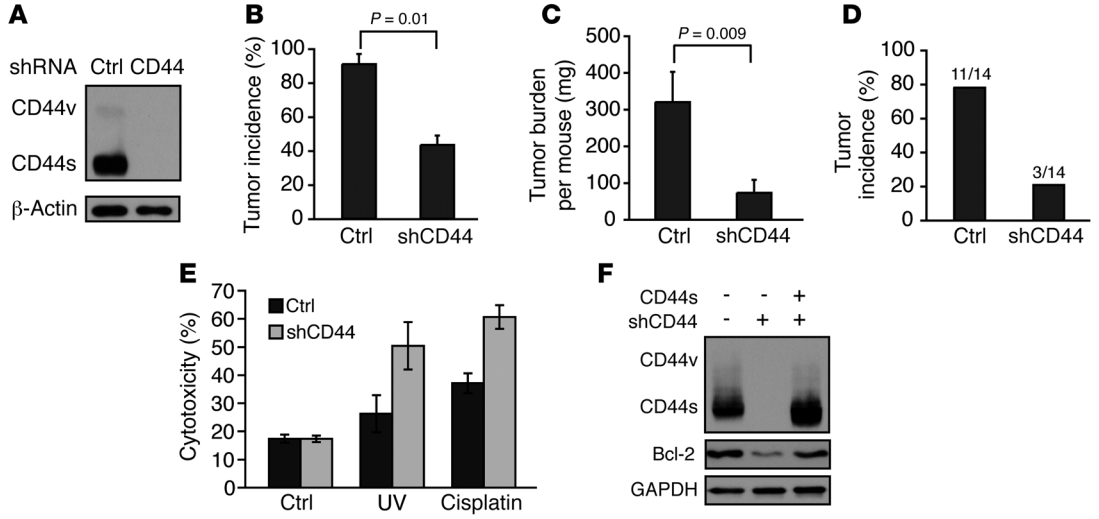

Figure 7

CD44s is required for EMT-associated tumor formation in vivo and confers resistance to cell death. (**A**) Immunoblot analysis of CD44 in recurrent tumor cells expressing nonspecific (Ctrl) or CD44 (shCD44) shRNA. (**B** and **C**) Depletion of CD44s inhibits the formation of EMT-associated tumors. Control or CD44 shRNA–expressing recurrent tumor cells (5×10^5) were injected into the fourth mammary fat pad of female FVB mice. Mice were sacrificed 3 weeks after inoculation, and tumors were removed and weighed. (**B**) Tumor incidence indicates percentage of mice in each group that formed tumors. Error bars indicate SD of 2 independent experiments (≥13 mice per group per experiment). (**C**) Tumor burden indicates average total tumor weight (mg) per mouse. Error bars represent SEM; $n = 27$ (control), $n = 26$ (shCD44). P values were calculated using a 2-tailed Student's t test. (**D**) Tumor incidence in FVB mice injected with 500 control or shCD44-expressing recurrent tumor cells into the fourth mammary fat pad. (**E**) Results of cytotoxicity assays indicating that knockdown of CD44s promotes cell death induced by UV irradiation (2 kJ/m^2) or cisplatin (500 μM). Error bars indicate SEM; $n = 3$. (**F**) Immunoblot analysis of CD44 and Bcl-2 expression in recurrent tumor cells expressing shCD44 and reconstituted with CD44s.

we tested whether silencing CD44s increases sensitivity to cell death–inducing stimuli. Treatment with UV irradiation or cisplatin resulted in increased cell death in recurrent tumor cells expressing CD44 shRNA (Figure 7E). This increased sensitivity to cell death may be explained, in part, by the observed 3.5-fold decrease in expression of the antiapoptotic Bcl-2 protein in the CD44-knockdown cells. The downregulation of Bcl-2 could be rescued by expressing human CD44s that is refractory to the CD44 shRNA (Figure 7F). Taken together, these results suggest that CD44s promotes EMT-associated tumor formation via the upregulation of pro-survival pathways.

