The non-coding 3′ UTR of CD44 induces metastasis by regulating extracellular matrix functions

Zina Jeyapalan Rutnam and Burton B. Yang

Journal of Cell Science 125, 2545

doi: 10.1242/jcs.113522

There was an error published in *J. Cell Sci.* **125**, 2075–2085.

The doi number for this paper was incorrect.

The correct doi number should be 10.1242/jcs.100818.

We apologise for this error.

The non-coding 3′ UTR of CD44 induces metastasis by regulating extracellular matrix functions

Zina Jeyapalan Rutnam[1,2] and Burton B. Yang[1,2,*]

[1]Sunnybrook Research Institute, Sunnybrook Health Sciences Centre, 2075 Bayview Avenue, Toronto, ON M4N 3M5, Canada
[2]Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON M5S 1A8, Canada
*Author for correspondence (byang@sri.utoronto.ca)

Accepted 12 December 2011
Journal of Cell Science 125, 2075–2085

doi: 10.1242/jcs100818

Summary

The importance of non-coding RNA transcripts in regulating microRNA (miRNA) functions, especially the 3′-untranslated region (3′ UTR), has been revealed in recent years. Genes encoding the extracellular matrix normally produce large mRNA transcripts including the 3′ UTR. How these large transcripts affect miRNA functions and how miRNAs modulate extracellular matrix protein expression are largely unknown. Here, we demonstrate that the overexpression of the CD44 3′ UTR results in enhanced cell motility, invasion and cell adhesion in human breast carcinoma cell line MDA-MB-231. Furthermore, we found that expression of the CD44 3′ UTR enhances metastasis in vivo. We hypothesize that increased expression of the CD44 3′ UTR affects miRNA binding and modulates synthesis of the extracellular matrix. Computational analysis indicated that miRNAs that interact with the CD44 3′ UTR also have binding sites in other matrix-encoding mRNA 3′ UTRs, including collagen type 1α1 (Col1α1) repressed by miR-328 and fibronectin type 1 (FN1) repressed by miR-512-3p, miR-491 and miR-671. Protein analysis demonstrated that expression of CD44, Col1α1 and FN1 were synergistically upregulated in vitro and in vivo upon transfection of the CD44 3′ UTR. The non-coding 3′ UTR of CD44 interacts with multiple miRNAs that target extracellular matrix properties and thus can be used to antagonize miRNA activities.

Key words: MicroRNA, 3′ UTR, Non-coding RNA, Extracellular matrix, Apoptosis, Angiogenesis, Tumorigenesis, Breast cancer

Introduction

In recent years, the importance of non-coding RNA transcripts and their connection with microRNAs has been revealed. This significance was demonstrated initially in our laboratory (Lee et al., 2009) by the finding that expression of the 3′-untranslated region (3′ UTR) of the protein versican resulted in altered cell and tissue adhesion. The non-coding transcript of versican 3′ UTR was able to antagonize the function of the microRNA miR-199a-3p in regulating the expression of versican and fibronectin. Further study showed that expression of versican 3′ UTR also lowers the steady-state expression of miR-199a-3p (Lee et al., 2010). Another class of non-coding RNA transcripts is the pseudogenes. The human genome contains a large number of pseudogenes, which have been discovered to be as abundant as functional genes and therefore represent a large component of the genome. It has been estimated that there are approximately 20,000 putative pseudogenes in the human genome (Torrents et al., 2003). Previously, pseudogenes were classified as ‘junk DNA’ based on the fact that they do not code for proteins. However, it has been recently shown that certain pseudogenes play important roles in maintaining homeostasis, specifically as modulators of miRNAs. Poliseno and colleagues recently described a PTEN pseudogene, PTENP1, that regulates PTEN cellular levels and suppresses cell growth (Poliseno et al., 2010). Furthermore, Chin and colleagues found that variation due to single nucleotide polymorphisms located in miRNA binding sites of the 3′ UTR can affect miRNA target expression and function (Chin et al., 2008). The 3′ UTR of KRAS contains a single nucleotide polymorphism correlated with an increased risk of non-small cell lung cancer (Chin et al., 2008). This single nucleotide polymorphism prevents let-7 from binding, which results in the overexpression of KRAS in lung cancer.

The widespread role of miRNAs in disease makes them ideal targets for therapeutic intervention (Mayr and Bartel, 2009; Negrini et al., 2007; Shimono et al., 2009). Because the base-pair interaction between the seed regions of miRNAs and mRNAs is essential for repressing mRNA function (Hua et al., 2006; Ye et al., 2008), a logical inhibitor is a nucleic acid that is antisense to the miRNA, which can compete with mRNAs. This has been demonstrated directly in cultured cells by transiently transfecting 2′-*O*-methyl-modified antisense RNAs into several independent miRNAs (Meister et al., 2004). Long-term research using antisense techniques has been performed and has led to the development of siRNA and miRNA therapeutics in vivo (Krützfeldt et al., 2005; Soutschek et al., 2004). Krützfeldt and colleagues recently performed the in vivo inhibition of four miRNAs using modified antisense RNAs, which they term ‘antagomirs’ (Krützfeldt et al., 2005). To date, the use of different non-coding oligonucleotides to regulate miRNA activity have been extensively investigated including decoys, sponges, locked nucleic acids, non-coding RNAs and antagomirs (Ebert et al., 2007; Haraguchi et al., 2009; Kahai et al., 2009; Krützfeldt et al., 2005; Lee et al., 2011; Ørom et al., 2006). These chemically modified antisense oligonucleotides are meant to antagonize specific miRNAs and are effective against a specific miRNA or members of a miRNA family. However, because multiple miRNAs can target one gene, and antagonizing one miRNA might not relieve enough translational repression exerted

Journal of Cell Science

by other miRNAs targeting the same gene, the ideal approach is to develop strategy to regulate multiple miRNAs.

Use of the 3′ UTR serves this purpose because one 3′ UTR can bind many miRNAs. The hypothesis is that a 3′ UTR can interact with an miRNA that also has many binding sites on other 3′ UTRs, with similar effects. Thus, the 3′ UTR can be used to antagonize miRNAs of interest and modulate the functions of genes with similar 3′ UTRs. In addition, miRNAs can be degraded through binding with the 3′ UTR, which will protect target mRNAs from translational inhibition. Previously, we demonstrated that a non-coding transcript, CD44 3′ UTR, was able to antagonize miRNAs involved in cell cycle regulation, which resulted in an increase in CDC42, a cell cycle regulator, to result in decreased tumorigenesis in the breast cancer cell line MT1 (Jeyapalan et al., 2011). Additionally, the CD44 3′ UTR was found to increase endothelial cell activities and angiogenesis in vivo. Analysis of these cells revealed significant differences in extracellular matrix (ECM) activities. Using this system, we applied the CD44 3′ UTR to the human metastatic cell line MDA-MB-231 in order to antagonize miRNAs involved in ECM activities and characterized the consequences in tumor metastasis.

Results

CD44 3′ UTR affects migration, invasion and adhesion of breast carcinoma cells

MDA-MB-231 cells were stably transfected with the CD44 3′ UTR and CD44E expression constructs and a control vector pcDNA3.1. To test expression of the CD44 3′ UTR, total RNA was extracted and subjected to real-time PCR analysis. MDA-MB-231 cells stably transfected with the CD44 3′ UTR construct (referred to hereafter as CD44 3′ UTR cells) produced a fivefold increase in the amount of CD44 3′ UTR compared with the control cells (Fig. 1A). Cell migration was measured in MDA-MB-231 cells transfected with CD44 3′ UTR, CD44E or pcDNA3.1, following creation of a scratch on the cultures. It was found that the CD44 3′ UTR cells had a faster rate of migration than the control and CD44-coding cells (Fig. 1B; supplementary material Fig. S1a). Cell invasion was examined using Matrigel-coated transwell chambers. When cells were placed in the chambers, the CD44 3′ UTR cells invaded through the Matrigel and membrane pores and spread out on the surface of the underside membrane significantly more than the CD44E-transfected and control cells (Fig. 1C; supplementary material Fig. S1b). Cell detachment was monitored and counted after cells were briefly treated with EDTA. The CD44 3′ UTR cells detached slower than the CD44E-transfected and control cells (Fig. 1D; supplementary material Fig. S1d).

