3026–3041 Nucleic Acids Research, 2011, Vol. 39, No. 8 Published online 10 December 2010
doi:10.1093/nar/gkq1003

# Expression of CD44 3′-untranslated region regulates endogenous microRNA functions in tumorigenesis and angiogenesis

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Received May 20, 2010; Revised October 4, 2010; Accepted October 7, 2010



## ABSTRACT

**The non-coding 3′-untranslated region (UTR) plays an important role in the regulation of microRNA (miRNA) functions, since it can bind and inactivate multiple miRNAs. Here, we show the 3′-UTR of CD44 is able to antagonize cytoplasmic miRNAs, and result in the increased translation of CD44 and downstream target mRNA, CDC42. A series of cell function assays in the human breast cancer cell line, MT-1, have shown that the CD44 3′-UTR inhibits proliferation, colony formation and tumor growth. Furthermore, it modulated endothelial cell activities, favored angiogenesis, induced tumor cell apoptosis and increased sensitivity to Docetaxel. These results are due to the interaction of the CD44 3′-UTR with multiple miRNAs. Computational algorithms have predicted three miRNAs, miR-216a, miR-330 and miR-608, can bind to both the CD44 and CDC42 3′-UTRs. This was confirmed with luciferase assays, western blotting and immunohistochemical staining and correlated with a series of siRNA assays. Thus, the non-coding CD44 3′-UTR serves as a competitor for miRNA binding and subsequently inactivates miRNA functions, by freeing the target mRNAs from being repressed.**

## INTRODUCTION

CD44 is a transmembrane glycoprotein that exists in several isoforms with different extracellular regions. It is involved in a wide range of cellular functions including cell–cell and cell–matrix interactions, lymphocyte activation and homing, haematopoiesis, tumor metastasis and cell migration (1–3). The various transcripts of CD44 are encoded for by one-gene locus on chromosome 11, which contains 20 exons (4). These transcripts undergo complex alternative splicing resulting in many functionally distinct isoforms (3). Thus, the alternative splicing is the basis for the structural and functional diversity of this protein, and may be related to tumor metastasis (5). The standard form of CD44, CD44H, plays a role in cell locomotion in the presence of glycosaminoglycan chains and is considered to enhance the tumorigenic properties of some lymphomas and melanomas (6). As opposed to the standard form of CD44, which is abundant in many tissues, isoforms encoded by the variant exons are highly restricted in their distribution in non-malignant tissues. In some cancers, CD44 upregulation is associated with a favorable outcome, such as in epithelial ovarian cancer; its increased expression is seen as an indicator of increased survival time (7). This is also the case with Burkitt's lymphoma, neuroblastoma and prostate cancer, where the loss of CD44 expression is accompanied with oncogenic transformation (8). Our recent study indicated that expression of CD44 is regulated by microRNA (miRNA) miR-328 (9).

The miRNAs are 18–24 nucleotides of single-stranded RNAs, which are transcribed from the genome and are regulators of mRNAs. The mRNAs that are bound by miRNAs are targeted for degradation and are transported to p-bodies leading to the translational repression of mRNAs (10). There are over 700 miRNAs that have been sequenced and reported, and it is estimated that one-third of genes are regulated by miRNAs as one miRNA can regulate the expression of many genes (11). By silencing various target mRNAs, miRNAs have key roles in controlling diverse regulatory pathways including development, apoptosis, protein secretion and cell proliferation (12–17). Furthermore, the deregulation of miRNAs has been implicated in a growing number of diseases, including cancer development (18,19). It has been reported that miRNAs are aberrantly expressed in human breast cancer (20). The expression of some

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miRNAs has been correlated with specific breast cancer biopathological features, such as estrogen and progesterone receptor expression, vascular invasion, lymph node metastasis, tumor stage and proliferation index (21–23).

Usually miRNAs function by targeting the 3′-UTRs of mRNAs. There are several regulatory sequences found in the 3′-UTR: a polyadenylation signal that marks the site of cleavage of the transcript ~30-nt downstream of the signal; binding sites for AU-rich element binding proteins, which can stabilize or destabilize the mRNA depending on the protein; binding sites for miRNAs (24,25). The 3′-UTR is well known to be involved in the stability, nuclear transport, cellular localization and translational efficiency of mRNAs (25,26). Furthermore, some 3′-UTRs are subjected to alternative splicing (27), which can be postulated to occur in order for the 3′-UTR to escape miRNA regulation in different biological activities. Recent studies have demonstrated that the 3′-UTRs are the most important target sites for miRNAs. We hypothesized that the 3′-UTR can also play a role in feedback regulation of miRNA functions. This study was designed to investigate whether the exogenous overexpression of CD44 3′-UTR could affect miRNA functions.

## MATERIALS AND METHODS

### Construct generation

To study the effect of CD44 3′-UTR on cell activities, we have cloned the 3′-UTR by using an existing construct that was previously made in the lab—Luc-CD44 3′-UTR (9). The CD44 3′-UTR fragment was digested with restriction enzymes NheI and ApaI and inserted into NheI- and ApaI-opened pcDNA3.1 vector. The protein expression construct of CD44E was also previously used in the lab (9) and was a kind gift from Dr Warren Knudson (28).

A luciferase reporter vector (pMir-Report; Ambion) was used to generate the luciferase constructs. The different lengths of the CDC42 3′-UTR was cloned using PCR. The PCR products were then digested with SacI and MluI and the fragment was inserted into a SacI- and MluI-digested pMir-Report Luciferase plasmid (Ambion), to obtain the different luciferase constructs. A mutant construct for each 3′-UTR was also generated using a similar approach with PCR. The control, a non-related sequence, was amplified from the coding sequence of the chicken versican G3 domain and was previously constructed in the lab (29). It is expected that there are no endogenous miRNAs that bind to this fragment as it is in the coding region.

Four different siRNA constructs complementary to CD44 3′-UTR sequences were generated. The CD44 3′-UTR MT-1 cells were seeded at the density of $2 \times 10^5$ cells per well in 6-well plates in 2 ml culture medium containing serum. After 24 h, cells were washed twice with serum-free medium, then transfected with the four different siRNA constructs using Lipofectamine™ 2000 (Invitrogen). Forty-eight hours after transfection, cells were harvested by exposure to trypsin and then subjected to the various assays.

### RNA analysis

Expression of the CD44 3′-UTR was confirmed by extraction of RNA (Qiagen RNAeasy™) from cells stably transfected with pcDNA3.1-CD44 3′-UTR and the empty vector control. Primers for the CD44 3′-UTR were used in a PCR reaction were previously used in our lab (9). RT–PCR for GAPDH was performed as endogenous loading control.

For real-time PCR, total RNA was extracted from cell cultures using the mirVana™ miRNA Isolation Kit (Ambion) according to the manufacturer's instructions. RT–PCR were performed as previously described (9). The primers for CD44 3′-UTR were previously used in our lab (9). The primers used as the real-time PCR controls were human-U6RNAf and human-U6RNAr.

### Cell proliferation assays

Transfected-MT-1 cells were seeded onto 100-mm tissue culture plates at a density of $1 \times 10^5$ cells/plate in DMEM containing 10% FBS and maintained at 37°C for up to 5 days. The cells were harvested and cell number was counted with a Coulter counter.

For cell-cycle analysis, cells were seeded at 90% confluence onto tissue culture plates in DMEM containing 5% FBS and maintained at 37°C for 24 h. Cell were trypsinized and resuspended in HBSS with 2% calf serum. Cells were then fixed with 80% ice cold ethanol for 30 min and resuspended in HBSS containing 0.1 mg/ml propidium iodide (PI), 0.6% of NP-40 and 2 mg/ml RNAseA (Bovine Pancreas Type II, Sigma) for 30 min. The DNA content was measured by flow cytometry (Beckman Coulter).

### Cell survival assays

For survival assays, cells were seeded at the density of $2 \times 10^5$ cells/well in 6-well plates in 2-ml culture medium containing serum. After 24 h, cells were washed twice with PBS, and then incubated in serum-free medium. At different time points, cells were harvested and the viable cells were counted after Trypan Blue staining.

For annexin V apoptosis assays, the cells were grown in low serum (1%) or serum-free conditions for 5 days. The cells were separately washed twice with cold PBS and stained with binding buffer containing fluorescein-labeled annexin V and with PI (APC Annexin V Apoptosis Detection Kit I, BD Pharmingen) following manufacturer's instructions. The cells that were positive for apoptosis (APC annexin V positive, PI negative or APC annexin V positive, PI positive) were analyzed by flow cytometry.

To test their drug sensitivity, the cells were cultured in DMEM medium supplemented with 10% FBS, and antibiotics. After cells adhered to the plate, 1 μM of Docetaxel (Sanofi-aventis Canada Inc.) was added. Cell numbers were counted 24 h after Docetaxel addition.

### Colony and tumor formation assays

Colony formation was assessed by plating $1 \times 10^3$ cells in 0.3% low-melting agarose (Seaplaque, FMC) and DMEM supplemented with 2% FBS. This mixture was placed on

0.66% agarose-coated 6-well tissue culture plates. Three weeks after plating, colonies were examined and photographed under a light microscope.

In the tumor formation experiments, CD44 3′-UTR and control MT-1 cells ($2 \times 10^6$ cells) were injected subcutaneously into 5-week-old CD1 strain nude mice (Jackson Laboratories) and tumor volume was measured every 5 days. BrdU was injected at a concentration of 50 mg/kg 24-h prior to the mice being sacrificed for tumor excision. Tumor volume ($V$) was measured using a caliper by measuring the length ($L$) and width ($W$), where $V = (L \times W^2)/2$. When the sizes of the tumors were above the limit described by the animal protocol approved by the Animal Care Committee at Sunnybrook Health Sciences Centre, the mice were sacrificed and the tumors were removed. Tumors were fixed in 10% buffered formalin, processed and embedded in paraffin.

### Immunoreaction

Western blot and immunohistochemistry were performed as previously described (9,29–31).

### Tube formation assay

YPEN-1 cells were mixed with the CD44 3′-UTR or control cells and cultured in BD Matrigel™ (Basement Membrane Matrix, BD Biosciences). The interaction of both types of cells and the formation of tube structures were examined after 24 h by light microscopy and photographed.

### Luciferase activity assays

MT-1 cells were cultured on 24-well tissue culture plates at a density of $3 \times 10^4$ cells/well in DMEM containing 10% FBS. The cultures were maintained at 37°C for 24 h, followed by co-transfection with the luciferase reporter constructs and miRNAs using Lipofectamine 2000 following the methods described by us recently (9,32).