CD44s expression is upregulated in advanced human breast tumors and correlates with N-cadherin expression. A critical question that arises from our in vitro and in vivo data is whether CD44s expression correlates with tumor progression in clinical breast tumor samples. To address this issue, we performed qRT-PCR analysis to assess mRNA levels of CD44s, as well as CD44v, in 5 normal human breast tissues and 27 patient breast tumors. The expression level of both CD44s and CD44v was upregulated in tumor samples (Supplemental Figure 12A), suggesting that both CD44 isoforms are important for breast tumorigenesis. Interestingly, we found that the expression of CD44s was significantly higher in advanced-grade tumors compared with normal breast tissues or lower-grade tumors (Figure 8A). The level of CD44s did not differ significantly between normal breast tissues and lower-grade tumors (grade 1, $P = 0.1994$). However, CD44s was elevated 7-fold in grade 2 and 3 tumors compared with grade 1 tumors ($P < 0.0001$). In contrast, there was no significant difference in CD44v5/v6 expression between grade 1 and grade 2/3 tumors (data not shown). These results indicate that expression of the CD44s isoform increases as tumors progress to a more advanced grade. Next, we sought to test whether tumors that show elevated levels of CD44s also display EMT characteristics. To address this issue, we performed Spearman correlation analysis to determine whether levels of CD44s correlate with levels of the mesenchymal marker N-cadherin in the tumor samples. Our results indicate that there was indeed a positive correlation between the expression levels of CD44s and N-cadherin ($r = 0.7814$, $P < 0.0001$), such that tumors that expressed higher levels of CD44s also expressed higher levels of N-cadherin. Conversely, tumors with lower levels of CD44s expressed lower levels of N-cadherin (Figure 8B). In contrast, analysis of the relationship between CD44v and N-cadherin did not show a significant correlation (Supplemental Figure 12B). These results provide a link between CD44s and a mesenchymal phenotype in human clinical tumor samples, further supporting our finding that CD44s promotes EMT. Together with our observations from the experimental model systems, these results indicate that the CD44s isoform is an essential mediator of EMT and breast cancer progression.

Discussion

Our data define a pivotal role for the CD44s isoform in accelerating EMT, a process that is frequently activated during tumor metastasis and recurrence. The central mechanism that we identified in this study is that cells utilize alternative splicing as a means of regulating EMT by producing a specific CD44 isoform that acts as a key mediator of EMT. We provide multiple lines of evidence, including analysis of experimentally induced EMT and tumors from a mouse model of breast cancer progression, to demonstrate that CD44 alternative splicing is dynamically regu-

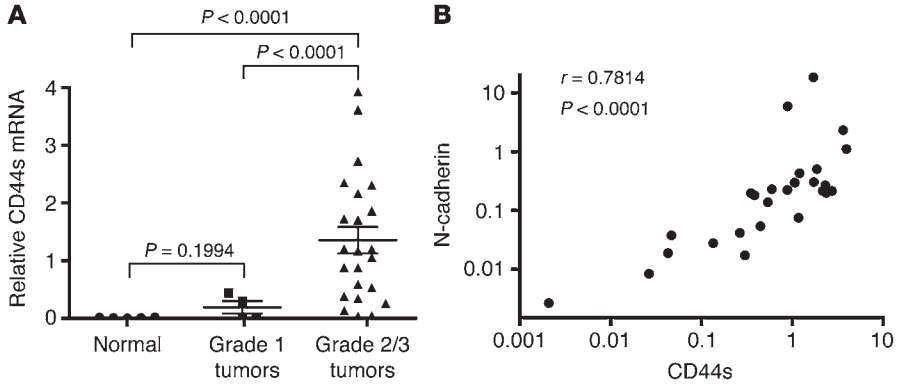

Figure 8

The CD44s isoform is enriched in advanced human breast cancers and correlates with N-cadherin expression. (**A**) The relative levels of CD44s expression in individual human samples from normal breast tissue or tumors were compared in different groups according to tumor grade. *P* values were calculated using a 2-tailed Student's *t* test. (**B**) The relative level of CD44s mRNA in each tumor sample was plotted against its corresponding level of N-cadherin mRNA. Spearman correlation analysis shows a significant correlation between CD44s and N-cadherin expression (Spearman correlation coefficient, $r = 0.7814$, with $P < 0.0001$).