CD44 3′ UTR enhances metastasis in vivo

The metastatic potential of the CD44 3′ UTR was investigated in vivo with the intravenous tail-vein injection of MDA-MB-231 cells transfected with CD44 3′ UTR and the control. The mortality rate for mice injected with the CD44 3′ UTR cells was increased because these mice developed edema and appeared

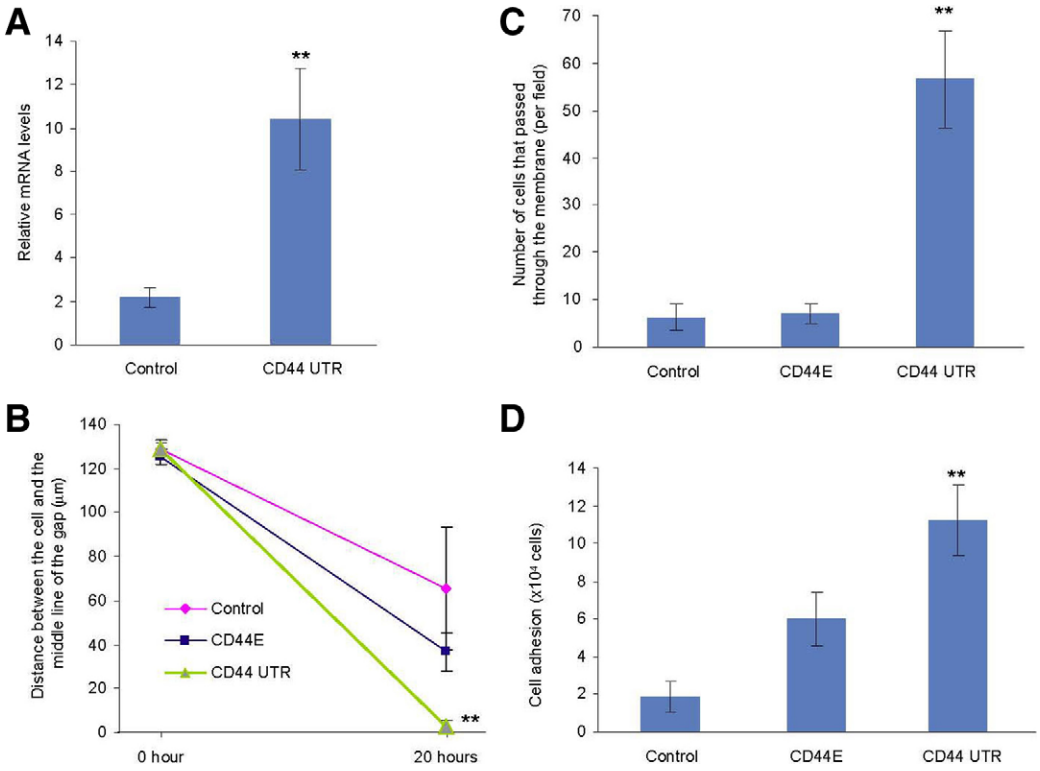

Fig. 1. CD44 3′ UTR affects migration, invasion and adhesion of breast carcinoma cells. (**A**) Total RNA isolated from CD44 3′ UTR- and vector-transfected cells were subjected to real-time PCR analysis. CD44 3′ UTR stable cells produced higher levels of CD44 3′ UTR than the control cells ($n=3$). (**B**) Cell migration was monitored using a scratch method. It was found that the MDA-MB-231 cells transfected with CD44 3′ UTR migrated faster than cells transfected with CD44E or control vector ($n=10$). (**C**) Cell invasion was examined using Matrigel-coated transwell chambers. Significantly more CD44 3′ UTR cells than CD44E and control cells travelled through the Matrigel and membrane pores and spread out on the underside of the membrane ($n=5$). (**D**) Cell detachment was monitored and counted after cells were treated with EDTA. The CD44 3′ UTR cells detached more slowly than did the CD44E and control cells ($n=5$). $^{**}P<0.01$.

frail and hunched and needed to be sacrificed based on the Animal Use Protocol starting at 8 weeks after the injection as compared with the control mice (Fig. 2A,B). Lung tissues were excised from these mice and stained with hematoxylin and eosin (H&E) and antibodies against CD44 and Ki67 (Fig. 2C). Lungs from mice injected with CD44 3′ UTR cells exhibited metastatic nodules that were not found in the control mice. These nodules were confirmed to be expressing an increased amount of CD44 because they stained for this molecule darker than the surrounding tissue. Additionally, these nodules had increased expression of Ki67, which is a common proliferating marker.

siRNAs against the CD44 3′ UTR affect adhesion of MDA-MB-231 cells

Four siRNAs against CD44 3′ UTR were transfected into the CD44 3′ UTR-transfected MDA-MB-231 cells and cell migration monitored. The siRNA sequences that bind to the CD44 3′ UTR are shown in Fig. 3A. We found that transfection with the siRNAs produced a decreased rate of migration compared with the control siRNA (Fig. 3B; supplementary material Fig. S2a). In cell invasion assays, we detected a lower number of cells invading through the Matrigel in the siRNA-treated cells than in the control cells treated with a non-related siRNA sequence (Fig. 3C; supplementary material Fig. S2b). In the detachment assays, the cells transfected with the siRNAs against CD44 3′ UTR were also monitored and we found that the siRNA-treated CD44 3′ UTR cells detached faster than the control cells (Fig. 3D; supplementary material Fig. S2c). To examine the effects of endogenous CD44 3′ UTR on binding miRNAs and mediating cell activities, we transfected the parental MDA-MB-231 cells with these siRNAs against the CD44 3′ UTR. We detected decreased rates of migration, invasion and adhesion in the siRNA-transfected cells compared with the controls (supplementary material Fig. S3a–c).

CD44 3′ UTR-interaction miRNAs target both FN1 and Col1α1 3′ UTRs

The increased migration and invasion and decreased detachment of the CD44 3′ UTR-transfected MDA-MB-231 cells led us to focus on investigating ECM proteins. Even though CD44 is a cell surface molecule involved in cell–cell interactions, adhesion and migration, the limited functional changes observed in the MDA-MB-231 cells suggested that the effect of the CD44 3′ UTR on cell activities was not the consequence of upregulation of CD44 protein alone; there must be other adhesion molecules involved in these actions. Analysis with computational algorithms showed that the 3′ UTRs of CD44, fibronectin type 1 (FN1) and collagen type 1 α1 (Col1α1) could have multiple miRNAs in common.

FN1 is involved in cell adhesion, growth and migration and its 3′ UTR was predicted to bind miR-512-3p, miR-491 and miR-671, in common with the CD44 3′ UTR (Fig. 4A). Col1α1 is a major ECM component that plays an important role in mediating

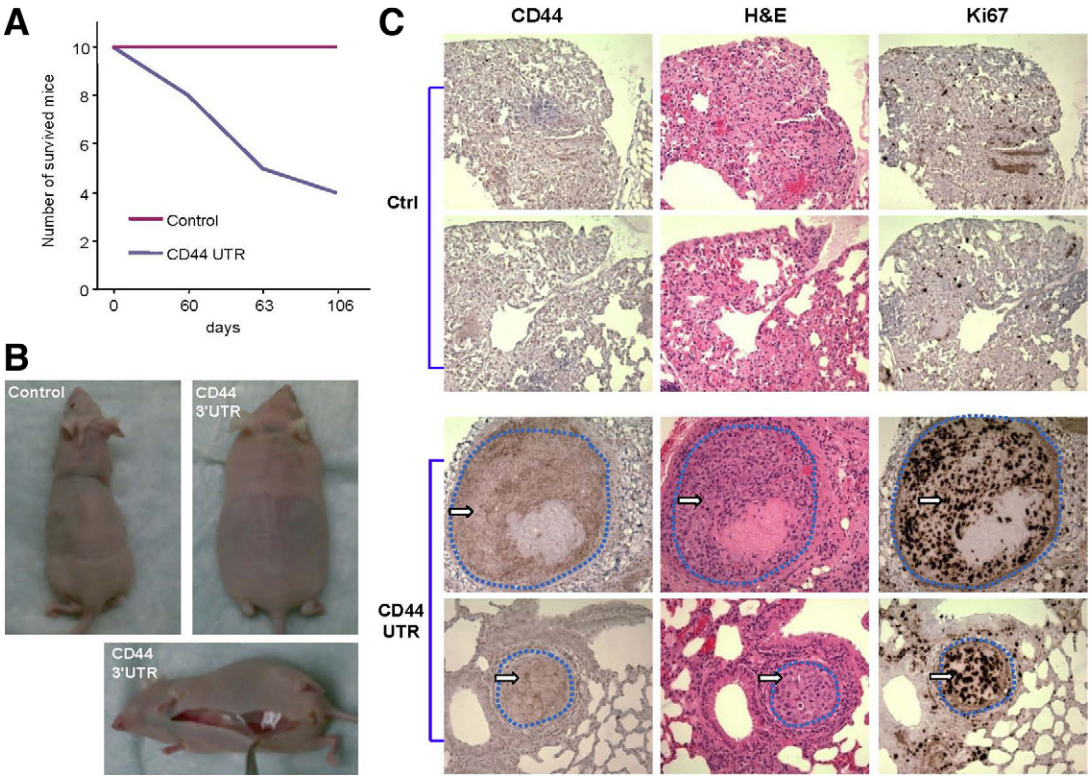

Fig. 2. Increased metastasis of CD44 3′ UTR cells in vivo. (**A**) Mice injected with CD44 3′ UTR-transfected MDA-MB-231 cells had a higher rate of mortality than mice injected with control cells. Survival rate is shown ($n=10$). (**B**) Mice injected with the CD44 3′ UTR cells exhibited characteristics of edema, which included enlarged body size and excess fluid in the peritoneal cavity. (**C**) Nodules were found in the lungs of CD44 3′ UTR group with H&E staining. These nodules expressed higher levels of CD44 and Ki67 than the control group. No nodules were found in the lungs of the control group. Tumor nodules are outlined with dotted lines and positive cells are labeled with arrows.