### Statistical analysis

The results (mean values ± SD) of all experiments were subjected to statistical analysis by $t$-test.

## RESULTS

### Expression of CD44 3′-UTR decreases tumor growth

To study the effect of the CD44 3′-UTR on miRNA function, we exogenously overexpressed the CD44 3′-UTR in human mammary carcinoma cells (MT-1). MT-1 is an established estradiol and progesterone receptor-negative breast cancer cell line, which was originally obtained from surgical material that was transplanted in nude mice (33). They are optimal cells for this study because they have been routinely used as a breast cancer xenograft model in tumor growth and breast cancer progression studies (34–36). Initially, the MT-1 cell line was stably transfected with a pcDNA3.1-CD44 3′-UTR expressing construct (Figure 1a). A control cell line was established with the stable transfection of an empty pcDNA3.1 vector. Another MT-1 cell line was also established that stably expressed CD44E, coding region of the epithelium form of CD44. Total RNA from pooled stable CD44 3′-UTR- and empty vector-transfected cells were subjected to RT–PCR using random primers (Figure 1a) and oligo-dT primer (Supplementary Figure S1a) for reverse transcription. The PCR products were analyzed on agarose gel electrophoresis to confirm expression of the 3′-UTR. Expression of CD44 3′-UTR were also analyzed by real-time PCR in MT-1 cells transfected with the 3′-UTR construct or control vector. The levels of CD44 3′-UTR in the CD44 3′-UTR-transfected cells were 3-fold over that of the control, which displayed the endogenous level of CD44 3′-UTR. This confirmed that the CD44 3′-UTR is being expressed from the CD44 3′-UTR plasmid in the stable cell line. The CD44 3′-UTR appeared to be stable in the cells, since we always detected much higher levels of the 3′-UTR in the CD44 3′-UTR transfected cells compared with the control.

A series of cell function assays were performed with the CD44 3′-UTR, CD44E and control cells. In cell proliferation assays, the CD44 3′-UTR cells showed a decreased proliferation rate compared with the controls (Figure 1b). In colony formation assays, cells were plated in low-melting agarose with 2% FBS. These conditions allowed cells to expand and form 3D colonies after 2 weeks. The control cells and CD44E cells formed bigger and more colonies per plate than the stable CD44 3′-UTR cells (Figure 1c). These results were correlated with cell-cycle analysis; the CD44 3′-UTR cells had a larger population of cells in the G1 (resting) phase than the control cells (Figure 1d). As well, the control cells had larger populations in the S (synthesis) and G2 (mitotic) phases, implying that they had an increased amount of cells actively dividing compared with the CD44 3′-UTR cells.

The tumorigenic potential of CD44 3′-UTR-transfected cells was tested in nude mice. Repeated experiments showed that expression of CD44 3′-UTR greatly decreased the tumorigenic potential of cancer cells compared with control mice (Figure 1e). Bromodeoxyuridine (BrdU), which is commonly used in the detection of proliferating cells in living tissues, was injected into the mice 24 h before sacrifice and tumor sections were stained with a BrdU-FITC antibody. A decrease in BrdU staining was observed in the CD44 3′-UTR tumors explaining their diminished growth rate compared with the controls (Supplementary Figure S1b).

### Overexpression of CD44 3′-UTR promotes cell death

The effect of the overexpression of exogenous CD44 3′-UTR on cell survival was monitored, when the CD44 3′-UTR and control MT-1 cells were maintained in tissue culture dishes in low serum and serum-free conditions. The CD44 3′-UTR cells had a decreased rate of cell survival. Surviving cells were harvested and counted for statistical analysis (Figure 2a). The cells were also subjected to apoptosis assays to confirm these results. The CD44 3′-UTR and control cells were cultured in low

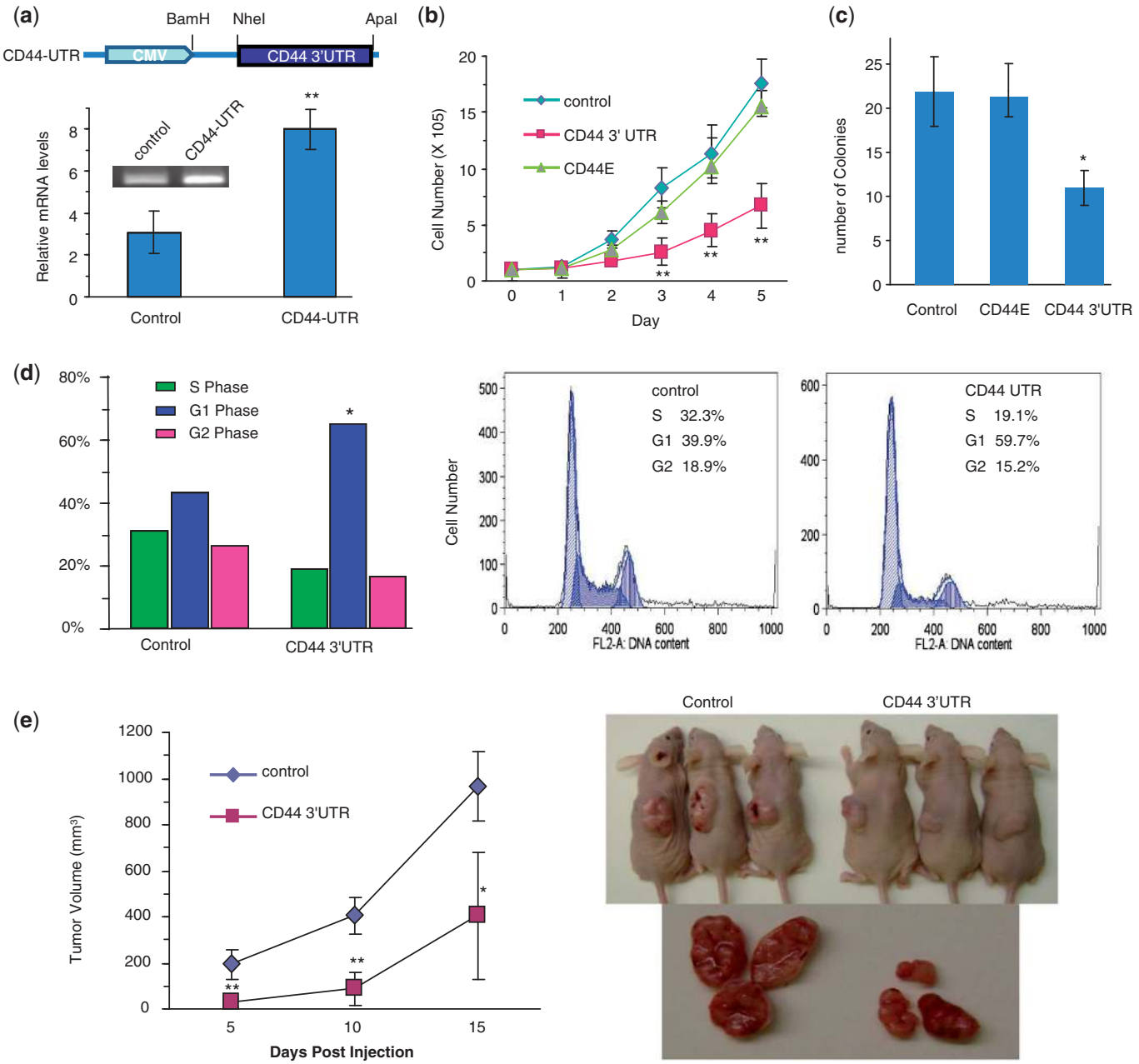


**Figure 1.** The CD44 3′-UTR decreases cell proliferation and tumor formation. (**a**) A fragment of CD44 3′-UTR (900 bp) was inserted into the pcDNA3.1 plasmid, downstream of the CMV promoter, NheI and ApaI sites, producing the CD44-UTR construct. Total RNA from CD44 3′-UTR and control cells were subjected to RT–PCR, and analyzed by agarose gel electrophoresis. CD44 3′-UTR cells had an increased expression of CD44 3′-UTR compared to control cells. There was a 3-fold increase in CD44 3′-UTR levels in the CD44 3′-UTR cells compared with the control cells as analyzed by real-time PCR. $^{**}P < 0.01$. (**b**) The CD44 3′-UTR, control and CD44E-transfected cells were plated and cell numbers were counted over a period of 5 days. The CD44 3′-UTR decreased cell proliferation of MT-1 cells. $n = 5$, $^{**}P < 0.01$. (**c**) Control and CD44E cells formed bigger and a higher amount of 3D colonies per plate than the CD44 3′-UTR cells when plated in low melting agarose with 2% FBS. $n = 3$, $^{*}P < 0.05$. (**d**) The CD44 3′-UTR cells had a larger population in the G1 phase and smaller populations in the S and G2 phases than the control cells as determined by cell cycle distribution through FACs analysis (left). Typical FACs profiles are shown (right). $n = 3$, $^{*}P < 0.05$. (**e**) MT-1 cells stably transfected with the CD44 3′-UTR or control plasmid were injected subcutaneously into nude mice and tumor growth was monitored for 15 days. Expression of the CD44 3′-UTR decreased tumor growth compared with the vector control (left). Typical mice and tumor sizes are shown (right). $n = 10$, $^{*}P < 0.05$, $^{**}P < 0.01$.

serum and serum-free conditions for 5 days, stained with annexin V and PI and analyzed by flow cytometry. The CD44 3′-UTR cells had a larger population of apoptotic cells (Annexin V positive, PI positive and Annexin V positive and PI positive) compared with the control cells in both serum-free (0%) and low-serum (1%) conditions (Figure 2b). Furthermore, the CD44 3′-UTR tumor sections exhibited more apoptotic cells than the control tumor sections when subjected to TUNEL staining (Figure 2c). In addition, cells expressing CD44 were