lated during EMT, resulting in a switch in isoform expression from CD44v to CD44s. Hence, these data provide compelling evidence that CD44 alternative splicing is altered during EMT and cancer progression.

Functionally, we identify a causal role for the CD44s isoform in both EMT and cancer progression. By silencing CD44 and subsequently reconstituting the CD44s isoform, we demonstrate that the specific CD44s isoform is necessary for the transition of epithelial cells to a mesenchymal phenotype during EMT. Our data reveal that the switch in CD44 isoform expression is governed by ESRP1, a splicing factor that stimulates CD44 alternative splicing and, therefore, the production of CD44v. We found that *ESRP1* mRNA was drastically decreased during EMT, which correlated with the downregulation of CD44v and upregulation of CD44s. Overexpression of ESRP1 in the Twist-inducible EMT system prevented the switch in CD44 isoform expression and blocked EMT. Conversely, silencing ESRP1 accelerated EMT, and this effect was diminished when CD44 was depleted in the ESRP1-silenced cells. These data reveal that ESRP1 negatively regulates EMT by preventing the shift in expression from CD44v to CD44s. They also indicate that CD44 isoform switching is functionally critical for cells to undergo EMT. We speculate that, in addition to ESRP1, there are other splicing factors that stimulate or inhibit CD44 alternative splicing, in turn affecting EMT. For example, the ESRP1 paralog ESRP2 (25) was also downregulated during EMT, and this coincided with the shift in expression of CD44 isoforms (data not shown). Other examples of splicing factors that may possibly regulate EMT include SRm160, Sam68, and the SWI/SNF subunit Brm (34–36). It has been shown that these factors promote CD44 alternative splicing, leading to variable exon inclusion in a manner reminiscent of ESRP1. It would be of interest to direct future studies toward the identification of additional splicing factors that may influence EMT by controlling CD44 alternative splicing and to determine the mechanisms that mediate the regulation of these splicing factors during EMT.

In addition, we have identified Akt as a critical downstream signaling effector of CD44s. The PI3K/Akt pathway has been shown to play a critical role in promoting EMT, in part through the downregulation of E-cadherin (26). In agreement with this notion, our data indicate that expression of CD44s leads to activation of Akt and results in impaired expression of E-cadherin in TGF-β–induced mesenchymal MCF10A cells. The ability of CD44s to accelerate EMT can also be attributed to our observation that CD44s promotes cell death resistance, a key feature of EMT. We demonstrate that CD44s expression results in increased cell survival and that this effect is dependent on Akt signaling. In contrast to CD44s, expression of CD44v does not potentiate Akt activity or accelerate EMT in TGF-β–treated MCF10A cells. We suspect that expression of different CD44 isoforms may dictate the interaction between CD44 and receptor tyrosine kinases on the cell surface, thus affecting distinct downstream signaling cascades in cells. Together, these data establish a connection between the CD44s isoform, Akt signaling, and EMT. They open up future investigations to identify receptor tyrosine kinases that interact with CD44s during EMT, further defining the mechanisms by which CD44s activates Akt signaling.