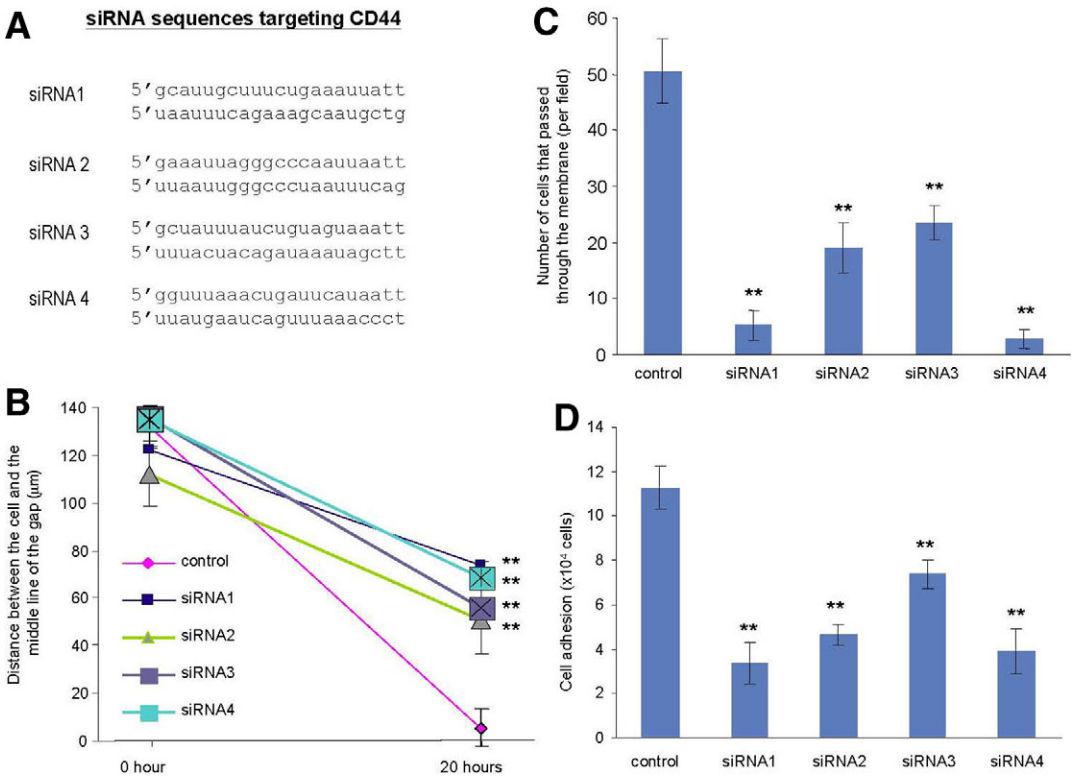

Fig. 3. siRNA against the CD44 3′ UTR affects adhesion of MDA-MB-231 cells. (**A**) siRNA sequences used to inhibit CD44 3′ UTR expression are shown. (**B**) CD44 3′ UTR-transfected MDA-MB-231 cells were co-transfected with siRNA sequences against CD44 3′ UTR. Cell migration was monitored and found to be decreased compared with the siRNA control ($n=10$). (**C**) The siRNA-treated CD44 3′ UTR cells showed reduced invasion through Matrigel and transwell inserts compared with the control ($n=3$). (**D**) The detachment of cells transfected with the siRNAs was also monitored and the siRNA-treated CD44 3′ UTR cells detached faster than the control siRNA-treated cells ($n=3$). $^{**}P<0.01$.

cell behavior and maintaining tissue architecture. Col1α1 is predicted to be a target of miR-328, which is also predicted to bind to CD44 3′ UTR (Fig. 4A).

We hypothesized that when the miRNAs in common were being absorbed by the CD44 3′ UTR, there would be an increased production of the adhesion molecules, FN1 and Col1α1 along with CD44 (Fig. 4B). The CD44 protein levels were upregulated in the CD44E and CD44 3′ UTR stable cells but not in the control MDA-MB-231 cells (Fig. 4C). This is because the binding of miRNAs to the overexpressed CD44 3′ UTR causes a decreased availability of miRNAs for binding to the endogenous CD44 3′ UTRs. This also resulted in increased production of FN1 protein, which was confirmed by western blot analysis (Fig. 4C). Because FN1 protein levels in the CD44E cells were similar to those in the control cells, the increase in FN1 was not due to downstream activation through the CD44 protein. The binding of miRNA-328 to the CD44 3′ UTR appeared to allow for an increased production of Col1α1 protein in the CD44 3′ UTR cells (Fig. 4C). Similarly, because there was no increase in Col1α1 in the CD44E cells, the increase in Col1α1 might also be due to the increase in CD44 3′ UTR and not due to downstream activation through the CD44 protein.

We confirmed the targeting of the four miRNAs to CD44 3′ UTR using luciferase assays. The 3′ UTR of CD44 was cloned into pMir-report vector. There was a decrease in luciferase activities when the construct was co-transfected along with miR-512-3p, miR-328, miR-491 and miR-671 into MDA-MB-231 cells (Fig. 4D).

Furthermore, we examined the effect of siRNAs against the CD44 3′ UTR on FN1 and Col1α1 expression. CD44 3′ UTR cells transfected with the four siRNAs binding to the CD44 3′ UTR were analyzed by western blot probed with anti-FN1 and anti-Col1α1 antibodies. FN1 and Col1α1 levels decreased in the siRNA-treated samples as compared with the control (Fig. 4E). This decrease in FN1 and Col1α1 was also seen when the parental MDA-MB-231 cells were transfected with the four siRNAs (supplementary material Fig. S3d).

Similar results were seen in tumors formed in mice injected with control and CD44 3′ UTR-transfected MT-1 cells when they were examined for FN1 and Col1α1 protein levels. Protein lystates from tumor tissue from CD44 3′ UTR and control tumors were subjected to western blot analysis with anti-FN1 and anti-Col1α1 antibodies (supplementary material Fig. S4a). FN1 and Col1α1 were upregulated in the CD44 3′ UTR tumors compared with the control tumors. Immunohistochemistry was also performed on the tumor sections probed with anti-FN1 and anti-Col1α1 antibodies (supplementary material Fig. S4b). There was an increase in FN1 and Col1α1 in CD44 3′ UTR tumor sections compared with the control sections.

The binding of miR-491, miR-671 and miR-512-3p to FN1 was confirmed using luciferase assays. The fragment of each binding site of FN1 3′ UTR was cloned into pMir-report vector to create three constructs, Luc-FN-491, Luc-FN-671 and Luc-FN-512-3p (supplementary material Fig. S5). There was a decrease in luciferase activities when each construct was co-transfected