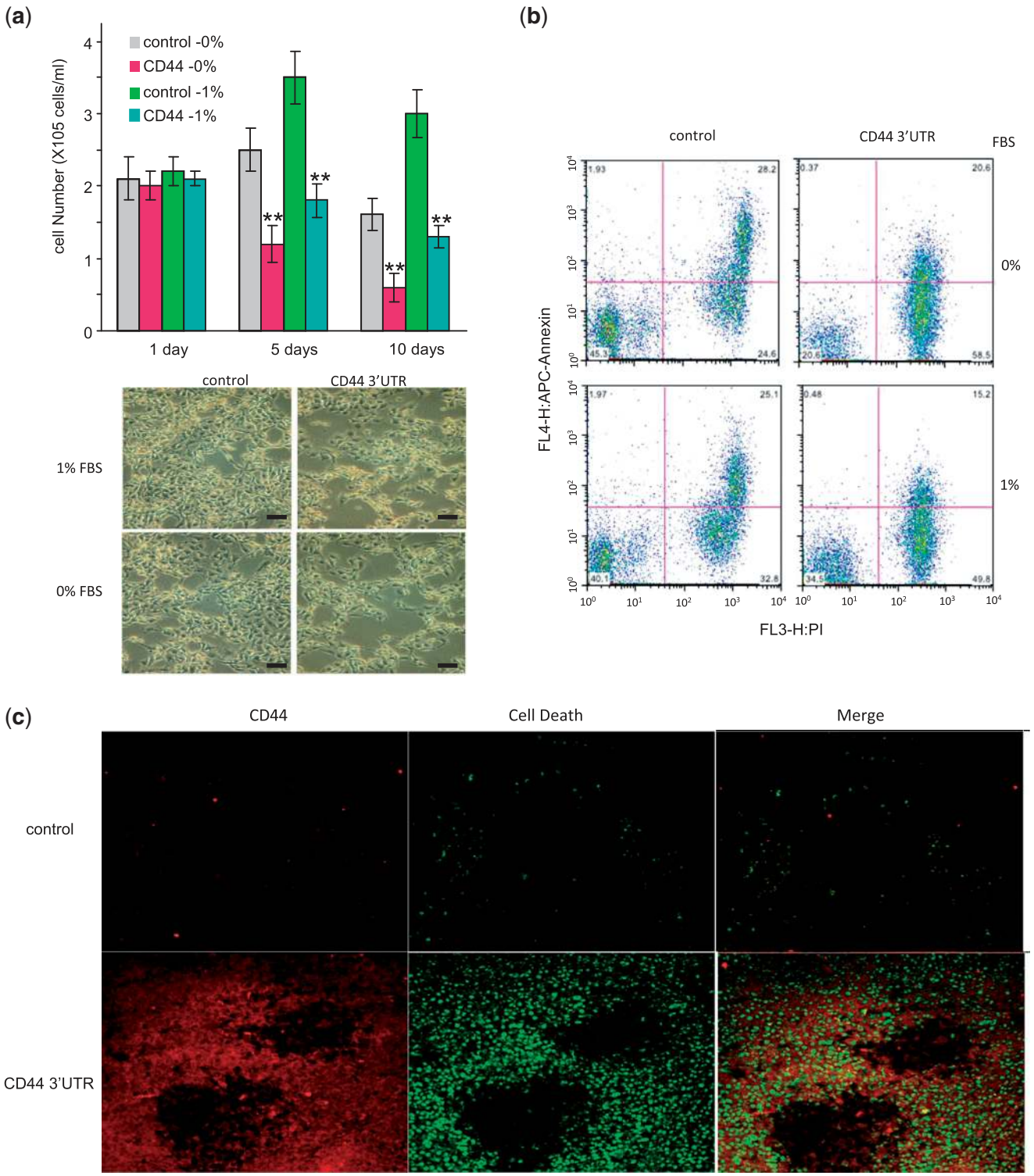


**Figure 2.** The CD44 3′-UTR promotes cell death. (**a**) CD44 3′-UTR and control cells were maintained in tissue culture dishes in low serum and serum-free conditions. Surviving cells were harvested and counted. The CD44 3′-UTR cells had a decreased rate of survival (upper). Cell survival was also monitored by light microscopy and photographs (lower). $n = 3$, $^{**}P < 0.01$, scale bars = 100 μm. (**b**) CD44 3′-UTR and control cells were cultured in low serum and serum-free conditions for 5 days. The cells were stained with Annexin V and PI and analyzed by flow cytometry. The 3′-UTR cells had a larger population of apoptotic cells (Annexin V positive, PI positive and Annexin V and PI positive) compared with the control cells in both 0% and 1% serum conditions. (**c**) Tumor sections of CD44 3′-UTR and the controls were co-immunostained for the expression of CD44 (red) and apoptotic cells (green). The areas of cells expressing high levels of CD44 were associated with apoptotic cells.

associated with staining for apoptotic cells. When tumor sections were subjected to H&E staining, nuclear condensation and fragmentation were present in the CD44 3′-UTR tumor sections, but not in the control tumor sections (Supplementary Figure S1c). The CD44 3′-UTR and control cells were also treated with Docetaxel, a common chemotherapeutic drug. The experiments indicated that the control cells were resistant to docetaxel treatment, which allowed them to survive longer compared with the CD44 3′-UTR cells (Supplementary Figure S1d).

### Expression of CD44 3′-UTR enhances endothelial cell activities and angiogenesis

Another series of cell function assays were performed to determine the endothelial cell activities affected by the expression of the CD44 3′-UTR. YPEN-1 is a rat endothelial cell line and can form tube-like structure in Matrigel™ (31,37). YPEN-1 cells have been previously used by our lab to examine tube formation. The CD44 3′-UTR and control cells were mixed with YPEN-1 cells and cultured in Matrigel™ to examine tube formation. The CD44 3′-UTR cells were able to form larger cell network complexes and longer tubes with YPEN-1 cells compared with the control cells (Figure 3a). To further test the endothelial functions *in vivo*, we examined blood vessels in tumor sections by immunostaining for CD34 expression, which is expressed in early hematopoietic and vascular-associated tissue and is a marker for blood vessels. There was an increase in blood vessels in the CD44 3′-UTR tumor sections compared with the control tumor sections (Figure 3b). Co-immunostaining for apoptotic cells and blood vessels were performed to determine whether or not the increase in blood vessels correlated with the presence of apoptotic cells. Indeed, the enhanced amount of blood vessels in CD44 3′-UTR tumor sections (red) did not result in a lower amount of cell death (green) as apoptotic cells were noticed in areas of large blood vessels compared with the control sections (Figure 3c). Thus, the increased number of blood vessels was unable to rescue the CD44 3′-UTR cells from death.

### Targeting analysis of CD44 3′-UTR

Analysis with computational algorithms showed that many miRNAs could potentially bind to the CD44 3′-UTR. Since each of these miRNAs in turn could target many mRNAs, we searched for mRNAs that encode proteins involved in cell proliferation, and miRNAs that could target these mRNAs with a high potential of translational repression and could bind to the CD44 3′-UTR with low binding energy. One of these is CDC42, a Rho-GTPase involved in the regulation of signaling pathways to control cell morphology, migration and cell-cycle progression. CDC42 has been shown to be able to alter growth factor signaling and inhibit cell cycle progression in G1 phase (38,39). Bioinformatical analysis identified three miRNAs, miR-216a, miR-330 and miR-608, which were predicted to target CDC42 expression and could bind to the CD44 3′-UTR with low energy (Figure 4a). We hypothesized that overexpression of CD44 3′-UTR by the CMV promoter would bind and arrest these three miRNAs and free the endogenous mRNAs of CD44 and CDC42 for translation (Figure 4b).

To confirm the targeting of miR-216a, miR-330 and miR-608 to the CDC42 3′-UTR, a series of luciferase assays were performed with each miRNA and CDC42 pMIR-report construct (Luc-CDC42-216, Luc-CDC42-330 and Luc-CDC42-608) or their mutant construct (Luc-CDC42-216-mut, Luc-CDC42-330-mut and Luc-CDC42-608-mut). The mutated sequences are shown in red in Figure 4a and the structures of the constructs are shown in Supplementary Figure S2. All predicted miRNAs were found to possess the repressive activities on CDC42 3′-UTR linked to a luciferase reporter construct, which resulted in a decrease in luciferase activities (Figure 4c and Supplementary Figure S3a). When the miRNA binding sites were mutated, there was a restoration in luciferase activities. This also occurred when the pcDNA3.1–CD44 3′-UTR was transfected along with each CDC42 3′-UTR and corresponding miRNA, demonstrating that the miRNA is able to be bound by the CD44 3′-UTR (Figure 4d for MT-1 cells and Supplementary Figure S3b for U343 cells).

To test whether multiple miRNAs could bind to the CD44 3′-UTR, a luciferase assay was performed with a luciferase vector containing the CD44 3′-UTR (Luc-CD44 3′-UTR) or a scrambled control sequence (control). With an increased amount of CD44 3′-UTR transfected, there was an increase in luciferase activities (Figure 4e). However, the luciferase activities of Luc-CD44 3′-UTR never reached the levels of the control. This suggests that endogenous miRNAs were targeting the CD44 3′-UTR. Due to increased supplies of the CD44 3′-UTR, some endogenous miRNAs were absorbed, which allowed luciferase translation to occur. Another set of luciferase experiments were used to examine the function of the CD44 3′-UTR in a competition assay. The Luc-CD44 3′-UTR and Luc-Ctrl constructs were co-transfected with the pcDNA3.1-CD44 3′-UTR (vector CD44 3′-UTR) construct at different amounts (Figure 4f). The increased amounts of CD44 3′-UTR bound more endogenous miRNAs freeing the translation of the luciferase protein, resulting in higher levels of luciferase activities.

### Upregulation of CD44 and CDC42 by expression of CD44 3′-UTR

We reasoned that the increased expression of CD44 3′-UTR should result in an increase in CD44 and CDC42 protein levels. This occurred because miRNAs would bind to the exogenously transfected CD44 3′-UTR freeing the endogenous mRNAs of CD44 and CDC42 (Figure 4b). This was confirmed by western blot using cell lysates from CD44 3′-UTR and control cells. We observed an elevated expression of CD44 and CDC42 proteins in the CD44 3′-UTR-transfected cells compared with the control cells (Figure 5a). To confirm that the CDC42 upregulation was a consequence of CD44 3′-UTR overexpression rather than a result of elevated CD44 levels, we exogenously expressed a CD44E-construct in the MT-1 cells. While we detected an

