Biochimica et Biophysica Acta 1842 (2014) 2087–2095

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Overexpression of Oct4 suppresses the metastatic potential of breast cancer cells via Rnd1 downregulation

Long Shen [a], Kunhua Qin [b], Dekun Wang [a], Yan Zhang [b], Nan Bai [b], Shengyong Yang [c], Yunping Luo [d], Rong Xiang [b], Xiaoyue Tan [a,*]

[a] Department of Pathology, Medical School of Nankai University, Tianjin 300071, China
[b] Department of Immunology, Medical School of Nankai University, Tianjin 300071, China
[c] West China Hospital, Molecular Medicine Research Centre, State Key Lab Biotherapy, Sichuan University, Chengdu 610064, China
[d] Department of Immunology, Beijing Union Medical School, Beijing 100010, China

ARTICLE INFO

Article history:
Received 24 March 2014
Received in revised form 7 July 2014
Accepted 21 July 2014
Available online 25 July 2014

Keywords:
Oct4
Metastasis
Breast cancer
E-cadherin
Rnd1

ABSTRACT

Although Oct4 is known as a critical transcription factor involved in maintaining "stemness", its role in tumor metastasis is still controversial. Herein, we overexpressed and silenced Oct4 expression in two breast cancer cell lines, MDA-MB-231 and 4T1, separately. Our data showed that ectopic overexpression of Oct4 suppressed cell migration and invasion *in vitro* and the formation of metastatic lung nodules *in vivo*. Conversely, Oct4 downregulation increased the metastatic potential of breast cancer cells both *in vitro* and *in vivo*. Furthermore, we identified Rnd1 as the downstream target of Oct4 by ribonucleic acid sequencing (RNA-seq) analysis, which was significantly downregulated upon Oct4 overexpression. Chromatin immunoprecipitation assays revealed the binding of Oct4 to the promoter region of Rnd1 by ectopic overexpression of Oct4. Dual luciferase assays indicated that Oct4 overexpression suppressed transcriptional activity of the Rnd1 promoter. Moreover, overexpression of Rnd1 partially rescued the inhibitory effects of Oct4 on the migration and invasion of breast cancer cells. Overexpression of Rnd1 counteracted the influence of Oct4 on the formation of cell adhesion and lamellipodia, which implied a potential underlying mechanism involving Rnd1. In addition, we also found that overexpression of Oct4 led to an elevation of E-cadherin expression, even in 4T1 cells that possess a relatively high basal level of E-cadherin. Rnd1 overexpression impaired the promoting effects of Oct4 on E-cadherin expression in MDA-MB-231 cells. These results suggest that Oct4 affects the metastatic potential of breast cancer cells through Rnd1-mediated effects that influence cell motility and E-cadherin expression.

1. Introduction

Metastasis remains intractable for the clinical treatment of breast cancer, although significant research progress has been made in recent years [1]. Some studies have revealed that transcription factors related to the maintenance of "stemness", such as Sox-2 and c-Myc, are involved in the regulation of metastasis [2,3]. Furthermore, a considerable number of studies have been carried out to investigate the relationship between metastasis and Oct4, an essential transcription factor that mediates the pluripotency of stem cells. So far, there is still no consensus on the influence of Oct4 on tumor metastasis [4,5]. In human pancreatic cancer, the expressional level of Oct4 was found negatively correlated with the prognostic value of patients [6]. However, our previous study showed that the downregulation of Oct4 increases the migration and invasion capabilities of MCF7 cells, a human breast cancer cell line with a low metastatic potential [7]. Therefore, the role and underlying mechanism of Oct4 in the metastasis of tumor is still far from clear.

Breast cancer metastasis is a multistep process involving numerous genes [8]. Among them, loss of E-cadherin expression is a critical and initial step for the occurrence of metastasis. E-cadherin is a transmembrane glycoprotein that is located in the adherent junctions of epithelial cells [9]. It interacts with the cytoskeleton through associations with cytoplasmic catenin proteins such as β-catenin, α-catenin, and p120 catenin (p120ctn) [10]. On the other hand, E-cadherin is also a regulator of epithelial junction formation and appears to be necessary for cell–cell adhesion [11]. In epithelial malignancy, the loss of E-cadherin plays a vital role in the alteration of adhesive properties as well as epithelial-to-mesenchymal transition, which are regarded as the key events for metastasis. A decrease in the expression of E-cadherin has been widely reported in the invasion of carcinomas, whereas increased expression of E-cadherin correlates with reduced invasiveness [12]. E-cadherin expression is regulated at both transcriptional and post-transcriptional levels. Interestingly and in contrast to other induced pluripotent stem