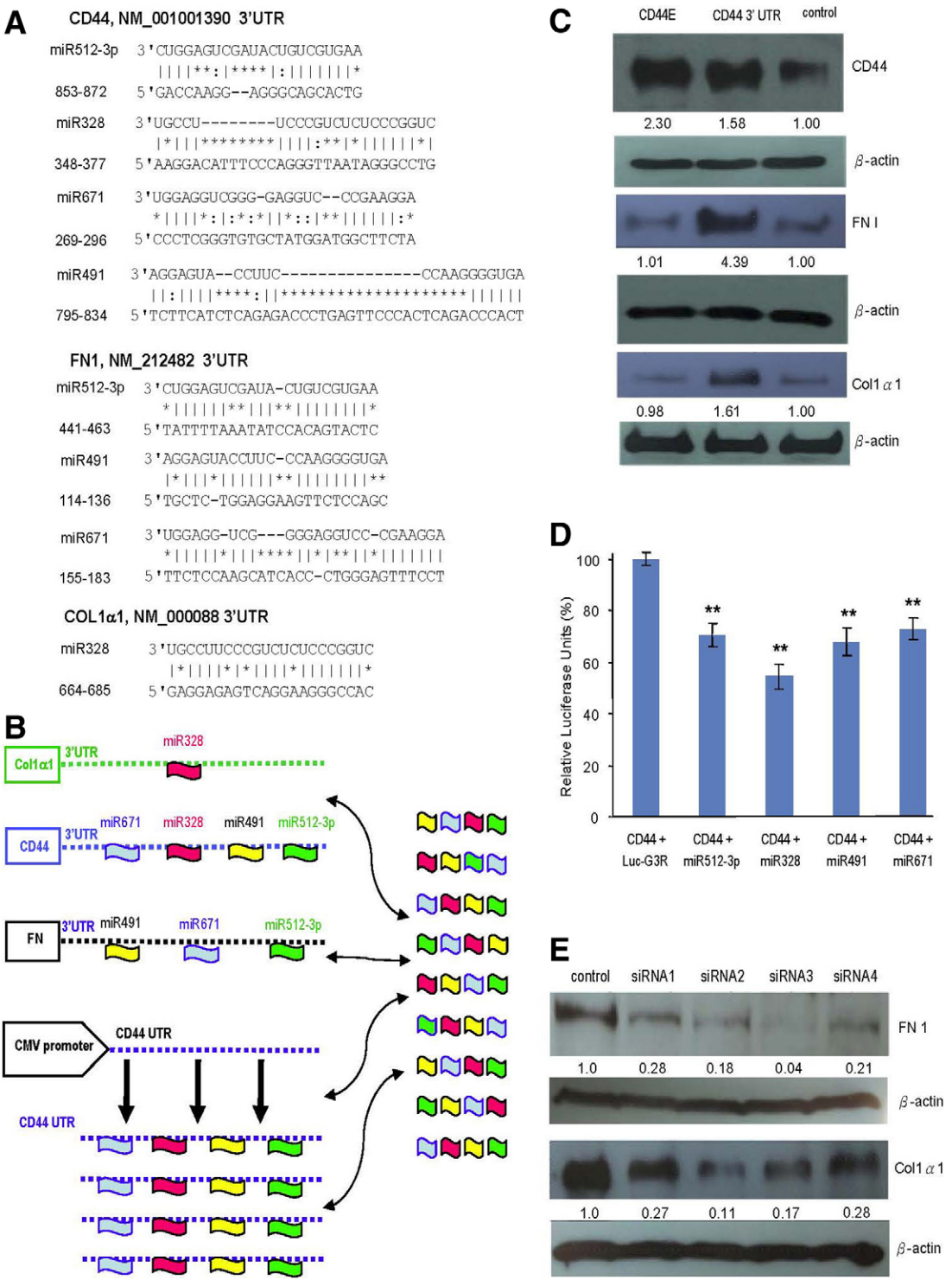

Fig. 4. CD44 3′ UTR modulates expression of FN1 and Col1a1 by binding miRNAs. (**A**) Computational analysis of the CD44 3′ UTR has shown that miR-512-3p, miR-491 and miR-671 interact with both CD44 and FN1 3′ UTRs and that miR-328 can interact with both CD44 and Col1α1. Predicted binding sequences of miR-512-3p, miR-491, miR-671 and miR-328 to CD44 (upper) and targeting of FN1 and Col1α1 3′ UTR by these miRNAs (lower) are shown. (**B**) The strategy employed in our study is shown. Overexpression of CD44 3′ UTR would attract endogenous miR-512-3p, miR-491 and miR-671, thus freeing mRNAs encoding CD44 and FN1 to be available for translation. This is also occurs with endogenous miR-328, which binds to the CD44 3′ UTR and allows increased amounts of mRNAs for CD44 and Col1α1 to undergo translation. (**C**) Protein lysates prepared from cells transfected with CD44 3′ UTR, CD44E or vector were subjected to western blot analysis with anti-CD44, anti-FN1 and anti-Col1α1 antibodies. Detection of β-actin on the same membranes served as a loading control. Increased expression of CD44, FN1 and Col1α1 was detected in the CD44 3′ UTR-transfected cells. Although transfection with CD44E increased CD44 expression, it did not increase FN1 and Col1α1 levels. (**D**) MDA-MB-231 cells were co-transfected with the four miRNAs and the luciferase reporter construct harboring the CD44 3′ UTR fragment. A non-related fragment of cDNA was used as a control. Luciferase activity assays indicated that all four miRNAs (miR-512-3p, miR-328, miR-491 and miR-671) repressed luciferase activity ($n=3$, $^{**}P<0.01$). (**E**) Cell lysates from siRNA-treated cells were subjected to western blot analysis with anti-FN1 and anti-Col1α1 antibodies. Both FN1 and Col1α1 levels decreased as a result of treatment with siRNA targeting CD44 3′ UTR.

along with its respective miRNA into MDA-MB-231 cells (Fig. 5B). The potential binding sites were also mutated to produce three mutation constructs, Luc-FN-mut-491, Luc-FN-mut-671 and Luc-FN-mut-512-3p (Fig. 5A). When the binding sites were mutated, there was a restoration of luciferase expression (Fig. 5B). The luciferase activities were also restored when pcDNA3.1-CD44 3′ UTR was co-transfected with each miRNA and Luc-FN construct because the miRNAs were bound by the CD44 3′ UTR (Fig. 5C). These activities were lower than for the control vector, because it is possible for other miRNAs to be working in the system, but higher than for the wild-type Luc-FN construct with miRNA alone.

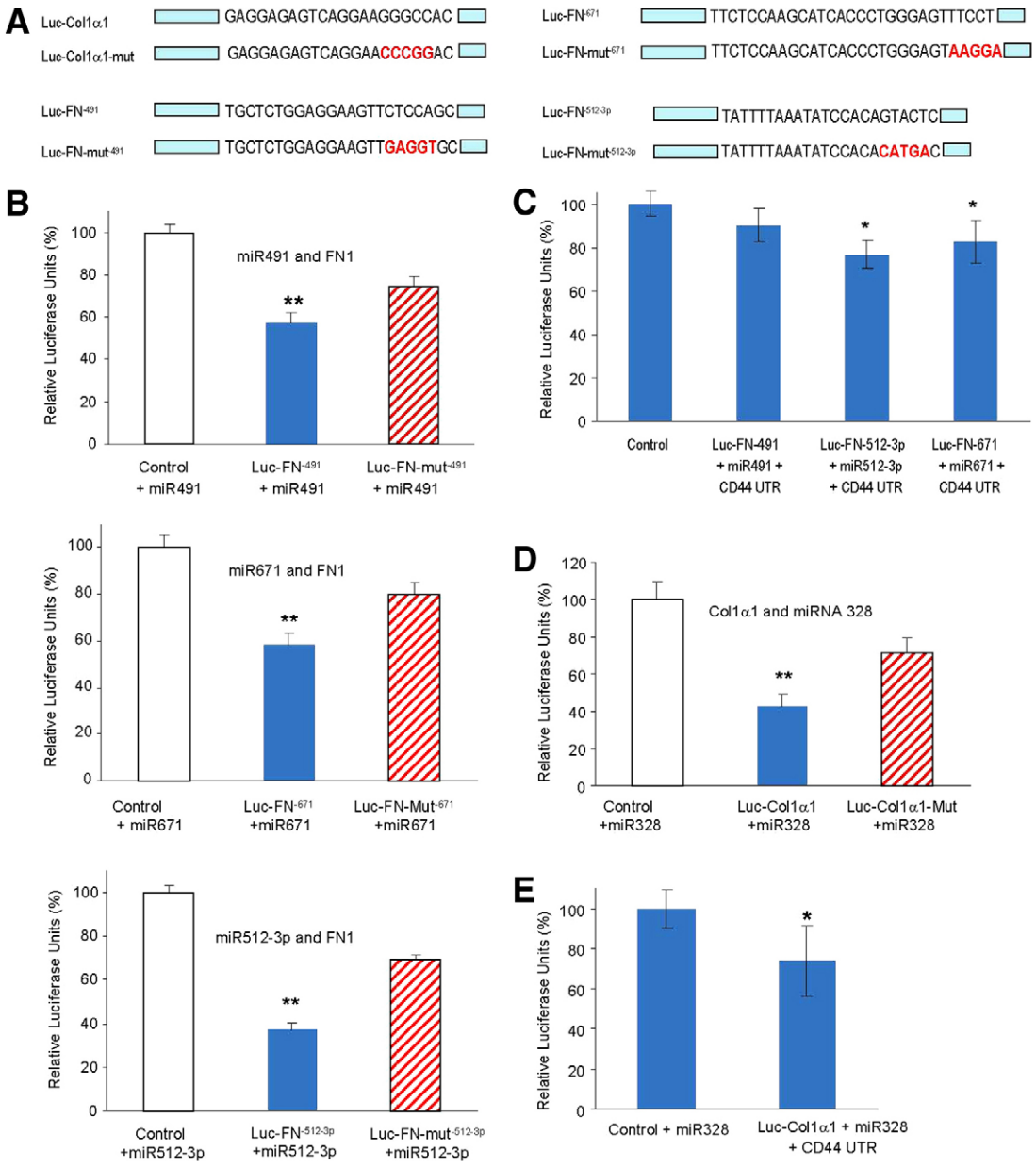

Fig. 5. Confirmation of miRNA targeting using luciferase activity assays. (**A**) FN1 and Col1α1 3′ UTR fragments were cloned into the luciferase reporter vector pMir-Report construct. Mutated sequences in the seed regions of FN1 and Col1α1 are shown in red. (**B**) MDA-MB-231 cells were co-transfected with different miRNAs and the luciferase reporter construct harboring the FN1 or mutant FN1 3′ UTR fragment. A non-related fragment of cDNA was used as a control. Luciferase activity assays indicated that all three miRNAs (miR-491, miR-671 and miR-512-3p) repressed luciferase activity when it harbored the corresponding FN1 3′ UTR; the effect was reversed when the potential miRNA target site was mutated ($n=3$). (**C**) The luciferase reporter plasmids were co-transfected into MDA-MB-231 cells with the corresponding miRNAs and pcDNA3.1–CD44 3′ UTR. The inhibitory effects of miRNAs could be recovered in the presence of pcDNA3.1-CD44 3′ UTR ($n=3$). (**D**) MDA-MB-231 cells were co-transfected with miR-328 and the luciferase reporter construct harboring the Col1α1 or mutant Col1α1 3′ UTR fragment. Luciferase activity assays indicated that miR-328 repressed luciferase activity of the Col1α1 3′ UTR, which was reversed when the miR-328 target site was mutated ($n=3$). (**E**) The luciferase reporter plasmid for Col1α1 was co-transfected with miR-328 and pcDNA3.1–CD44 3′ UTR into MDA-MB-231 cells. The inhibitory effects of the miRNA could be recovered in the presence of pcDNA3.1-CD44 3′ UTR ($n=3$). *$P<0.05$, **$P<0.01$.