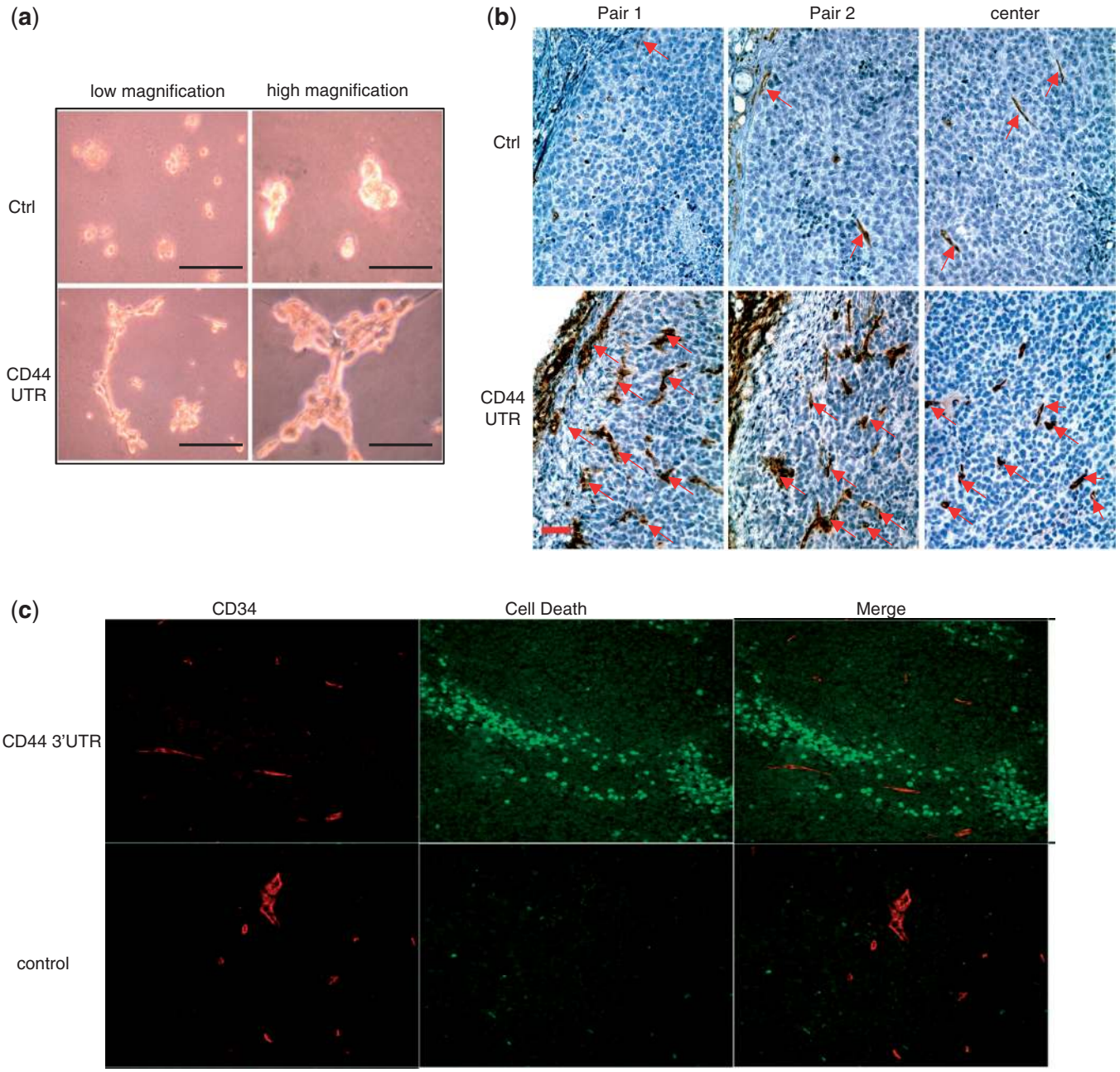


**Figure 3.** CD44 3′-UTR expression promotes endothelial-cell activities and angiogenesis. (**a**) YPEN-1 cells were mixed with CD44 3′-UTR and control cells and inoculated in Matrigel™. Tube formation was examined with light microscopy. CD44 3′-UTR mixed with YPEN-1 cells formed larger complexes and longer tubes compared with the control cells mixed with YPEN-1 cells. Low magnification scale bars = 400 μm, high-magnification scale bars = 100 μm. (**b**) Pairs of CD44 3′-UTR and control tumor sections were stained with anti-CD34 antibody. There was an increased number of blood vessels (arrows) in the CD44 3′-UTR tumor sections compared with the controls. Scale bars = 50 μm. (**c**) The tumor sections were co-immunostained with TUNEL for apoptotic cells (green) and CD34 to detect blood vessels (red). The CD44 3′-UTR tumor sections had an increased amount of apoptotic cell death and blood vessels compared with the control.

increased expression of CD44, reaching levels equal to that of CD44 in the CD44 3′-UTR-transfected cells, we did not detect an increased expression of CDC42 (Figure 5b). These results indicated that increased expression of CDC42 was a consequence of the overexpression of the CD44 3′-UTR and not due to the CD44 protein upregulation resulting in downstream-signaling pathways. Increased expression of CD44 and CDC42 was further confirmed by immunostaining the tumor sections and western blot analysis of tumor tissue lysates. An elevated expression of CD44 and CDC42 proteins in the 3′-UTR tumor was observed in both immunostaining (Figure 5c) and western blotting (Figure 5d). The mRNA levels of CDC42 were analyzed in the CD44 3′-UTR and control cells by RT-PCR. The equal levels of mRNAs suggest that the increased levels of

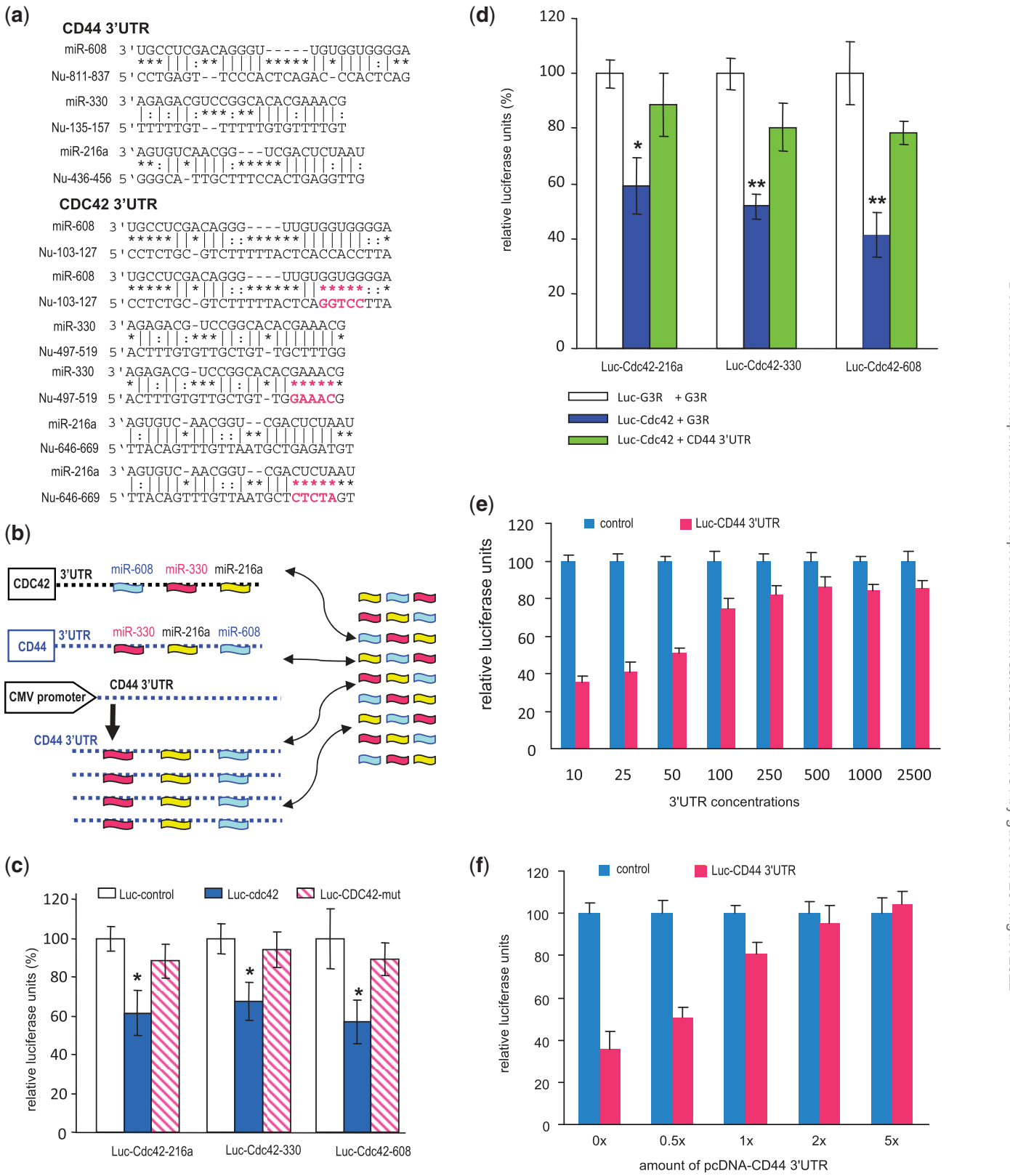


**Figure 4.** Targeting of CDC42 by miR-216a, miR-330 and miR-608. (**a**) Computational analysis of the CD44 3′-UTR has shown that miR-216a, miR-330 and miR-608 could interact with both CD44 and CDC42 3′-UTRs. Predicted binding sequences of miR-216a, miR-330 and miR-608 to CD44 (upper) and targeting of CDC42 3′-UTR by these miRNAs (lower) are shown. Mutated sequences in the seed regions of CDC42 are in red. (**b**) We hypothesized that the overexpression of the CD44 3′-UTR would attract endogenous miR-216a, miR-330 and miR-608, thus freeing CD44 and CDC42 mRNAs to be available for translation. (**c**) The CDC42 3′-UTR fragments were cloned into the luciferase reporter vector pMir-Report. MT-1 cells were co-transfected with different miRNAs and the luciferase reporter construct harboring the CDC42 3′-UTR or mutant CDC42

Continued

CDC42 protein was regulated at the translation level rather than the transcription level (Supplementary Figure S3c).

As MT-1 is a human breast carcinoma cell line, we sought to examine CD44 levels in human breast cancer samples. Tumor sections from random patients were immunostained with an anti-CD44 antibody. The levels of CD44 were attenuated in the cells with a cancer phenotype, large and multiple nuclei, correspondingly with an increase in CD44 protein levels in adjacent normal tissues (Figure 6). It suggests that CD44 may function as a tumor suppressor. Since the sections were randomly chosen, the consistent results suggest that the association of tumor progression with decreased expression of CD44 might be important in clinical studies.