* Corresponding author at: Department of Pathology, Medical School of Nankai University, 94 Weijin Road, Tianjin 300071, China. Tel.: +86 22 23504447; fax: +86 22 23502554.
E-mail address: xiaoyuetan@nankai.edu.cn (X. Tan).

http://dx.doi.org/10.1016/j.bbadis.2014.07.015
0925-4439/

cell-related transcription factors, a study by Li et al. revealed that Oct4 possesses the unique capacity to suppress the EMT mediator Snail and activate the epithelial program including inducing E-cadherin [13].

Rnd1 belongs to a unique subfamily of RhoGTPases and is associated with five other GTPases: Rnd2, Rnd3, RhoH, RhoBTB1, and RhoBTB2. These GTPases bind to GTP but do not show any detectable hydrolyzation. Rnd1 and Rnd3 are involved in the regulation of E-cadherin and the rearrangement of the cytoskeleton [14,15]. The expression of Rnd1 or Rnd3 is accompanied by the loss of actin stress fibers [16,17], which reduces cadherin-based cell–cell adhesion. Rnd3 can be downregulated by Nanog, thus suppressing cell migration [18]. In addition, overexpression of Rnd3 downregulates the expression of E-cadherin in gastric cancer cells [14]. Rnd1 affects cell morphology mainly through an interaction with p190RhoGAP and increase in the GTPase-activating protein (GAP) activity of RhoA, thereby reducing RhoA-GTP levels [19,20]. RhoA-GTP levels correlate with E-cadherin expression [21]. Moreover, RhoA functions as a regulator of cadherin-based cell–cell adhesion that has a central role in the maintenance of cell adhesion in the epithelium [22]. Upon activation, RhoA increases the accumulation of actin filaments and adhesion of cadherin to cell walls [23].

In this study, we aimed to elucidate the role and underlying mechanism of Oct4 in breast cancer metastasis. We also investigated whether Rnd1 expression is regulated by Oct4 and its role in cadherin-based cell–cell adhesion and tumor invasion. Our results provide novel insights for the development of therapeutics targeting Oct4.

2. Material and methods

2.1. Cell lines and lentivirus production

4T1 cells were purchased from the Cell Culture Center of the Shanghai Institute for Biological Sciences (Chinese Academy of Science, Shanghai, China) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). T47D, MCF7, 4TO7 and EMT6 cells were obtained from the American Type Culture Collection (Manassas, VA). MDA-MB-231 and 293T cells were kind gifts from Dr. Ralph Reisfeld (Scripps Research Institute, La Jolla, CA) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS. 293T cells were used to produce lentiviruses for stable transfection of cell lines. Briefly, 293T cells were seeded in six-well plates (1×10^6 cells/well) and then transfected with pLV-cDNA or pLV-shRNA together with VSV-G, Rev, and Gag-Pol vectors by Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 16 h, the transfection solutions were exchanged with fresh medium. The culture supernatants containing viruses were collected after 40 h. A total of 1×10^5 MDA-MB-231 or 4T1 cells in 2 ml medium with 8 μg/ml polybrene were infected with 1 ml lentivirus-containing supernatant by centrifugation at 1100 *g* for 1 h. After 48 h of culture, the cells were transferred to fresh medium containing blasticidin or puromycin for selection. Control cell lines containing the empty vector control or scrambled shRNA (sc) were established by the same method.

2.2. Plasmids

To construct human OCT4, mouse Oct4, and human RND1 overexpression vectors, human or mouse cDNAs were amplified by PCR and then cloned into pLV-EF1-MCS-IRES-Bsd (Biosettia, San Diego, CA). To silence mouse Oct4, three shRNAs were designed by the online application at the Invitrogen website (https://rnaidesigner.invitrogen.com/rnaiexpress/index.jsp). To silence human RND1, we designed two shRNAs using the same website. A scrambled sequence was used as a control for knockdown analysis. The shRNAs were cloned into pLV-H1-EF1α-puro vector (Biosettia) to generate pLV-shRNA plasmids. The primers and shRNA sequences are summarized in Table 1.