Furthermore, luciferase activity assays were also performed with the miR-328 binding site on Col1α1 3′ UTR. This fragment was cloned into pMir-report vector generating a luciferase construct Luc-Col1α1 (supplementary material Fig. S5). There was a decrease in luciferase activity when the Luc-Col1α1 and miR-328 were co-transfected into MDA-MB-231 cells (Fig. 5D). When this binding site was mutated (Luc-Col1α1-mut), luciferase activities were restored. A restoration of luciferase activities also occurred when the pcDNA3.1-CD44 3′ UTR was co-transfected along with the Luc-Col1α1 and miR-328, compared with Luc-Col1α1 and miR-328 transfection alone (Fig. 5E). This demonstrated that the miR-328 could be bound by the CD44 3′ UTR, thus allowing for increased luciferase expression; however, these levels were lower than those for the control vector suggesting that other miRNAs play a role in this system.

Corroboration of miRNA function in MDA-MB-231 cells

The functions of miR-328, miR-491, miR-671 and miR-512-3p were confirmed by transfecting these miRNAs into MDA-MB-231 cells stably transfected with the CD44 3′ UTR. Migration assays showed that there was a decrease in cell migration in the miRNA-transfected CD44 3′ UTR cells compared with the control CD44 3′ UTR cells (Fig. 6A; supplementary material Fig. S6a). The functions of these miRNAs were further confirmed by invasion assays. There was a decrease in the number of miRNA-transfected cells that invaded through the Matrigel and the transwell membrane pores as compared

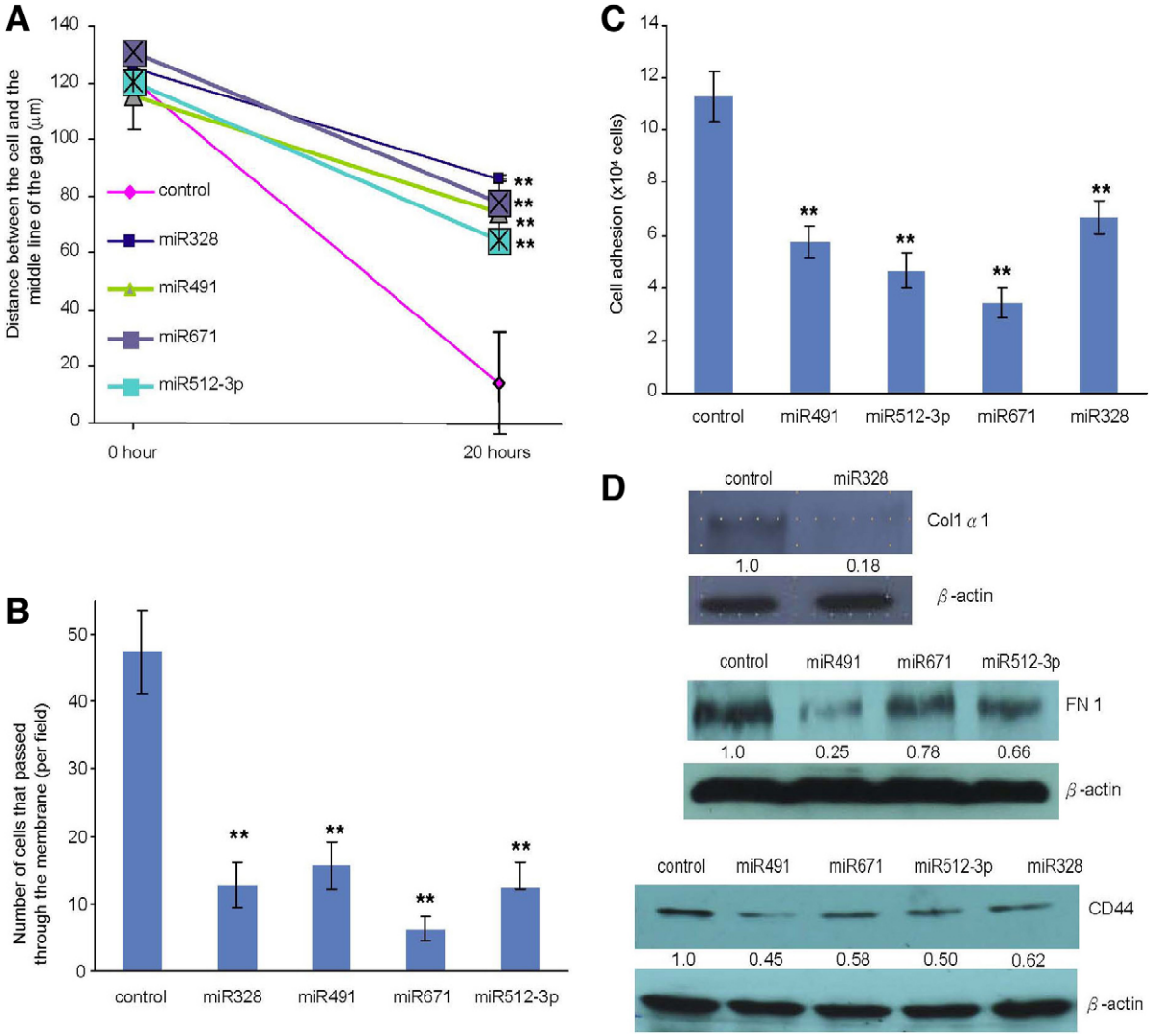

Fig. 6. Corroboration of miRNA function in MDA-MB-231 cells. (**A**) Stable CD44 3′ UTR-transfected MDA-MB-231 cells were co-transfected with miR-328, miR-491, miR-671, miR-512-3p or control oligonucleotide. There was a decrease in cell migration for all the miRNA-transfected cells compared with the control ($n=10$). (**B**) Invasion of the miRNA-transfected cells was examined. There was a decrease in the number of cells that invaded through the Matrigel and transwell membrane pores for miRNA-transfected cells as compared with the control cells ($n=3$). (**C**) In cell adhesion assays, there was an increase in detachment of the miRNA-transfected CD44 3′ UTR cells compared with the control ($n=5$). (**D**) Western blot was used to confirm the downregulation of Col1α1 and FN1 by the overexpression of the corresponding miRNAs. When CD44 3′ UTR cells were transfected with the miRNAs, there was a decrease in levels of collagen (miR-328) and FN1 (miR-491, miR-671 and miR-512-3p). CD44 protein levels also decreased when the CD44 3′ UTR cells were transfected with miR-328, miR-491, miR-671 and miR-512-3p. $^{**}P<0.01$.

with the control cells (Fig. 6B; supplementary material Fig. S6b). Also, there was an increase in detachment of the miRNA-transfected CD44 3′ UTR cells compared with the control cells (Fig. 6C; supplementary material Fig. S6c). Western blot analysis confirmed that there was a downregulation of Col1α1 and FN1 by the overexpression of these miRNAs (Fig. 6D). When the CD44 3′ UTR cells were transfected with the miRNAs, there was a decrease in Col1α1 (due to miR-328) and FN1 (due to miR-491, miR-671 and miR-512-3p). Furthermore, these miRNAs targeted CD44, as shown by the decrease in expression of CD44 in the CD44 3′ UTR cells when they were transfected with miR-328, miR-491, miR-671 and miR-512-3p (Fig. 6D). These results demonstrated that miR-328, miR-491, miR-671 and miR-512-3p are involved in migration, invasion and detachment and confirmed CD44 3′ UTR function in MDA-MB-231 cells. However, some miRNAs have a stronger effect on the cell matrix properties than others. For example, in vitro, miR-328 had a stronger effect on the inhibition of cell migration than the other microRNAs but miR-671 had a stronger effect on the inhibition of invasion and cell adhesion.

Discussion

We have previously demonstrated that expression of the CD44 3′ UTR inhibited tumor growth and angiogenesis, which upregulated CD44 and CDC42 expression by binding to the miRNAs miR-216a, miR-330 and miR-608 (Jeyapalan et al., 2011). We hypothesize that the CD44 3′ UTR can also regulate the expression of many other proteins because a 3′ UTR has the capacity to bind many miRNAs, which can affect other mRNAs downstream. This study was initiated by our observation that the breast cancer cell line MDA-MB-231 transfected with CD44 3′ UTR displayed an increase in metastasis. As a result of CD44 3′ UTR expression, cells and tumors had a lower rate of migration and invasion than control samples.