### Corroboration of miRNA function

The function of miR-216a, miR-330 and miR-608 were confirmed by transfecting these miRNAs into stable CD44 3′-UTR and control MT-1 cells, followed by a series of cell-functional assays. Proliferation rates were monitored in miRNA transfected CD44 and control cells over a period of 5 days. It was found that miR-330 increased the proliferation of control and CD44 MT-1 cells significantly compared with the non-coding control and miR-216a and miR-608 (Figure 7a and Supplementary Figure S4a). This was further confirmed with cell cycle analysis in which miR-330 transfection also resulted in a larger population of cells in the S and G2 phases and decreased G1 phase population compared with miR-216a, miR-608 and the non-coding control (Figure 7b and Supplementary Figure S4b). These results suggest that miR-330 is involved in cell proliferation and has the function to increase the proliferation rate of MT-1 cells; thus, increasing the tumorigenic properties of the cell line, while miR-216a and miR-608 decrease the tumor forming potential. In addition, cell survival of miR-216a-, miR-330- and miR-608-transfected CD44 3′-UTR cells in low-serum conditions was examined. There was an increased amount of cells that survived when the CD44 3′-UTR MT-1 cells were transfected with the miRNAs compared with the control sequence (Figure 7c). Endothelial tube formation was analyzed in CD44 3′-UTR MT-1 cells transfected with the miRNAs which were mixed with YPEN-1 cells and it was found that miR-216a and miR-608 inhibited the formation of tubule structures while miR-330 allowed the tubule like structures to form similar to the CD44 3′-UTR. This experiment indicated that miR-608 and miR-216a were involved in tubule formation (Figure 7d). Furthermore, it was confirmed that miR-216a, miR-330 and miR-608 were able to down regulate CDC42 protein levels in the control MT-1 cells (Figure 7e). The over-expression of the miRNAs allowed for the direct confirmation of the CD44 3′-UTR function in MT-1 cells.

### Confirmation of CD44 3′-UTR targeting

Small interfering RNA (siRNA) sequences were designed to bind to the CD44 3′-UTR region (Supplementary Figure S4c). An unrelated sequence was used as a control. The CD44 siRNAs were used to silence CD44 3′-UTR expression to confirm the functionality of the CD44 3′-UTR. The effectiveness of the siRNA treatments were tested by RT–PCR for CD44 mRNA from the CD44 3′-UTR cells transfected with the different siRNAs. The treatments decreased the levels of CD44 mRNA (Figure 8a). Western blot analysis was performed and the siRNA-treated cells were found to have decreased CD44 and CDC42 protein levels (Figure 8b). Cell-cycle distribution was analyzed using FACs analysis. We detected an increase in S and G2 phases in CD44 3′-UTR siRNA-treated cells compared with control cells which had a higher level of G1 phase population (Figure 8c). We also detected lower levels of apoptosis in the cells treated with siRNA-187, siRNA-199, siRNA-541 and siRNA-650 (Figure 8d). This observation concurred with the increase in cell proliferation and survival of siRNA treatment (Supplementary Figures S4d and S4e).

## DISCUSSION

This study was designed to test our hypothesis that overexpression of CD44 3′-UTR in human breast-carcinoma cells would function as a decoy when interacting with endogenous miRNAs, thereby, arresting their function in mRNA targeting. The mRNAs would be relieved of inhibition resulting in enhanced protein synthesis, and a change in cellular activity or phenotype would be expected. Our experiments provided strong evidence that the overexpression of the CD44 3′-UTR did indeed affect the activities of the breast-carcinoma cells MT-1 in terms of cell proliferation, cell cycle progression, survival, apoptosis, formation of tube-like structures *in vitro* and blood vessel formation and tumor growth *in vivo*.

Initially, we observed the overexpression of the CD44 3′-UTR inhibited the proliferation rate of MT-1 cells. We speculated that the exogenous CD44 3′-UTR mechanistically decreased the proliferation of MT-1 cells by binding

Figure 4. Continued
fragment in MT-1 cells. A non-related fragment of cDNA was used as a control. Luciferase activity assays indicated that all three miRNAs repressed luciferase activities when it harbored the corresponding CDC42 3′-UTR, which were reversed when the potential miRNA target site was mutated. $n = 3$, $^{*}P < 0.05$. (**d**) The luciferase reporter plasmids were co-transfected with corresponding miRNAs or control sequence and the pcDNA3.1–CD44 3′-UTR or pcDNA3.1-G3R into MT-1 cells. The inhibitory effects of miRNAs could be significantly recovered in the presence of pcDNA3.1-CD44 3′-UTR. $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$. (**e**) The luciferase reporter vector harboring the CD44 3′-UTR (Luc-CD44 3′-UTR) or a control was transfected at different concentrations in MT-1 cells. Luciferase activities were normalized using the control as 100%. The luciferase activities of Luc-CD44 3′-UTR never reached the control values. However, the luciferase activities increased with higher doses of plasmids. An increase in CD44 3′-UTR concentrations results in increasing luciferase activities. $n = 3$. (**f**) The Luc-CD44 3′-UTR or control was co-transfected with the pcDNA3.1-CD44 3′-UTR construct at different amounts in MT-1 cells. With an increase of pcDNA3.1-CD44 3′-UTR, more endogenous miRNAs were bound, freeing the translation of luciferase protein and resulting in higher levels of luciferase activities. $n = 3$.

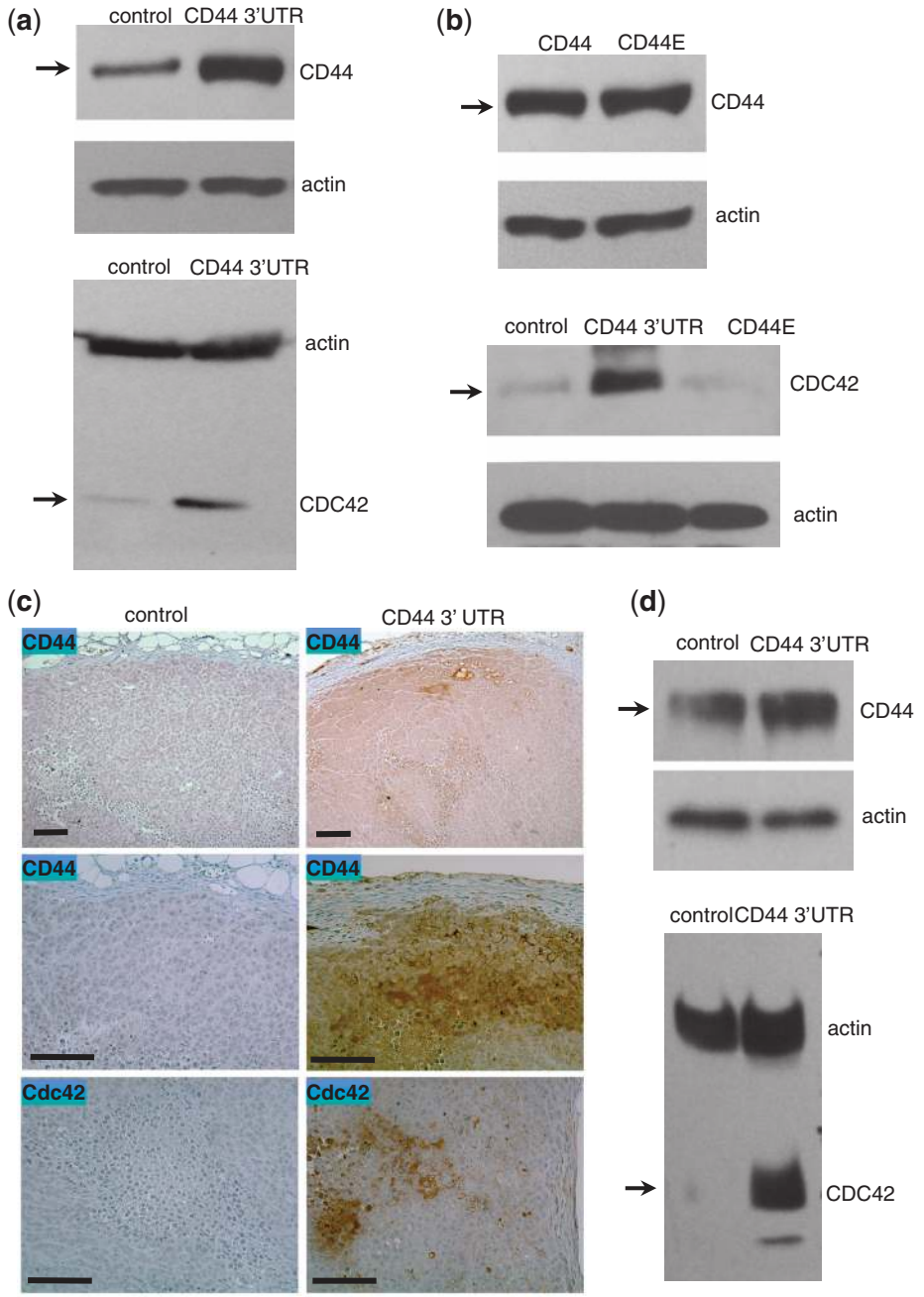


**Figure 5.** Up-regulation of CD44 and CDC42 expression in cells expressing CD44 3′-UTR. (**a**) Protein lysates were prepared from CD44 3′-UTR- and vector-transfected cells and subjected to western-blot analysis probed with anti-CD44 and -CDC42 antibodies. Detection of β-actin on the same membranes served as a loading control. Increased expression of CD44 and CDC42 was detected in the CD44 3′-UTR-transfected cells. (**b**) Protein lysates were prepared from CD44 3′-UTR- and CD44E-transfected cells and subjected to western-blot analysis probed with anti-CD44 and -CDC42 antibodies. Although transfection with CD44E increased CD44 expression, it did not increase CDC42 level. (**c**) Immunohistochemistry was performed on tumor sections with anti-CD44 and -CDC42 antibodies. There was an increase in CD44 and CDC42 in the CD44 3′-UTR tumor sections compared to the control sections. Scale bars = 100 μm. (**d**) Protein lysates were prepared from CD44 3′-UTR and control tumors and subjected to western-blot analysis probed with anti-CD44 and -CDC42 antibodies. β-actin detection on the same membranes served as a loading control. Both CD44 and CDC42 were up-regulated in the CD44 3′-UTR tumors.

to endogenous miRNAs and inhibiting downstream proliferative activities. Data supporting this observation was obtained from cell-cycle progression assays. We found that cells overexpressing CD44 3′-UTR displayed significantly higher levels of G1 population than the cells transfected with an empty vector. Since these experiments were performed in a human breast-carcinoma cell line MT-1, we tested how these effects could be translated to an *in vivo* phenotype. Our findings from the tumor formation experiments were consistent with that of cell proliferation and cell cycle progression. It should be noted that cell proliferation only represents one aspect of tumorigenicity,