Table 1
Primers for plasmids and polymerase chain reaction (PCR) assay.

Gene name	Sequence (5′ → 3′)
Primers for plasmids	
hOct4	
Sense	GCTCTAGAGCCACCATGGCGGGACACCTGGCTT
Antisense	CGGGATCCTCAGTTTGAATGCATGGGAGAG
hRnd1	
Sense	GCTCTAGAGCCACCATGAAGGAGAGACGGGCCCCC
Antisense	CGGGATCCTCACATAATGGAACAGCTTTTGGCCTT
mOct4	
Sense	GCTCTAGAGCCACCATGGCTGGACACCTGGCTTCAGA
Antisense	GCTAGCTAGCTCAGTTTGAATGCATGGGAGAGCC
shRNA sequences for plasmids	
mOct4-sh1	AAAAGGAGGAAGCCGACAACAATTTGGATCCAAATTGTTGTCGGCT TCCTCC
mOct4-sh2	AAAAGAGCACGAGTGGAAAGCAATTGGATCCAAGCCTTAAGAACAT GTGTAA
mOct4-sh3	AAAAGCCTTAAGAACATGTGTAATTGGATCCAATTACACATGTTCTTA AGGC
hRND1-sh1	AAAAGCGAACAGACCTGAGTACTCTTTGGATCCAAAGAGTACTCAG GTCTGTTCGC
hRND1-sh2	AAAAGGCCAGATGTAAGCTCGTTCTTTGGATCCAAAGAACGAGCTT ACATCTGGCC
Scramble	AAAAGCTACACTATCGAGCAATTTTGGATCCAAAATTGCTCGATAG
Control	TGTAGC
qRT-PCR primers	
Human Oct4	
Sense	TGAGGGCGAAGCAGGAGTC
Antisense	GGAAAGGGACCGAGGAGTA
Human E-cadherin	
Sense	CTTCAATCCCACCACG
Antisense	AAATGCCATCGTTGTTC
Human Rnd1	
Sense	TACGATAATGTCCGTCCACTC
Antisense	CTTTGCTATTGCACAACCCT
Human GAPDH	
Sense	CGGGAAACTGTGGCGTGAT
Antisense	AGTGGGTGTCGCTGTTGAAGT
ChIP assay primers	
Sense	CAAACACCCCTAAGCAGCTT
Antisense	TAGCTGGGATTACAGGCATG
Dual luciferase assay	
Sense	CTAGCTAGCCACAAATCTCCAGGAGCCCTTAT
Antisense	CCCAAGCTTCGTCTCAGCACGCCAATCAA

2.3. Migration, invasion, and wound healing assays

Cell migration and invasion assays were performed as described previously [7]. For migration assays, 1×10^5 MDA-MB-231 or 5×10^4 4T1 cells were re-suspended in serum-free medium and seeded into Boyden chamber inserts with an 8-μm pore membrane (BD Bioscience, Franklin Lakes, NJ). Medium containing 10% FBS was added to the lower chamber. For invasion assays, the upper chamber of the insert was coated with Matrigel (BD Biosciences), and 1×10^6 MDA-MB-231 or 5×10^5 4T1 cells were added to the inserts. For wound healing assays, 1×10^6 MDA-MB-231 or 3×10^5 4T1 cells were seeded and grown to 90% confluence. A sterile pipette tip was then used to create scratch wounds. Images were then taken at 0, 24 and 48 h with an IX71 inverted microscope (Olympus, Tokyo, Japan). Three independent experiments were performed.

2.4. Adhesion assays

96-well plates were coated with 50 μl/well collagen I (sc-136154, Santa Cruz Biotechnology, Santa Cruz, CA). MDA-MB-231 cells were detached with 10 mM ethylene diamine tetraacetic acid/PBS (phosphate-buffered saline) and dispersed as single cells. A cell suspension of 2×10^5 cells/ml (100 μl) was added to each well. After incubation

for 60 min at 37 °C, the unattached cells were removed by washing with PBS. The attached cells were fixed with 4% formaldehyde and then stained with crystal violet. Each condition was evaluated in quadruplicate. Attached cells were counted under a 20× objective.