Initially, the invasion and migration observations were surprising because we reported previously that the elevated expression of CD44 inhibits tumor growth (Jeyapalan et al., 2011). However, the dual nature of CD44 (as a tumor suppressor and activator) in breast cancer progression has been reported in many studies (Hill et al., 2006; Lopez et al., 2005; Marangoni et al., 2009). Because CD44 can express a great number of isoforms and miRNAs can function in different cellular environments, the key point to this variation in CD44 effects appears to be dependent on the isoforms of CD44 expressed, the type of cellular environment it is involved in and the type of cancer involved. Our results show that the CD44 3′ UTR is important in metastasis because MDA-MB-231 cells transfected with CD44 3′ UTR have increased migration and invasion characteristics (they are able to migrate faster than the control cells in scratch assays and invade through Matrigel-coated trans-membrane pores). This suggests that these cells have the increased ability to pass through epithelial tissues and invade surrounding tissues. This has been reported to occur due to the capability that CD44 has to promote the attachment of matrix metalloproteinase 9 to the leading edge of migrating cells (Yu and Stamenkovic, 1999).

Because the ECM plays crucial roles in cancer metastasis, we investigated whether CD44 3′ UTR could upregulate expression of the ECM molecules by modulating miRNA function. After extensive examination, we found that the CD44 3′ UTR had several miRNAs in common with the 3′ UTRs of FN1 (miR-512-3p, miR-491 and miR-671) and Col1α1 (miR-328). Due to binding of endogenous miRNAs to the exogenously overexpressed CD44 3′ UTR, we found not only an increased expression of CD44, but also an increased expression of FN1 and Col1α1. FN1 is an important ECM molecule and is known to be involved in matrix remodeling, cell adhesion and migration processes, which affect cell motility by regulating actin polymerization. Recently, Howe and co-workers reported that the inhibition of FN1 by miR-200c resulted in the suppression of cell migration (Howe et al., 2011). The involvement of FN1 in cancer cell migration and metastasis has also been well documented (Hanamura et al., 1997; Waalkes et al., 2010). Additionally, Col1α1 is a major ECM component that plays a significant role in influencing cell behavior and maintaining tissue architecture. The upregulation of collagen genes in primary tumors with metastatic potential has been widely reported (O'Carroll et al., 2007; Oneyama et al., 2011; Ramaswamy et al., 2003). Furthermore, high levels of type 1 collagen have also been found in metastatic lesions (Brown et al., 1999; Jensen et al., 2002). Recently, Soikkeli and co-workers reported that both FN1 and Col1α1 are involved in promoting cell migration, invasion and metastasis due to their increased expression and interaction with other matrix network proteins (Soikkeli et al., 2010). However, similar to reports on the dual nature of CD44, there have also been reports of a decreased synthesis of collagen by cells in culture upon oncogenic transformation through treatment with viruses or chemical carcinogens (Adams et al., 1977; Smith and Niles, 1980).

MDA-MB-231 cells stably transfected with CD44 3′ UTR were co-transfected with siRNAs and miRNAs targeting the 3′ UTR to corroborate the function of CD44 3′ UTR. The siRNAs and miRNAs were found to decrease CD44, FN1 and Col1α1 levels in the stable CD44 3′ UTR cells and in the parental MDA-MB-231 cells. Furthermore, they were able to reverse the cell activities, resulting in decreased migration and invasion of the MDA-MB-231 cells. This shows that the phenotypic changes associated with the CD44 3′ UTR in the MDA-MB-231 cells is due to the direct effects of the 3′ UTR of CD44. Admittedly, because there are many miRNAs that are capable of binding to the 3′ UTR of CD44, there might be other miRNAs and other ECM molecules that contribute to the enhanced cell migration and metastatic phenotype seen here, along with miR-328, miR-491, miR-671 and miR-512-3p and CD44, FN1 and Col1α1. Further investigations could reveal that these other miRNAs and matrix molecules also play a significant role in the development of metastasis when the CD44 3′ UTR is overexpressed.

The 3′ UTR has been shown to be involved in multiple roles in addition to translational regulation. A study by Nagasaki and co-workers has shown that potentially 3% of all genes are alternatively spliced within their 3′ UTR to escape miRNA regulation in various biological activities (Nagasaki et al., 2005). Furthermore, a 3′ UTR mutation located within the binding site of miR-189 has been reported to result in Tourette's syndrome (Abelson et al., 2005). The importance of the interplay between 3′ UTR and miRNA during translational regulation is gradually being revealed.

Our study demonstrates that, as usual, the 3′ UTR of CD44 allows translational regulation by miRNAs. Conversely, the 3′ UTR can regulate miRNA activities by binding to them. A study by Gentner and co-workers revealed that imperfect miRNA binding sites are better targets and are more efficient in repressing gene expression than perfect binding sites (Gentner et al., 2009). The 3′ UTR non-coding transcript is a naturally

evolved decoy because it contains imperfect binding sites for many miRNAs. Our experiments indicate that the similar functions of miRNAs can be regulated by a fragment of the non-coding transcript of a 3′ UTR. This might represent the more natural way of regulation of gene expression, allowing co-regulation of genes with related functions to CD44 to occur in a finely tuned manner. In the 3′ UTR overexpression system we investigate here, we tried to artificially simulate a naturally occurring non-coding RNA. The 3′ UTR can act as a pseudogene to regulate miRNA and other mRNAs similarly to the pseudogene of PTEN recently discovered (Poliseno et al., 2010).

Non-coding and mutant transcripts have been found to exist after genomic deletion and truncation experiments in which translational silencing produced mutant phenotypes (Bala et al., 2007; van Rooij et al., 2006). However, in knockout experiments when the protein of interest is no longer expressed but no detectable phenotypes are seen, it is believed that the mutated or deleted proteins are compensated by other proteins. With the knowledge of miRNA functions regulated by non-coding RNAs, we might also need to consider that, in some cases, the knockout mice can express a mutated or truncated mRNA that can modulate miRNA function. Thus, even though other related proteins can compensate for function, the non-coding transcript might play an important role in the compensation. In other words, a gene can execute its function via the protein or its transcript to regulate miRNA activities.

Our results demonstrate a functional alteration in cellular activities induced by expression of the non-coding transcript of CD44. Expression of the 3′ UTR altered the translation of other mRNAs such as FN1 and Col1α1, which were targeted by the same miRNAs. Thus, it is possible that the 3′ UTR can play diverse roles in the fine tuning of gene expression in a very effective way. Future therapeutic strategies might be developed by such approach.

Materials and Methods

Construct generation

To study the effect of CD44 3′ UTR on cell activities, we used the CD44 3′ UTR construct described previously (Jeyapalan et al., 2011). The protein expression construct CD44E has been successfully used in our laboratory (Wang et al., 2008) and was originally generated and kindly donated by Warren Knudson's group (Rush Medical College, Chicago, IL) (Jiang et al., 2002).

A luciferase reporter vector (pMir-Report; Ambion) was used to generate the luciferase constructs. The Col1α1 3′ UTR and different lengths of the FN1 3′ UTR were cloned using RT-PCR. The PCR products were digested with *Sac*I and *Mlu*I and the fragment inserted into a *Sac*I- and *Mlu*I-digested pMir-Report Luciferase plasmid (Ambion) to obtain different luciferase constructs. A mutant construct for each luciferase–3′ UTR construct was generated using a similar approach. Similarly, the PCR products were digested with *Sac*I and *Mlu*I and the fragment was inserted into a *Sac*I- and *Mlu*I-digested pMir-Report Luciferase plasmid to obtain different mutant luciferase constructs. A non-related sequence amplified from the coding sequence of the chicken versican G3 domain and inserted into the luciferase construct was used as a control, as previously described (Lee et al., 2009). It was expected that there would be no endogenous miRNAs targeting this fragment because it is in the coding region. The CD44 3′ UTR luciferase plasmid used was previously constructed in this laboratory (Wang et al., 2008).

Cell migration

Cell migration was measured using a culture scratching assay as described previously (Shatseva et al., 2011). In brief, 3×10^5 cells were seeded in each well of a six-well plate. The cell cultures were scratched using a 200-µl pipette tip. The cultures were washed with PBS to remove cell debris and then the culture medium was replaced with serum-free medium. Cell migration was monitored and photographed after 20 hours. Distance traveled from the initial scratch site was measured and the migration distance quantified. For the parental cells transfected with siRNA targeting CD44 3′ UTR, cells were photographed and the migration distance measured after 30 hours.

Cell invasion

Cell transwell membrane inserts were placed in a 24-well plate and coated with 100 µl of Matrigel (diluted 1:10). 1×10^3 cells were placed in serum-free medium inside the inserts. Medium supplemented with 10% FBS was placed at the bottom of the inserts and in the wells of the plates. Cells were incubated at 37℃ and were allowed to invade and migrate through the Matrigel and the membrane pores in the inserts. After 20 hours, the upper Matrigel layer and cells were removed, and the cells on the surface of the lower side of the membrane were fixed and stained. Cells that migrated onto the lower surface were counted from representative areas for quantification. For the parental cells transfected with siRNA targeting CD44 3′ UTR, cells were photographed and quantified after 48 hours.

Cell detachment

Cells were plated at 1×10^5 cells per well in a six-well plate and treated with 5 mM EDTA. Cell detachment was monitored, photographed and quantified 10 minutes after the addition of EDTA. For the parental cells transfected with siRNA targeting CD44 3′ UTR, cells were photographed and quantified after 5 minutes.