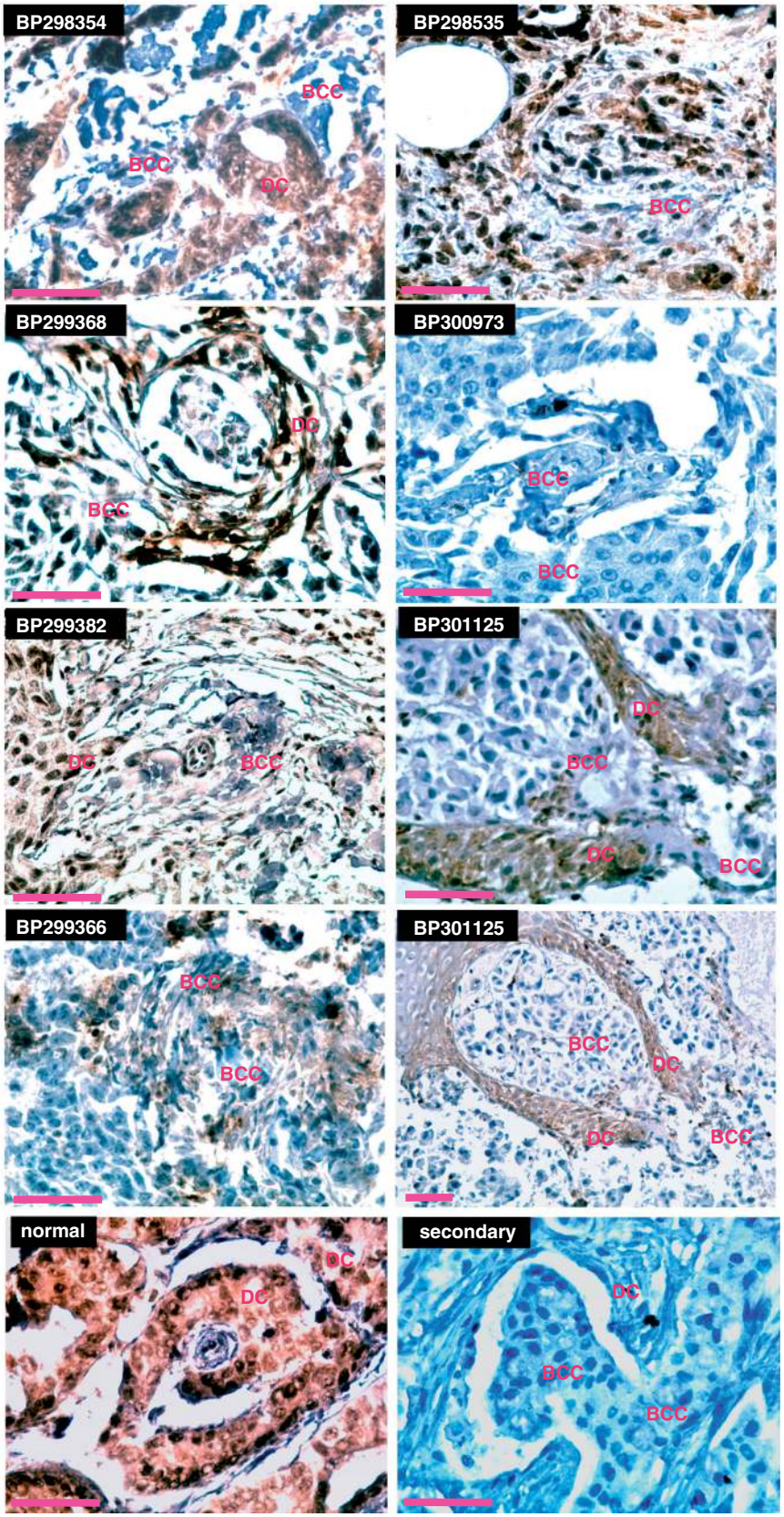


**Figure 6.** Decreased CD44 expression in human breast cancer cells. Seven human breast cancer specimens were probed for CD44 expression. CD44 levels greatly decreased in the ductal cells (DC) which showed phenotypes of breast cancer cells (BCC). One photo showing the normal ducts of the breast and one photo showing the secondary antibody staining are provided (lower). Scale bars = 50 μm.

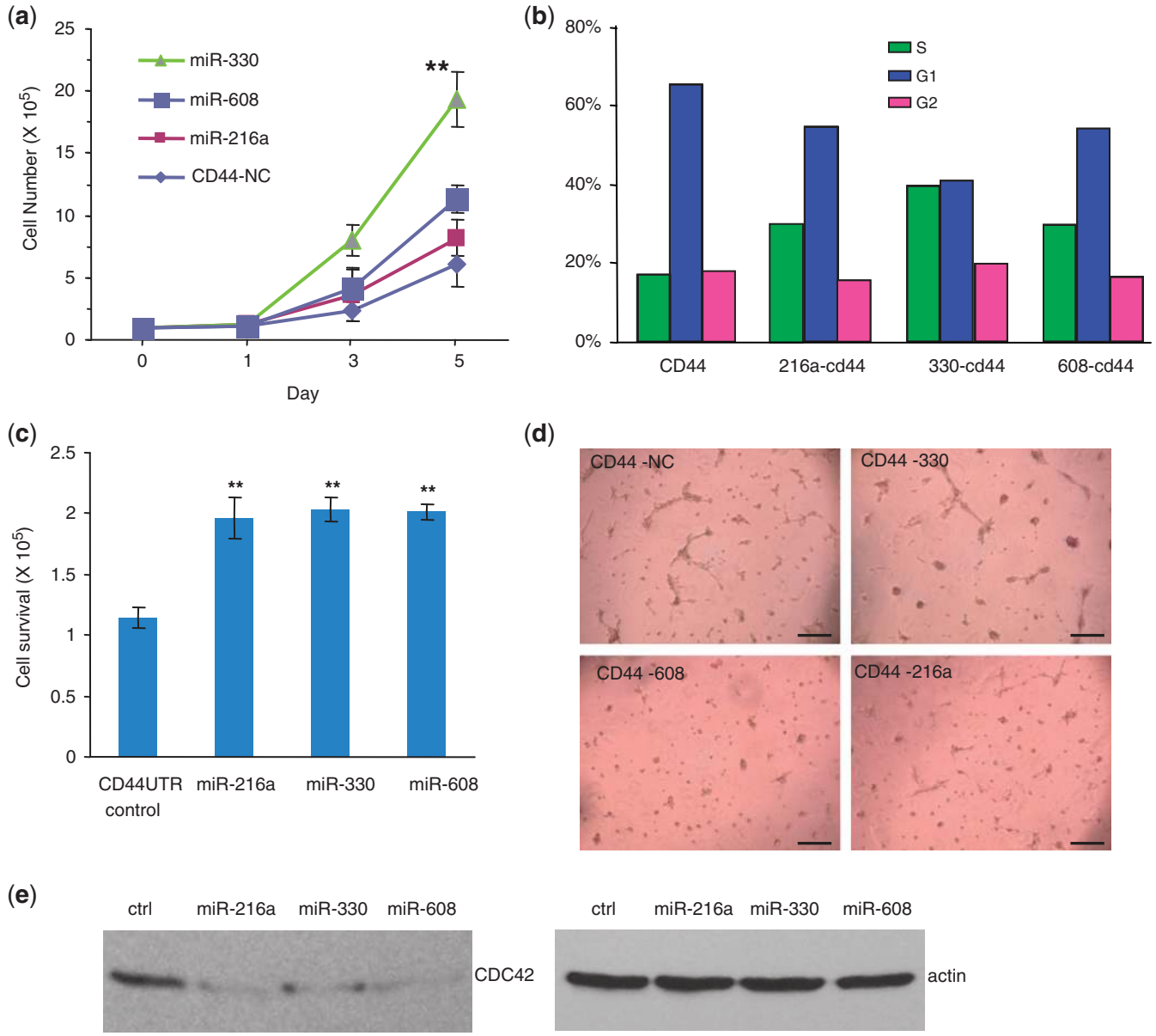


**Figure 7.** Functions of miR-216a, miR-330 and miR-608 in MT-1 cell line. (**a**) The proliferation rate was measured in the CD44 3′-UTR cells transfected with miR-216a, miR-330 and miR-608 over a period of 5 days. Expression of miR-330 increased proliferation of CD44 3′-UTR cells. $n = 5$, $^{**}P < 0.01$. (**b**) Cell-cycle progression of the miRNA transfected CD44 3′-UTR cells were analyzed by FACs analysis. Transfection with miR-330 decreased G1 population but increased G2 and S populations in the CD44 3′-UTR cells. $n = 3$. (**c**) Cell survival was examined in the CD44 3′-UTR MT-1 cells transfected with miR-216a, miR-330 and miR-608. Expression of miR-216a, miR-330 and miR-608 increased cell viability. $n = 5$, $^{**}P < 0.01$. (**d**) Tube formation was analyzed in the CD44 3′-UTR cells transfected with miR-216a, miR-330 and miR-608. Expression of miR-216a and miR-608 inhibited the formation of tubule structures in the CD44 3′-UTR MT-1 cell line. Scale bars = 250 μm. (**e**) Cell lysates of miRNAs transfected into MT-1 cells were analyzed by western blot. Expression of these three miRNAs repressed CDC42 levels.

and increased cell proliferation cannot always be translated to enhanced tumorigenesis. On the other hand, cell survival, especially in poor conditions such as in serum-free medium, is always correlated with tumorigenicity (32). Thus, a decreased cell survival *in vitro* can normally be translated to decreased tumorigenesis.