Our finding that CD44s accelerates EMT was recapitulated in vivo. Using a mouse model of breast tumor formation and recurrence, we identified a drastic change in CD44 isoform expression from CD44v in the epithelial primary tumors to CD44s in the recurrent tumors that display EMT properties. We further demonstrate that CD44s is required for formation of EMT-associated recurrent tumors in mice. An important feature of tumor recurrence is that residual tumor cells must maintain the ability to survive environmental insults, such as treatment with chemotherapy or radiation. Our observation that CD44s mediates cell death resistance in recurrent tumor cells logically explains the requirement of CD44s for EMT-associated recurrent tumor formation in mice. Furthermore, by analyzing the expression of CD44s in human breast tumor specimens, we identified a statistically significant correlation between CD44s expression and tumor grade. Additionally, our results reveal that tumors expressing high levels of CD44s also expressed elevated levels of the mesenchymal marker N-cadherin. This positive correlation of CD44s with N-cadherin adequately corroborates our experimental observations that expression of CD44s is essential for EMT. Since EMT is associated with tumor progression and poor prognosis (37, 38), a potentially important and useful implication is that therapeutic targeting of the CD44s isoform might be beneficial in inhibiting EMT, thereby halting tumor progression.

Previous studies of the role of CD44 in vivo have shown that *Cd44*-null mice are viable, possibly due to functional compensation for the loss of CD44 by other molecules during embryonic development (22, 39, 40). Pronounced phenotypes of *Cd44*-null mice were observed only when these animals were challenged; for example, *Cd44*-null mice succumb to unremitting inflammation resulting from lung injury (41). When *Cd44*-null mice were

crossed with mice that carried one mutant allele of *p53*, a decrease in metastatic osteosarcoma was noted (41). In contrast, breast tumor metastasis increased when *Cd44*-null mice were crossed with transgenic MMTV-PyVmT mice (42). These seemingly contradictory observations highlight the complex nature of the functions of CD44 and its isoforms in carcinogenesis. The discrepancy between these observations could be due to isoform- or tissue-specific effects, which had not previously been addressed. The knockout mice lack all forms of CD44, including CD44v and CD44s, in both epithelial and stromal cells; different CD44 isoforms likely have diverse functions in these compartments. Our study suggests that isoform specificity must be considered when dissecting the role of CD44 in tumorigenesis.

The process of RNA alternative splicing, which is estimated to occur in 92%–94% of human genes (12, 13), must be precisely regulated to ensure the expression of functionally different splice isoforms in a spatial and temporal manner. Conversely, deregulated alternative splicing may result in disease (43, 44). Our data demonstrate that cells utilize alternative splicing to orchestrate a switch in isoform expression from CD44v to CD44s, which in turn controls EMT and cancer progression. These findings also suggest that a change in cellular phenotype, such as from the epithelial state to the mesenchymal state, can be modulated at the level of alternative splicing. Interestingly, we previously showed that CD44 alternative splicing is coupled to Ras/MAPK signaling via a positive feedback loop (18). Activation of this feed-forward loop triggers the transition from a nonproliferative to a proliferative cellular state, highlighting another way in which alternative splicing is able to alter cellular state. These findings illustrate that the function of a gene is not a fixed property of a cell, but is dynamically regulated by alternative splicing. This tightly controlled regulation allows for the production of functionally distinct protein isoforms that play diverse biological roles. Thus, our findings suggest that gene regulation at the level of alternative splicing serves as an indispensable mechanism governing normal and pathological processes. Investigating the potential role of alternative splicing in cancer progression may therefore lead to the development of novel therapeutic interventions that target specific splice isoforms for the treatment of advanced cancer.

Methods

Cell lines and EMT induction. Maintenance of the immortalized human mammary epithelial cells HMLE, HMLE/Twist-ER, and HMLE/Snail-ER, as well as tamoxifen induction of EMT in HMLE/Twist-ER or HMLE/Snail-ER cells were performed as previously described (23). Induction of EMT by TGF-β in HMLE cells was performed by culturing cells in DMEM/F12 medium containing 5% calf serum, 10 ng/ml EGF, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, and 5 ng/ml TGF-β. TGF-β–induced EMT in MCF10A cells was performed by addition of 1 ng/ml TGF-β to MCF10A medium (DMEM/F12 containing 5% horse serum, EGF [20 μg/ml], hydrocortisone [0.5 μg/ml], cholera toxin [0.1 μg/ml], and insulin [10 μg/ml]). MDCK and 293 cells were grown in DMEM supplemented with 10% fetal bovine serum and antibiotics. Mouse primary and recurrent breast tumor cell lines were maintained as previously described (1).