2.5. Animal models of tumors

6- to 8-week-old female NOD/SCID and BABL/c mice were purchased from Beijing HFK Bio-Technology (Peking, China). Experiments were conducted according to Nankai University Guidelines for Animal Experiments. Mice were anaesthetized with 7% trichloroacetaldehyde hydrate before injection. To establish tumors using MDA-MB-231 cells, 100 μl of 3×10^7 cells/ml was injected into the fourth fat pad of NOD/SCID mice. To establish 4T1 tumors, mice were divided randomly into five groups ($n = 5$): vector control-overexpressing, Oct4-overexpressing, scrambled control, Oct4 shRNA-2 and Oct4 shRNA-3. Stably transfected 4T1 cells (1×10^5) were injected into the fourth fat pad of BABL/c mice. Mice were sacrificed at day 30 after injection. Primary tumors and lungs were separated for tissue sectioning and homogenate preparation.

2.6. Tissue processing, hematoxylin and eosin staining, and immunohistochemistry

Tumor tissues and lungs were fixed with paraformaldehyde, embedded in paraffin, and then sectioned. Some sections were incubated with hematoxylin and eosin (H&E), dehydrated, and mounted. For E-cadherin or RND1 staining, sections were incubated at 4 °C overnight with anti-E-cadherin (610182, BD Biosciences) or -RND1 (sc-25029, Santa Cruz Biotechnology) antibodies. After incubation with a biotinylated secondary antibody for 90 min and then an avidin–peroxidase complex for 30 min, the sections were visualized with DAB or AEC and counterstained with hematoxylin. Images were obtained with a BX53 research microscope (Olympus).

2.7. RNA extraction, PCR, and RNA-seq analysis

Total RNA was extracted using a TRIzol reagent [24]. Products of semi-quantitative PCR were detected by agarose gel electrophoresis. The primers used are listed in Table 1. For RNA-seq analysis, 1×10^7 Oct4-overexpressing or control MDA-MB-231 cells were collected to extract total RNA. mRNA was purified by Oligo(dT) magnetic bead adsorption. Transcription data were profiled and compared according to standard protocols (digital gene expression, Beijing Genomics Institute, Beijing, China).

2.8. Protein isolation and Western blotting

Cell lysates were prepared with cold radioimmunoprecipitation assay lysis buffer as described previously [25]. Equivalent total protein from each lysate was loaded onto 10–12% Tris–acrylamide gels, electrophoresed, and then transferred to nitrocellulose membranes. After blocking nonspecific binding sites, the membranes were probed with primary antibodies, including anti-Oct4 (sc-25029), anti-Rnd1 (sc-5279, Santa Cruz Biotechnology), anti-E-cadherin (610182, BD Biosciences), and then horseradish peroxidase-conjugated secondary antibodies. Labeled proteins were detected by an ECL chemiluminescence kit (Millipore, Billerica, MA).

2.9. Immunofluorescence and cytoskeleton staining

E-cadherin was detected by immunofluorescence [7] using an antibody against E-cadherin (610182, BD Biosciences). For cytoskeleton staining, cells were fixed with 4% formaldehyde for 15 min and then blocked with 1% bovine serum albumin for 1 h. After rinsing, the cells were incubated with 5 μg/ml FITC-phalloidin (P2141, Sigma, St. Louis, MO) at room temperature for 1 h. The lamellipodia of each cell was counted to represent the cell morphology after gene expression changes. Images were obtained with a FV10000 confocal microscope (Olympus).

2.10. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed using an EZ-ChIP kit (Millipore) following the manufacturer's instructions. In brief, cells grown in 100-mm dishes were cross-linked with 1% formaldehyde for 10 min at room temperature. The reaction was stopped by the addition of 1 ml glycine. Fixed cells were washed twice and then transferred into a 1.5-ml centrifuge tube. After centrifugation, the cell pellet was resuspended in 1 ml SDS lysis buffer. About 1×10^6 cells were used for each immunoprecipitation by using antibody agaist Oct4 (2750, Cell Signal Technology, Danvers, MA). Semi-quantitative RT-PCR was performed using TransStart FastPfu DNA Polymerase (TransGen Biotech, Peking, China). Chromatin binding was compared with the amount of input.