Cell death is another important aspect of tumor growth. In non-optimal conditions and in the presence of chemotherapeutics, we detected an increased sensitivity of CD44 3′-UTR cells to nutrition depletion as compared with the control cells. Furthermore, this phenotype was also observed in our *in vivo* studies. The presence of nuclear condensation and fragmentation and increased TUNEL staining observed in the tumors formed by the CD44 3′-UTR -transfected cells demonstrated the occurrence of extensive cell death. This could explain why CD44 3′-UTR tumors displayed a slower growth rate than the control tumors. The increase in cell survival with the overexpression of miRNAs in CD44 3′-UTR -transfected cells revealed that the cell death could be a result of the

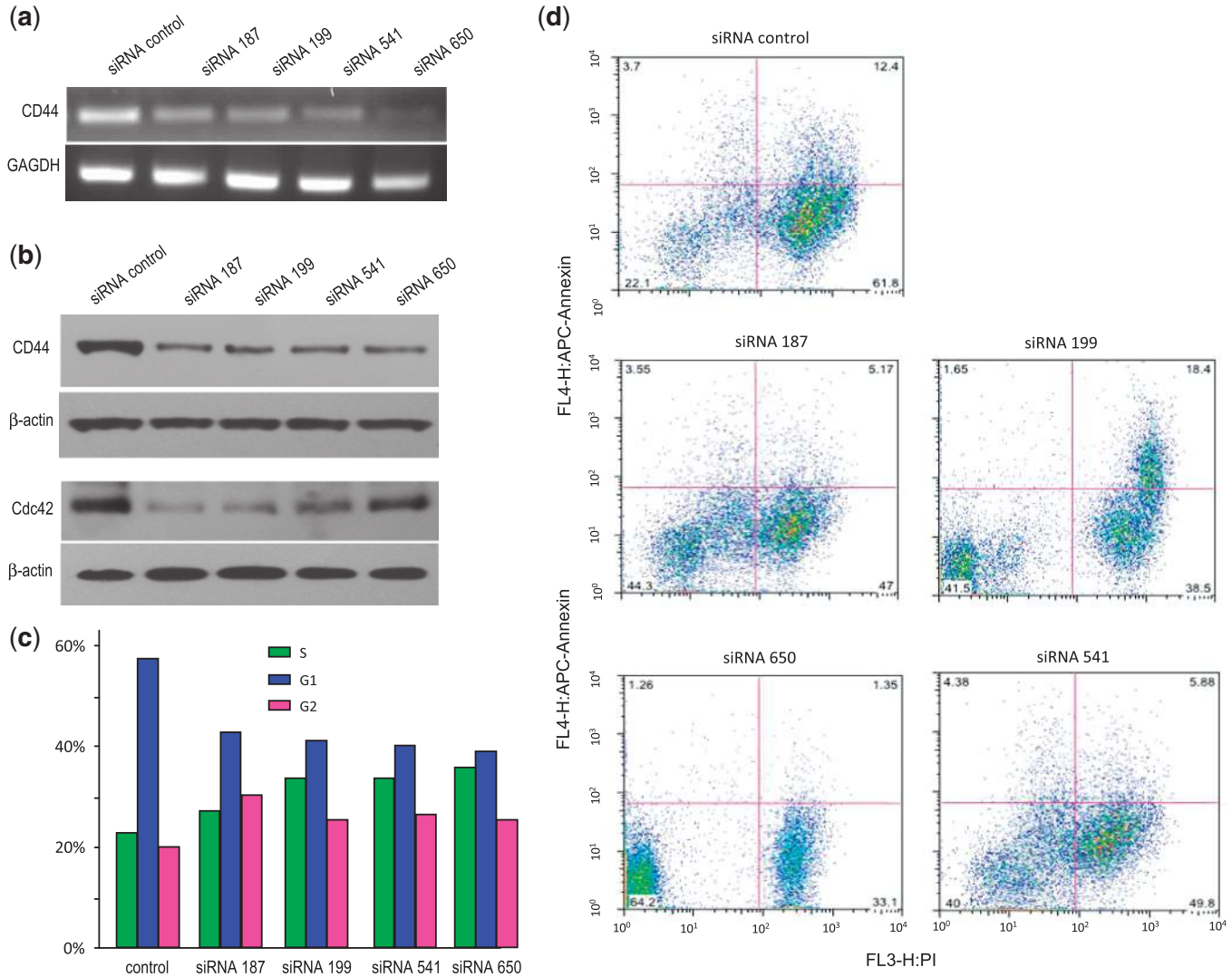


**Figure 8.** siRNA against the CD44 3′-UTR enhances proliferation and decreases CD44 and CDC42 protein levels and apoptosis. (**a**) Total RNA was collected from CD44 3′-UTR cells transfected with different siRNAs targeting CD44 3′-UTR. These samples were used in RT–PCR to measure CD44 mRNA levels. All siRNAs were found to decrease the levels of CD44 mRNA. (**b**) CD44 3′-UTR and control cells were transfected with different siRNAs targeting the CD44 3′-UTR. Cell lysates were subjected to western-blot analysis. Both CD44 and CDC42 levels decreased as a result of siRNA targeting CD44 3′-UTR. (**c**) Cell-cycle distribution was analyzed. There was an increase in the S and G2 phases of the siRNA treated cells compared with the control, which had an increased G1 population. $n = 3$. (**d**) Apoptosis of the siRNA treatment was analyzed using Annexin V staining. Treatments with siRNA-187, siRNA-541 and siRNA-650 decreased apoptosis compared with the control. $n = 5$.

increasing amount of apoptotic proteins due to CD44 3′-UTR expression, since increased CD44 3′-UTR levels could bind and arrest endogenous miRNA functions in MT-1 cells. These downstream apoptotic effects require further investigation in order to determine the identity of the proteins involved.

To understand the mechanism of how the CD44 3′-UTR works in a cellular context, we analyzed potential miRNAs that could bind to CD44 3′-UTR with low energy and found that there are many miRNAs that can possibly do so. Since each miRNA can potentially target a great number of mRNAs with sites that perfectly match their seed regions, it is impossible to analyze all mRNAs with sites that match the seed regions of these potential CD44 3′-UTR-interacting miRNAs. Rather, we focused on genes that play an important role in cell proliferation, cell cycle progression, cell survival and apoptosis. Interestingly, we found that miR-216a, miR-330 and miR-608 can potentially target both the CD44 and CDC42 3′-UTRs.

CD44 is a cell surface receptor for hyaluronan binding and plays an important role in mediating cell adhesion (4,40). Increased expression of CD44 is expected to modulate the activities of nearby cells including endothelial cells, affecting angiogenesis. We have previously demonstrated that expression of a microRNA (miR-328) controlled the morphogenesis of cells and tissues by targeting CD44 expression (9). Decreased CD44 expression would reduce hyaluronan-binding capacity and cell–cell and cell–matrix interactions. This is in consistent with our current report that increased expression of CD44-promoted angiogenesis. Recently, we also found

that the expression of miR-93 repressed expression of integrin β8, a cell-surface receptor modulating cell adhesion and proliferation. As a consequence, endothelial-cell activities and angiogenesis were affected (41), suggesting that cell surface receptors play important roles in mediating endothelial-cell activities.

It is conceivable that the overexpression of the CD44 3′-UTR would enhance CD44 expression, since the expressed CD44 3′-UTR binds identical miRNAs as compared with the endogenous CD44 mRNA. This is supported by our recent study showing that expression of versican 3′-UTR promoted versican expression (29). The effect of the CD44 3′-UTR on the expression of other genes is more complicated, since there may be many miRNAs that can bind to both 3′-UTRs with a high affinity. Computational analysis indicated that miR-216a, miR-330 and miR-608 potentially targeted both CD44 and CDC42 3′-UTRs. We thus focused on CDC42, as we expected that, with more miRNAs (three in our study) potentially targeting CDC42 and binding to CD44 3′-UTR, the decoy effect of CD44 3′-UTR would be more evident. Indeed, our data demonstrated that overexpression of the CD44 3′-UTR not only up-regulated CD44 expression but also increased CDC42 protein levels. The CD44 3′-UTR functioned as a decoy sponge binding to miR-216a, miR-330 and miR-608, resulting in increased CD44 and CDC42 protein levels. This conclusion was supported by the detection of equal levels of CDC42 mRNAs in both the control and CD44 3′-UTR-transfected cells, suggesting that the increased levels of CDC42 protein were due to regulation at the translational level. An additional result to support this finding was obtained by exogenously expressing a CD44E construct, showing that the increased expression of CDC42 was not due to CD44 protein upregulation. Rather, it was due to the overexpression of CD44 3′-UTR, which arrested the functions of relevant miRNAs.

The tube-formation assays performed with Matrigel™ appeared to be an ideal approach to test the effect of CD44 3′-UTR on angiogenesis, since the results obtained by this method and the results obtained by studying angiogenesis in tumors were consistent. While angiogenesis in an *in vivo* setting is the consequence of the activity of many genes, studying angiogenesis via tube-formation assay performed in Matrigel™ is relatively simple. From this study, it appears that CD44, a major cell-surface glycoprotein involved in cell–cell and cell–matrix interactions, mediated the effects that the tumor cells had on the endothelial cells. This result is in agreement with our previous report that CD44 plays a role in zonation morphogenesis and in the formation of tube-like structures (9). The ability of the CD44 3′-UTR cells to modulate tubule-like-structure formation when mixed with YPEN-1 cells and the corresponding data displaying increased formation of blood vessels in CD44 3′-UTR tumors demonstrated the potential of CD44 3′-UTR in angiogenic and anti-angiogenic therapies.

During our analysis of tumor angiogenesis, we unexpectedly observed more CD34 staining and larger blood vessels in the CD44 3′-UTR tumor sections than in the control group, although these tumors were smaller than those formed by the control cells. Usually, larger tumors would require more blood vessels to provide nutrients, allowing the tumor cells to survive and grow. As such, angiogenesis is an important criterion of tumorigenesis. In this aspect, we were also surprised to detect extensive cell death surrounding the blood vessels. These results indicate that the increased amount of blood vessel formation was unable to rescue the CD44 3′-UTR cells from death. Our results suggest that there are different sets of genes in regulating angiogenesis and tumorigenesis. CD44 3′-UTR may be adopted in future studies to dissect genes that regulate angiogenesis and/or tumorigenesis.

When we found that miR-216a, miR-330 and miR-608 can all interact with CD44 3′-UTR as well as target CDC42 3′-UTR, we tested the possibility that all of these miRNAs may function together in mediating CD44 3′-UTR effects. This idea is related to the concept that microRNAs with similar functions cluster together in various disease processes (41). However, in our function dissecting experiments, we found that increased levels of miR-330 promoted cell proliferation, suggesting that the arrest of miR-330 function inhibits cell proliferation, a result observed when CD44 3′-UTR was expressed. On the other hand, up-regulation of miR-216a and miR-608 inhibited formation of tube-like structures, suggesting that arrest of miR-216a and miR-608 (by the overexpression of CD44 3′-UTR) would increase formation of tube-like structures, a result observed when CD44 3′-UTR expressing cells were employed. This could explain why we observed enhanced angiogenesis and inhibited tumor growth when the CD44 3′-UTR was overexpressed. It appears that the effects of a 3′-UTR are very complex as compared with the effects induced by a gene or a miRNA. Considering the number of miRNAs that can interact with a 3′-UTR, we conclude that 3′-UTR can not only function as a mediator of miRNA-regulating gene expression, but also as a modulator-regulating miRNA functions. In the latter case, the great number of 3′-UTRs in a cell may also function as a buffer system to modulate and to fine-tune miRNA activity.