Plasmids and shRNA. The human CD44 shRNA that targets all CD44 isoforms and control luciferase shRNA were obtained from S. Godar, University of Cincinnati, Cincinnati, Ohio, USA (24). The mouse CD44-targeted shRNA sequence (GACAGGCTTTCAACAGTACCTTA) and a nonspecific control shRNA sequence (GCCCGAATTAGCTGGACACTCAA) were flanked by human miRNA-30 sequences and cloned into the XhoI and EcoRI sites of the LMP MicroRNA-adapted Retroviral shRNA Vector (Open Biosystems). Human CD44s and CD44v cDNAs were cloned into the EcoRI restriction site of the pBabe-hygro vector. Mouse ESRP1 was amplified from cDNA isolated from a murine epithelial cell line and cloned into the BamHI and SalI restriction sites of the pBRIT-HA/FLAG vector (Addgene). The two ESRP1 shRNA plasmids (clones V2LHS_155253 and V2LHS_155255 in the pGIPZ vector) were obtained from Open Biosystems. The CD44v5 luciferase reporter construct was described previously (35).

Luciferase assays. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System from Promega as directed by the manufacturer's instructions. Briefly, ESRP1 and CD44v5 minigene constructs were cotransfected into 293 cells along with a *Renilla* luciferase construct, which served as an internal control for transfection efficiency. Cells were assayed for luciferase expression 24 hours after transfection.

Semiquantitative and real-time RT-PCR. RNA was isolated from cells using the RNeasy mini kit (QIAGEN) or TRIzol reagent (Invitrogen). De-identified patient RNA samples were approved for use by the Northwestern University Institutional Review Board. cDNA was generated by reverse transcription with Omniscript RT (QIAGEN). Hot StarTaq DNA polymerase (QIAGEN) was used for semiquantitative RT-PCR, and PCR products were run on 1.5% agarose gels. qRT-PCR was performed using Power SYBR Green Master Mix (Applied Biosystems). TATA-binding protein (TBP) served as a loading control.

Antibodies. Antibodies used for Western blotting were as follows: CD44H (R&D Systems), CD44 IM7 (Santa Cruz Biotechnology Inc.), E-cadherin and γ-catenin (Cell Signaling Technology), N-cadherin (BD), vimentin (Zymed, Invitrogen), occludin (Santa Cruz Biotechnology Inc.), HA (Covance), β-actin (Sigma-Aldrich), and GAPDH (Chemicon). E-cadherin antibody (Cell Signaling Technology) was used for immunofluorescence and immunohistochemistry. Alexa Fluor 488–labeled phalloidin (Invitrogen) was used to stain for F-actin.

Cell death assays. Apoptosis was measured by analyzing DNA fragmentation using the cell death detection ELISA^{plus} kit (Roche). Cells were treated with cisplatin (100 μM, Sigma-Aldrich) or grown in low-attachment plates (Sigma-Aldrich) to induce apoptosis. Cells were collected and analyzed according to the manufacturer's protocol. Cytotoxicity was measured using the Cytotoxicity Detection Kit (LDH) (Roche). Cells were exposed to UV irradiation or cisplatin at indicated doses, and cytotoxicity was measured as per the manufacturer's protocol.

Animal studies. Injections of recurrent tumor cells into the fourth mammary fat pad were performed on 9-week-old female FVB mice. Mice were anesthetized, and the skin covering the mammary gland was incised. Tumor cells were injected into the exposed mammary gland. Groups of 13 or more mice were sacrificed 3 or 5 weeks after tumor cell injection. Mammary tumors were removed and weighed individually. All animal experiments were approved by the IACUC of Northwestern University.