2.11. Dual luciferase assay

The human RND1 promoter predicted by software was cloned downstream of the firefly luciferase (FL) coding region into a pGL3-basic luciferase reporter vector (Promega, Madison, WI). Cells were co-transfected with pGL3-Rnd1 promoter and pLV-control or pLV-hOct4 plasmids. A pRL Renilla luciferase (RL) reporter vector (pRL-TK) was used as the internal control. At 48 h after transfection, cell lysates were collected and the activation of the RND1 promoter was quantified as the ratio of FL/RL activity following the manufacturer's instructions.

2.12. Statistical analysis

Data analysis was performed using the GraphPad Prism 5 software. All results are presented as the mean ± S.E.M. The differences between two groups were analyzed by two-way analysis of variance. A *P*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Oct4 expression levels affect the metastatic potential of breast cancer cells

To determine the effect of Oct4 on the metastasis of breast cancer, we first detected the basal expression level of Oct4 in a variety of human and mouse breast cancer cell lines with different metastatic potentials (Fig. 1A). Comparing with the relatively low metastatic efficient cell lines, MDA-MB-231 and 4T1 exert a lower Oct4 expression level. We then established stable Oct4-overexpressing MDA-MB-231 and 4T1 cell lines, as well as a stable Oct4-silenced 4T1 cell line. Results of wound healing and transwell assays suggested that exogenous overexpression of Oct4 suppressed the migration and invasion capacities of MDA-MB-231 and 4T1 cells (Fig. 1B–D), whereas the downregulation of Oct4 increased the migration of 4T1 cells (Fig. 1G & H). Consistent with the results of *in vitro* experiments, there were significantly less metastatic lung nodules in mice injected with Oct4-overexpressing MDA-MB-231 or 4T1 cells compared with those in the controls (Fig. 1E). Furthermore, there were more metastatic lung nodules in mice injected with Oct4-silenced 4T1 cells compared with those in the control (Fig. 1I). However, no detectable difference in tumor initiation and growth could be found between Oct4-overexpressing or Oct-silenced groups with their respective control (Fig. 1F & H). Combined with our previous data showing that the downregulation of Oct4 increases the migration and invasion capabilities of MCF7 cells, our *in vivo* and *in vitro* experimental data here suggested that the exogenous overexpression of