In summary, we have found that CD44 3′-UTR inhibits proliferation, colony formation, and tumor formation. It also induces apoptosis, promotes endothelial-cell activities, favors angiogenesis and shows an increased sensitivity to chemotherapeutics as a result of endogenous miRNAs binding to the exogenously introduced CD44 3′-UTR. Furthermore, it was found that miR-330 is imperative in regulating cell proliferation and MT-1 endothelial cell activities. Due to the interaction of the CD44 3′-UTR and CDC42 3′-UTR with miR-216a, miR-330 and miR-608, expression of exogenous CD44 3′-UTR increased CD44 and CDC42 protein levels. This overexpression of a non-coding transcript freed the potential targets of the miRNAs and led to an up-regulation of multiple protein levels. The 3′-UTR can thus be used as a functional miRNA inhibitor that is capable of modulating multiple miRNAs rather than just one. In this capacity, the 3′-UTR can be used to bind to multiple oncogenic miRNAs, which is a characteristic that can be exploited and potentially applied to gene therapy.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

## FUNDING

National Sciences and Engineering Research Council of Canada (227937-01); Heart and Stroke Foundation of Ontario (NA6282 to B.B.Y.) who is the recipient of a Career Investigator Award (CI5958) from the Heart and Stroke Foundation of Ontario. Funding for open access charge: my research grant.

*Conflict of interest statement*. None declared.

## REFERENCES

1. Taher,T.E., van der Voort,R., Smit,L., Keehnen,R.M., Schilder-Tol,E.J., Spaargaren,M. and Pals,S.T. (1999) Cross-talk between CD44 and c-Met in B cells. *Curr. Top Microbiol. Immunol.*, **246**, 31–37.
2. Weber,G.F., Ashkar,S., Glimcher,M.J. and Cantor,H. (1996) Receptor-ligand interaction between CD44 and osteopontin (Eta-1). *Science*, **271**, 509–512.
3. Fox,S.B., Fawcett,J., Jackson,D.G., Collins,I., Gatter,K.C., Harris,A.L., Gearing,A. and Simmons,D.L. (1994) Normal human tissues, in addition to some tumors, express multiple different CD44 isoforms. *Cancer Res.*, **54**, 4539–4546.
4. Terpe,H.J., Tajrobehkar,K., Gunthert,U. and Altmannsberger,M. (1993) Expression of cell adhesion molecules alpha-2, alpha-5 and alpha-6 integrin, E-cadherin, N-CAM and CD-44 in renal cell carcinomas. An immunohistochemical study. *Virchows Arch. A Pathol. Anat. Histopathol.*, **422**, 219–224.
5. Li,H., Liu,J. and Hofmann,M. (1995) [CD44 expression patterns in primary and secondary brain tumors]. *Zhonghua Yi Xue Za Zhi*, **75**, 525–528, 573.
6. Manten-Horst,E., Danen,E.H., Smit,L., Snoek,M., Le Poole,I.C., Van Muijen,G.N., Pals,S.T. and Ruiter,D.J. (1995) Expression of CD44 splice variants in human cutaneous melanoma and melanoma cell lines is related to tumor progression and metastatic potential. *Int. J. Cancer*, **64**, 182–188.
7. Voutilainen,K., Anttila,M., Sillanpaa,S., Tammi,R., Tammi,M., Saarikoski,S. and Kosma,V.M. (2003) Versican in epithelial ovarian cancer: relation to hyaluronan, clinicopathologic factors and prognosis. *Int. J. Cancer*, **107**, 359–364.
8. Herrlich,P., Morrison,H., Sleeman,J., Orian-Rousseau,V., Konig,H., Weg-Remers,S. and Ponta,H. (2000) CD44 acts both as a growth- and invasiveness-promoting molecule and as a tumor-suppressing cofactor. *Ann. NY Acad. Sci.*, **910**, 106–118.
9. 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.
10. Liu,J., Valencia-Sanchez,M.A., Hannon,G.J. and Parker,R. (2005) MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. *Nat. Cell Biol.*, **7**, 719–723.
11. Lewis,B.P., Burge,C.B. and Bartel,D.P. (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*, **120**, 15–20.
12. Chen,Y. and Stallings,R.L. (2007) Differential patterns of microRNA expression in neuroblastoma are correlated with prognosis, differentiation, and apoptosis. *Cancer Res.*, **67**, 976–983.
13. Chendrimada,T.P., Gregory,R.I., Kumaraswamy,E., Norman,J., Cooch,N., Nishikura,K. and Shiekhattar,R. (2005) TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature*, **436**, 740–744.
14. Gauthier,B.R. and Wollheim,C.B. (2006) MicroRNAs: 'ribo-regulators' of glucose homeostasis. *Nat. Med.*, **12**, 36–38.
15. Hatfield,S.D., Shcherbata,H.R., Fischer,K.A., Nakahara,K., Carthew,R.W. and Ruohola-Baker,H. (2005) Stem cell division is regulated by the microRNA pathway. *Nature*, **435**, 974–978.
16. Huang,T.H., Zhu,M.J., Li,X.Y. and Zhao,S.H. (2008) Discovery of porcine microRNAs and profiling from skeletal muscle tissues during development. *PLoS ONE*, **3**, e3225.
17. Johnston,R.J. and Hobert,O. (2003) A microRNA controlling left/right neuronal asymmetry in Caenorhabditis elegans. *Nature*, **426**, 845–849.
18. Dalmay,T. and Edwards,D.R. (2006) MicroRNAs and the hallmarks of cancer. *Oncogene*, **25**, 6170–6175.
19. Ruvkun,G. (2006) Clarifications on miRNA and cancer. *Science*, **311**, 36–37.
20. Iorio,M.V., Ferracin,M., Liu,C.G., Veronese,A., Spizzo,R., Sabbioni,S., Magri,E., Pedriali,M., Fabbri,M., Campiglio,M. *et al.* (2005) MicroRNA gene expression deregulation in human breast cancer. *Cancer Res.*, **65**, 7065–7070.
21. Wickramasinghe,N.S., Manavalan,T.T., Dougherty,S.M., Riggs,K.A., Li,Y. and Klinge,C.M. (2009) Estradiol downregulates miR-21 expression and increases miR-21 target gene expression in MCF-7 breast cancer cells. *Nucleic Acids Res.*, **37**, 2584–2595.
22. Lowery,A.J., Miller,N., Devaney,A., McNeill,R.E., Davoren,P.A., Lemetre,C., Benes,V., Schmidt,S., Blake,J., Ball,G. *et al.* (2009) MicroRNA signatures predict oestrogen receptor, progesterone receptor and HER2/neu receptor status in breast cancer. *Breast Cancer Res.*, **11**, R27.
23. Foekens,J.A., Sieuwerts,A.M., Smid,M., Look,M.P., de Weerd,V., Boersma,A.W., Klijn,J.G., Wiemer,E.A. and Martens,J.W. (2008) Four miRNAs associated with aggressiveness of lymph node-negative, estrogen receptor-positive human breast cancer. *Proc. Natl Acad. Sci. USA*, **105**, 13021–13026.
24. Mazumder,B., Seshadri,V. and Fox,P.L. (2003) Translational control by the 3′-UTR: the ends specify the means. *Trends Biochem. Sci.*, **28**, 91–98.
25. Linsen,S.E., de Wit,E., Janssens,G., Heater,S., Chapman,L., Parkin,R.K., Fritz,B., Wyman,S.K., de Bruijn,E., Voest,E.E. *et al.* (2009) Limitations and possibilities of small RNA digital gene expression profiling. *Nat. Methods*, **6**, 474–476.
26. Gebauer,F. and Hentze,M.W. (2004) Molecular mechanisms of translational control. *Nat. Rev. Mol. Cell Biol.*, **5**, 827–835.
27. 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.
28. 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.
29. 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.
30. 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.
31. 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.
32. Lee,D.Y., Deng,Z., Wang,C.H. and Yang,B.B. (2007) MicroRNA-378 promotes cell survival, tumor growth, and angiogenesis by targeting SuFu and Fus-1 expression. *Proc. Natl Acad. Sci. USA*, **104**, 20350–20355.
33. Fichtner,I., Dehmel,A., Naundorf,H. and Finke,L.H. (1997) Expression of CD44 standard and isoforms in human breast cancer xenografts and shedding of soluble forms into serum of nude mice. *Anticancer Res.*, **17**, 3633–3645.
34. Engebraaten,O. and Fodstad,O. (1999) Site-specific experimental metastasis patterns of two human breast cancer cell lines in nude rats. *Int. J. Cancer*, **82**, 219–225.
35. Ram,T.G., Schelling,M.E. and Hosick,H.L. (2000) Blocking HER-2/HER-3 function with a dominant negative form of

HER-3 in cells stimulated by heregulin and in breast cancer cells with HER-2 gene amplification. *Cell Growth Differ.*, **11**, 173–183.
36. Yee,A.J., Akens,M., Yang,B.L., Finkelstein,J., Zheng,P.S., Deng,Z. and Yang,B. (2007) The effect of versican G3 domain on local breast cancer invasiveness and bony metastasis. *Breast Cancer Res.*, **9**, R47.
37. Yamazaki,K., Lehr,J.E., Rhim,J.S. and Pienta,K.J. (1995) Establishment of immortalized Copenhagen rat prostate endothelial cell lines. *In Vivo*, **9**, 421–426.
38. Aspberg,A., Miura,R., Bourdoulous,S., Shimonaka,M., Heinegard,D., Schachner,M., Ruoslahti,E. and Yamaguchi,Y. (1997) The C-type lectin domains of lecticans, a family of aggregating chondroitin sulfate proteoglycans, bind tenascin-R by protein-protein interactions independent of carbohydrate moiety. *Proc. Natl Acad. Sci. USA*, **94**, 10116–10121.
39. Molnar,A., Theodoras,A.M., Zon,L.I. and Kyriakis,J.M. (1997) Cdc42Hs, but not Rac1, inhibits serum-stimulated cell cycle progression at G1/S through a mechanism requiring p38/RK. *J. Biol. Chem.*, **272**, 13229–13235.
40. Aruffo,A., Stamenkovic,I., Melnick,M., Underhill,C.B. and Seed,B. (1990) CD44 is the principal cell surface receptor for hyaluronate. *Cell*, **61**, 1303–1313.
41. 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.* (2010) MiR-93 promotes tumor growth and angiogenesis. *Oncogene* (in press).
42. Kim,Y.K., Yu,J., Han,T.S., Park,S.Y., Namkoong,B., Kim,D.H., Hur,K., Yoo,M.W., Lee,H.J., Yang,H.K. *et al.* (2009) Functional links between clustered microRNAs: suppression of cell-cycle inhibitors by microRNA clusters in gastric cancer. *Nucleic Acids Res.*, **37**, 1672–1681.