Statistics. All statistical analyses (2-tailed Student's *t* test, Spearman correlation) were performed using GraphPad Prism software. *P* values less than 0.05 were considered significant.

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1. Moody SE, et al. The transcriptional repressor Snail promotes mammary tumor recurrence. *Cancer Cell*. 2005;8(3):197–209.
2. Thiery JP, Sleeman JP. Complex networks orchestrate epithelial-mesenchymal transitions. *Nat Rev Mol Cell Biol*. 2006;7(2):131–142.
3. Yang J, et al. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell*. 2004;117(7):927–939.
4. Yang J, Weinberg RA. Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. *Dev Cell*. 2008;14(6):818–829.
5. Bolos V, Peinado H, Perez-Moreno MA, Fraga MF, Esteller M, Cano A. The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors. *J Cell Sci*. 2003; 116(pt 3):499–511.
6. Comijn J, et al. The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. *Mol Cell*. 2001;7(6):1267–1278.
7. Eger A, et al. DeltaEF1 is a transcriptional repressor of E-cadherin and regulates epithelial plasticity in breast cancer cells. *Oncogene*. 2005; 24(14):2375–2385.
8. Gregory PA, et al. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat Cell Biol*. 2008; 10(5):593–601.
9. Park SM, Gaur AB, Lengyel E, Peter ME. The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. *Genes Dev*. 2008;22(7):894–907.
10. Kong W, et al. MicroRNA-155 is regulated by the transforming growth factor beta/Smad pathway and contributes to epithelial cell plasticity by targeting RhoA. *Mol Cell Biol*. 2008;28(22):6773–6784.
11. Ma L, et al. miR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and cancer metastasis. *Nat Cell Biol*. 2010;12(3):247–256.
12. Wang ET, et al. Alternative isoform regulation in human tissue transcriptomes. *Nature*. 2008; 456(7221):470–476.
13. Pan Q, Shai O, Lee LJ, Frey BJ, Blencowe BJ. Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nat Genet*. 2008;40(12):1413–1415.
14. Brickell PM, Latchman DS, Murphy D, Willison K, Rigby PW. Activation of a Qa/Tla class I major histocompatibility antigen gene is a general feature of oncogenesis in the mouse. *Nature*. 1983; 306(5945):756–760.
15. Sharma S, Lichtenstein A. Aberrant splicing of the E-cadherin transcript is a novel mechanism of gene silencing in chronic lymphocytic leukemia cells. *Blood*. 2009;114(19):4179–4185.
16. Shtivelman E, Lifshitz B, Gale RP, Roe BA, Canaani E. Alternative splicing of RNAs transcribed from the human abl gene and from the bcr-abl fused gene. *Cell*. 1986;47(2):277–284.
17. Venables JP. Unbalanced alternative splicing and its significance in cancer. *Bioessays*. 2006;28(4):378–386.
18. Cheng C, Yaffe MB, Sharp PA. A positive feedback loop couples Ras activation and CD44 alternative splicing. *Genes Dev*. 2006;20(13):1715–1720.
19. Orian-Rousseau V, Chen L, Sleeman JP, Herrlich P, Ponta H. CD44 is required for two consecutive steps in HGF/c-Met signaling. *Genes Dev*. 2002; 16(23):3074–3086.
20. Sherman LS, Rizvi TA, Karyala S, Ratner N. CD44 enhances neuregulin signaling by Schwann cells. *J Cell Biol*. 2000;150(5):1071–1084.
21. Bourguignon LY, Zhu H, Chu A, Iida N, Zhang L, Hung MC. Interaction between the adhesion receptor, CD44, and the oncogene product, p185HER2, promotes human ovarian tumor cell activation. *J Biol Chem*. 1997;272(44):27913–27918.
22. Ponta H, Sherman L, Herrlich PA. CD44: from adhesion molecules to signalling regulators. *Nat Rev Mol Cell Biol*. 2003;4(1):33–45.