RNA analysis

Expression of the CD44 3′ UTR was confirmed by real-time PCR. In brief, total RNA was extracted from cell cultures using the mirVana miRNA Isolation Kit (Ambion) according to the manufacturer's instructions. Real-time PCR was performed as previously described (Fang et al., 2011). The primers for CD44 3′ UTR were previously used in our laboratory (Wang et al., 2008). The primers used as the real-time PCR controls were human-U6RNAf and human-U6RNAr (Shan et al., 2009).

Tail injection protocol

Eight-week old CD1 strain nude mice were injected in a tail vein with 1×10^6 cells. Mice were monitored weekly for changes in appearance and behavior. Protocols established with the Animal Care Facility at Sunnybrook Research Institute dictated when mice were to be sacrificed for humane reasons. All animal experiments were performed according to the relevant regulatory standards.

siRNA assays

Four different siRNA sequences complementary to CD44 3′ UTR were used as previously described (Jeyapalan et al., 2011). The CD44 3′ UTR-transfected MDA-MB-231 cells were seeded at a density of 2×10^5 cells per well in six-well plates in 2 ml culture medium containing 10% FBS. Cells were washed twice with serum-free medium 24 hours after the inoculation, followed by transfection with the four different siRNAs using Lipofectamine 2000 (Invitrogen). The cells were harvested by exposure to trypsin 48 hours after the transfection and then subjected to various assays.

Tumor formation assay

The tumor formation assay was performed as described (LaPierre et al., 2007). Briefly, 6-week-old CD1 strain nude mice (Jackson Laboratories) were subcutaneously injected with CD44 3′ UTR-transfected MT1 cells and control cells (1×10^6 cells). Tumors were retrieved by the end of the fourth week and fixed in 10% formalin, processed, embedded in paraffin and sectioned for immunohistochemistry.

Immunohistochemistry

Immunohistochemistry was performed on the paraffin-embedded tissue sections. The tumor sections were processed for antigen retrieval, blocked with 10% goat serum and incubated overnight with primary antibodies against CD44, Ki67, Col1α1 and FN1 in TBS containing 10% goat serum. The sections were then washed and labeled with biotinylated secondary antibody, followed by avidin-conjugated horseradish peroxidase provided by the Vectastain ABC kit (Vector, PK-4000). The staining was developed according to the manufacturer's instructions. The slides were subsequently stained with Mayer's hematoxylin for counterstaining, followed by slide mounting.

Western blotting

Western blot was performed on cell and tissue lysates. Briefly, cells were seeded onto a six-well plate at 2×10^5 cells per well and left overnight. They were then transfected with 1 µg of CD44 3′ UTR or the control vector to create stable cell lines. In addition, the CD44 3′ UTR cells were transfected with siRNA against CD44 3′ UTR or different miRNA mimics. The control was a scramble RNA provided in the kit. Proteins were extracted 48 hours after transfection by lysing in 60 µl lysis buffer containing protease inhibitors (150 mM NaCl, 25 mM Tris-HCl, pH 8.0, 0.5 M EDTA, 20% Triton X-100, 8 M urea, and 1× protease inhibitor cocktail).

Tissues were weighed and ground in a quantity of lysis buffer according to weight. All samples were subjected to SDS-PAGE and then transferred to nitrocellulose membranes. They were then incubated overnight at 4℃ with

antibodies against CD44, FN1 or Col1α1 at a dilution of 1:500. Appropriate secondary antibodies at 1:2000 dilution were incubated with the membranes at room temperature for 2 hours. After detection of the protein bands, the blots were stripped and re-probed with mouse monoclonal antibody against β-actin (A5316, Sigma) to confirm equal sample loading. After secondary antibody incubation, the blot was washed and detected using an ECL kit (Millipore) and autoradiography. Band quantification was performed using Syntools. Band values took into account each corresponding β-actin band and were compared with the controls, which were set at baseline 1.0. The calculation formula used was band value = (sample band intensity/sample β-actin intensity) ÷ (control band intensity/control β-actin intensity).

Luciferase activity assays

Luciferase activity assays were performed using a dual-luciferase reporter system developed by Promega (E1960). In brief, MDA-MB-231 cells were seeded onto 24-well tissue culture plates at a density of 3×10^4 cells per well in medium containing 10% FBS and incubated overnight. The cells were co-transfected with the luciferase reporter constructs, corresponding miRNA mimics and Renilla luciferase construct using Lipofectamine 2000. The cells were then lysed using 100 µl of passive lysis buffer per well, placed on a shaker for 30 minutes and centrifuged for supernatant collection. Supernatent (20 µl) was then mixed with 100 µl of LAR II, and firefly luciferase activities measured using a luminometer. For the internal control, 100 µl of Stop & Go reagent was added to the samples. Renilla luciferase activities were then measured in the same tube. Luciferase activities after different treatments were compared after normalization with Renilla luciferase activities. The miRNAs were used at a concentration of 100 nM.

For the CD44 3′ UTR competition experiments, the same luciferase system was used. Cell were transfected with 10 µg of luciferase construct containing FN1 or Col1α1 3′ UTR in each well. The cells were also transfected with CD44 3′ UTR or the control vector at a concentration of 10 µg plasmid per well.

Statistical analysis

The results (mean±s.d.) of all experiments were subjected to statistical analysis using the Student's *t*-test; $P<0.05$ was considered significant.

Acknowledgements

The authors thank Michael Sefton for valuable discussion and Zhaoqun Deng, Haoran Li and Jennifer Sun for technical assistance.

Funding

This work was supported by a grant from Canadian Institutes of Health Research [grant number MOP-102635] to B.B.Y. Z.J. is the recipient of an Ontario Graduate Scholarship. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.100818/-/DC1

References

Abelson, J. F., Kwan, K. Y., O'Roak, B. J., Baek, D. Y., Stillman, A. A., Morgan, T. M., Mathews, C. A., Pauls, D. L., Rasin, M. R., Gunel, M. et al. (2005). Sequence variants in SLITRK1 are associated with Tourette's syndrome. *Science* **310**, 317-320.

Adams, S. L., Sobel, M. E., Howard, B. H., Olden, K., Yamada, K. M., de Crombrugghe, B. and Pastan, I. (1977). Levels of translatable mRNAs for cell surface protein, collagen precursors, and two membrane proteins are altered in Rous sarcoma virus-transformed chick embryo fibroblasts. *Proc. Natl. Acad. Sci. USA* **74**, 3399-3403.

Bala, A. D., Spitzer, M. W. and Takahashi, T. T. (2007). Auditory spatial acuity approximates the resolving power of space-specific neurons. *PLoS ONE* **2**, e675.

Brown, L. F., Guidi, A. J., Schnitt, S. J., Van De Water, L., Iruela-Arispe, M. L., Yeo, T. K., Tognazzi, K. and Dvorak, H. F. (1999). Vascular stroma formation in carcinoma in situ, invasive carcinoma, and metastatic carcinoma of the breast. *Clin. Cancer Res.* **5**, 1041-1056.

Chin, L. J., Ratner, E., Leng, S., Zhai, R., Nallur, S., Babar, I., Muller, R. U., Straka, E., Su, L., Burki, E. A. et al. (2008). A SNP in a let-7 microRNA complementary site in the KRAS 3′ untranslated region increases non-small cell lung cancer risk. *Cancer Res.* **68**, 8535-8540.

Ebert, M. S., Neilson, J. R. and Sharp, P. A. (2007). MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells. *Nat. Methods* **4**, 721-726.

Fang, L., Deng, Z., Shatseva, T., Yang, J., Peng, C., Du, W. W., Yee, A. J., Ang, L. C., He, C., Shan, S. W. et al. (2011). MicroRNA miR-93 promotes tumor growth and angiogenesis by targeting integrin-â8. *Oncogene* **30**, 806-821.

Gentner, B., Schira, G., Giustacchini, A., Amendola, M., Brown, B. D., Ponzoni, M. and Naldini, L. (2009). Stable knockdown of microRNA in vivo by lentiviral vectors. *Nat. Methods* **6**, 63-66.

Hanamura, N., Yoshida, T., Matsumoto, E., Kawarada, Y. and Sakakura, T. (1997). Expression of fibronectin and tenascin-C mRNA by myofibroblasts, vascular cells and epithelial cells in human colon adenomas and carcinomas. *Int. J. Cancer* **73**, 10-15.

Haraguchi, T., Ozaki, Y. and Iba, H. (2009). Vectors expressing efficient RNA decoys achieve the long-term suppression of specific microRNA activity in mammalian cells. *Nucleic Acids Res.* **37**, e43.

Hill, A., McFarlane, S., Mulligan, K., Gillespie, H., Draffin, J. E., Trimble, A., Ouhtit, A., Johnston, P. G., Harkin, D. P., McCormick, D. et al. (2006). Cortactin underpins CD44-promoted invasion and adhesion of breast cancer cells to bone marrow endothelial cells. *Oncogene* **25**, 6079-6091.

Howe, E. N., Cochrane, D. R. and Richer, J. K. (2011). Targets of miR-200c mediate suppression of cell motility and anoikis resistance. *Breast Cancer Res.* **13**, R45.