23. Mani SA, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell*. 2008;133(4):704–715.
24. Godar S, et al. Growth-inhibitory and tumor- suppressive functions of p53 depend on its repression of CD44 expression. *Cell*. 2008;134(1):62–73.
25. Warzecha CC, Sato TK, Nabet B, Hogenesch JB, Carstens RP. ESRP1 and ESRP2 are epithelial cell-type-specific regulators of FGFR2 splicing. *Mol Cell*. 2009;33(5):591–601.
26. Grille SJ, et al. The protein kinase Akt induces epithelial mesenchymal transition and promotes enhanced motility and invasiveness of squamous cell carcinoma lines. *Cancer Res*. 2003;63(9):2172–2178.
27. Larue L, Bellacosa A. Epithelial-mesenchymal transition in development and cancer: role of phosphatidylinositol 3′ kinase/AKT pathways. *Oncogene*. 2005; 24(50):7443–7454.
28. Bakin AV, Tomlinson AK, Bhowmick NA, Moses HL, Arteaga CL. Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration. *J Biol Chem*. 2000; 275(47):36803–36810.
29. Julien S, et al. Activation of NF-kappaB by Akt upregulates Snail expression and induces epithelium mesenchyme transition. *Oncogene*. 2007; 26(53):7445–7456.
30. Nath AK, Brown RM, Michaud M, Sierra-Honigmann MR, Snyder M, Madri JA. Leptin affects endocardial cushion formation by modulating EMT and migration via Akt signaling cascades. *J Cell Biol*. 2008;181(2):367–380.
31. Testa JR, Tsichlis PN. AKT signaling in normal and malignant cells. *Oncogene*. 2005;24(50):7391–7393.
32. Feng Q, et al. PDK1 regulates vascular remodeling and promotes epithelial-mesenchymal transition in cardiac development. *Mol Cell Biol*. 2010; 30(14):3711–3721.
33. Moody SE, et al. Conditional activation of Neu in the mammary epithelium of transgenic mice results in reversible pulmonary metastasis. *Cancer Cell*. 2002;2(6):451–461.
34. Batsche E, Yaniv M, Muchardt C. The human SWI/SNF subunit Brm is a regulator of alternative splicing. *Nat Struct Mol Biol*. 2006;13(1):22–29.
35. Cheng C, Sharp PA. Regulation of CD44 alternative splicing by SRm160 and its potential role in tumor cell invasion. *Mol Cell Biol*. 2006;26(1):362–370.
36. Matter N, Herrlich P, Konig H. Signal-dependent regulation of splicing via phosphorylation of Sam68. *Nature*. 2002;420(6916):691–695.
37. Barrallo-Gimeno A, Nieto MA. The Snail genes as inducers of cell movement and survival: implications in development and cancer. *Development*. 2005; 132(14):3151–3161.
38. Peinado H, Olmeda D, Cano A. Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat Rev Cancer*. 2007; 7(6):415–428.
39. Protin U, Schweighoffer T, Jochum W, Hilberg F. CD44-deficient mice develop normally with changes in subpopulations and recirculation of lymphocyte subsets. *J Immunol*. 1999;163(9):4917–4923.
40. Schmits R, et al. CD44 regulates hematopoietic progenitor distribution, granuloma formation, and tumorigenicity. *Blood*. 1997;90(6):2217–2233.
41. Weber GF, Bronson RT, Ilagan J, Cantor H, Schmits R, Mak TW. Absence of the CD44 gene prevents sarcoma metastasis. *Cancer Res*. 2002;62(8):2281–2286.
42. Lopez JI, Camenisch TD, Stevens MV, Sands BJ, McDonald J, Schroeder JA. CD44 attenuates metastatic invasion during breast cancer progression. *Cancer Res*. 2005;65(15):6755–6763.
43. Cooper TA, Wan L, Dreyfuss G. RNA and disease. *Cell*. 2009;136(4):777–793.
44. Srebrow A, Kornblihtt AR. The connection between splicing and cancer. *J Cell Sci*. 2006; 119(pt 13):2635–2641.