Hua, Z., Lv, Q., Ye, W., Wong, C. K., Cai, G., Gu, D., Ji, Y., Zhao, C., Wang, J., Yang, B. B. et al. (2006). MiRNA-directed regulation of VEGF and other angiogenic factors under hypoxia. *PLoS ONE* **1**, e116.

Jensen, B. V., Johansen, J. S., Skovsgaard, T., Brandt, J. and Teisner, B. (2002). Extracellular matrix building marked by the N-terminal propeptide of procollagen type I reflect aggressiveness of recurrent breast cancer. *Int. J. Cancer* **98**, 582-589.

Jeyapalan, Z., Deng, Z., Shatseva, T., Fang, L., He, C. and Yang, B. B. (2011). Expression of CD44 3′-untranslated region regulates endogenous microRNA functions in tumorigenesis and angiogenesis. *Nucleic Acids Res.* **39**, 3026-3041.

Jiang, H., Peterson, R. S., Wang, W., Bartnik, E., Knudson, C. B. and Knudson, W. (2002). A requirement for the CD44 cytoplasmic domain for hyaluronan binding, pericellular matrix assembly, and receptor-mediated endocytosis in COS-7 cells. *J. Biol. Chem.* **277**, 10531-10538.

Kahai, S., Lee, S. C., Lee, D. Y., Yang, J., Li, M., Wang, C. H., Jiang, Z., Zhang, Y., Peng, C. and Yang, B. B. (2009). MicroRNA miR-378 regulates nephronectin expression modulating osteoblast differentiation by targeting GalNT-7. *PLoS ONE* **4**, e7535.

Krützfeldt, J., Rajewsky, N., Braich, R., Rajeev, K. G., Tuschl, T., Manoharan, M. and Stoffel, M. (2005). Silencing of microRNAs in vivo with 'antagomirs'. *Nature* **438**, 685-689.

LaPierre, D. P., Lee, D. Y., Li, S. Z., Xie, Y. Z., Zhong, L., Sheng, W., Deng, Z. and Yang, B. B. (2007). The ability of versican to simultaneously cause apoptotic resistance and sensitivity. *Cancer Res.* **67**, 4742-4750.

Lee, D. Y., Shatseva, T., Jeyapalan, Z., Du, W. W., Deng, Z. and Yang, B. B. (2009). A 3′-untranslated region (3′UTR) induces organ adhesion by regulating miR-199a* functions. *PLoS ONE* **4**, e4527.

Lee, D. Y., Jeyapalan, Z., Fang, L., Yang, J., Zhang, Y., Yee, A. Y., Li, M., Du, W. W., Shatseva, T. and Yang, B. B. (2010). Expression of versican 3′-untranslated region modulates endogenous microRNA functions. *PLoS ONE* **5**, e13599.

Lee, S. C., Fang, L., Wang, C. H., Kahai, S., Deng, Z. and Yang, B. B. (2011). A non-coding transcript of nephronectin promotes osteoblast differentiation by modulating microRNA functions. *FEBS Lett.* **585**, 2610-2616.

Lopez, J. I., Camenisch, T. D., Stevens, M. V., Sands, B. J., McDonald, J. and Schroeder, J. A. (2005). CD44 attenuates metastatic invasion during breast cancer progression. *Cancer Res.* **65**, 6755-6763.

Marangoni, E., Lecomte, N., Durand, L., de Pinieux, G., Decaudin, D., Chomienne, C., Smadja-Joffe, F. and Poupon, M. F. (2009). CD44 targeting reduces tumour growth and prevents post-chemotherapy relapse of human breast cancers xenografts. *Br. J. Cancer* **100**, 918-922.

Mayr, C. and Bartel, D. P. (2009). Widespread shortening of 3′UTRs by alternative cleavage and polyadenylation activates oncogenes in cancer cells. *Cell* **138**, 673-684.

Meister, G., Landthaler, M., Dorsett, Y. and Tuschl, T. (2004). Sequence-specific inhibition of microRNA- and siRNA-induced RNA silencing. *RNA* **10**, 544-550.

Nagasaki, H., Arita, M., Nishizawa, T., Suwa, M. and Gotoh, O. (2005). Species-specific variation of alternative splicing and transcriptional initiation in six eukaryotes. *Gene* **364**, 53-62.

Negrini, M., Ferracin, M., Sabbioni, S. and Croce, C. M. (2007). MicroRNAs in human cancer: from research to therapy. *J. Cell Sci.* **120**, 1833-1840.

O'Carroll, D., Mecklenbrauker, I., Das, P. P., Santana, A., Koenig, U., Enright, A. J., Miska, E. A. and Tarakhovsky, A. (2007). A Slicer-independent role for Argonaute 2 in hematopoiesis and the microRNA pathway. *Genes Dev.* **21**, 1999-2004.

Oneyama, C., Ikeda, J., Okuzaki, D., Suzuki, K., Kanou, T., Shintani, Y., Morii, E., Okumura, M., Aozasa, K. and Okada, M. (2011). MicroRNA-mediated down-regulation of mTOR/FGFR3 controls tumor growth induced by Src-related oncogenic pathways. *Oncogene* **30**, 3489-3501.

Ørom, U. A., Kauppinen, S. and Lund, A. H. (2006). LNA-modified oligonucleotides mediate specific inhibition of microRNA function. *Gene* **372**, 137-141.

Poliseno, L., Salmena, L., Zhang, J., Carver, B., Haveman, W. J. and Pandolfi, P. P. (2010). A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. *Nature* **465**, 1033-1038.

Ramaswamy, S., Ross, K. N., Lander, E. S. and Golub, T. R. (2003). A molecular signature of metastasis in primary solid tumors. *Nat. Genet.* **33**, 49-54.

Shan, S. W., Lee, D. Y., Deng, Z., Shatseva, T., Jeyapalan, Z., Du, W. W., Zhang, Y., Xuan, J. W., Yee, S. P., Siragam, V. et al. (2009). MicroRNA MiR-17 retards tissue growth and represses fibronectin expression. *Nat. Cell Biol.* **11**, 1031-1038.

Shatseva, T., Lee, D. Y., Deng, Z. and Yang, B. B. (2011). MicroRNA miR-199a-3p regulates cell proliferation and survival by targeting caveolin-2. *J. Cell Sci.* **124**, 2826-2836.

Shimono, Y., Zabala, M., Cho, R. W., Lobo, N., Dalerba, P., Qian, D., Diehn, M., Liu, H., Panula, S. P., Chiao, E. et al. (2009). Downregulation of miRNA-200c links breast cancer stem cells with normal stem cells. *Cell* **138**, 592-603.

Smith, B. D. and Niles, R. (1980). Characterization of collagen synthesized by normal and chemically transformed rat liver epithelial cell lines. *Biochemistry* **19**, 1820-1825.

Soikkeli, J., Podlasz, P., Yin, M., Nummela, P., Jahkola, T., Virolainen, S., Krogerus, L., Heikkilä, P., von Smitten, K., Saksela, O. et al. (2010). Metastatic outgrowth encompasses COL-I, FN1, and POSTN up-regulation and assembly to fibrillar networks regulating cell adhesion, migration, and growth. *Am. J. Pathol.* **177**, 387-403.

Soutschek, J., Akinc, A., Bramlage, B., Charisse, K., Constien, R., Donoghue, M., Elbashir, S., Geick, A., Hadwiger, P., Harborth, J. et al. (2004). Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* **432**, 173-178.

Torrents, D., Suyama, M., Zdobnov, E. and Bork, P. (2003). A genome-wide survey of human pseudogenes. *Genome Res.* **13**, 2559-2567.

van Rooij, E., Sutherland, L. B., Liu, N., Williams, A. H., McAnally, J., Gerard, R. D., Richardson, J. A. and Olson, E. N. (2006). A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. *Proc. Natl. Acad. Sci. USA* **103**, 18255-18260.

Waalkes, S., Atschekzei, F., Kramer, M. W., Hennenlotter, J., Vetter, G., Becker, J. U., Stenzl, A., Merseburger, A. S., Schrader, A. J., Kuczyk, M. A. et al. (2010). Fibronectin 1 mRNA expression correlates with advanced disease in renal cancer. *BMC Cancer* **10**, 503.

Wang, C. H., Lee, D. Y., Deng, Z., Jeyapalan, Z., Lee, S. C., Kahai, S., Lu, W. Y., Zhang, Y. and Yang, B. B. (2008). MicroRNA miR-328 regulates zonation morphogenesis by targeting CD44 expression. *PLoS ONE* **3**, e2420.

Ye, W., Lv, Q., Wong, C. K., Hu, S., Fu, C., Hua, Z., Cai, G., Li, G., Yang, B. B. and Zhang, Y. (2008). The effect of central loops in miRNA:MRE duplexes on the efficiency of miRNA-mediated gene regulation. *PLoS ONE* **3**, e1719.

Yu, Q. and Stamenkovic, I. (1999). Localization of matrix metalloproteinase 9 to the cell surface provides a mechanism for CD44-mediated tumor invasion. *Genes Dev.* **13**, 35-48.