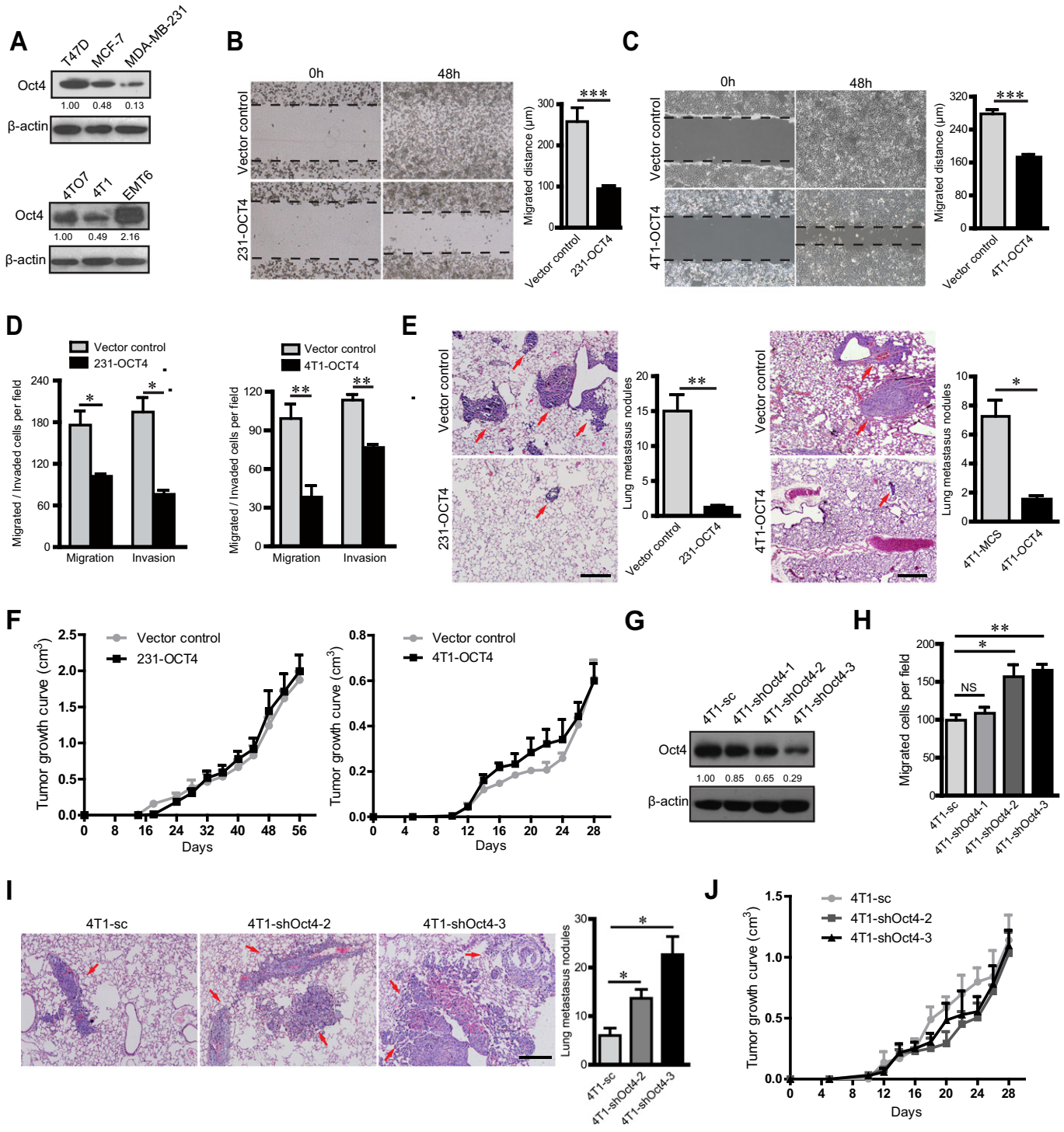

Fig. 1. Oct4 expression levels influence the metastatic potential of breast cancer cells. We compared the expression level of Oct4 in a variety of breast cancer cell lines with different metastatic potentials. (A) The results of Western blotting by using an antibody against Oct4. We established stable Oct4-overexpressing MDA-MB-231 and 4T1 cell lines as well as their respective stable vector control-overexpressing cell lines. (B & C) Representative images of the wound healing assay and statistic results on the migrated distances are shown. $^{***}P < 0.001$. (D) Results of invasion assays. Data represent the mean ± S.E.M. of triplicate wells. $^{*}P < 0.05$, $^{**}P < 0.01$. A total of 3×10^6 Oct4-overexpressing or control MDA-MB-231 cells were injected into the fat pad of 6–8-week-old NOD/SCID mice (n = 5). Primary tumors and lungs were isolated at day 56 after injection. In addition, 1×10^5 Oct4-overexpressing or control 4T1 cells were injected into the fat pad of 6–8-week-old Balb/c mice (n = 5). Primary tumors and lungs were isolated at day 30 after injection. (E) Representative images of H&E staining and the number of metastatic nodules in the lungs are shown. Scale bar = 20 μm. Data represent the mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$. Tumor growth curves are shown in (F). 4T1 cells were stably transfected with scrambled shRNA (sc) or Oct4 shRNAs (sh1, sh2, or sh3). (G) Western blotting was used to detect the efficiency of Oct4 silencing in the stably transfected cell lines. (H) Migrated cell counts in the transwell assay. Data represent the mean ± S.E.M. of triplicate wells. $^{*}P < 0.05$, $^{**}P < 0.01$. A total of 1×10^5 4T1-sc, 4T1- sh2, or 4T1-sh3 cells were injected into the fat pad of 6–8-week-old Balb/c mice (n = 5). Primary tumors and lungs were isolated at day 30 after inoculation. (I) Representative images of H&E staining of the lungs and the numbers of metastatic lung nodules are shown. Scale bar = 50 μm, $^{*}P < 0.05$. Tumor growth curves are shown in (J).

Oct4 suppressed these effects, whereas the downregulation of Oct4 increased the metastatic potential of breast cancer cells.

3.2. Overexpression of Oct4 suppresses Rnd1 transcription in breast cancer cells

To investigate the mechanism underlying the role of Oct4 in breast cancer metastasis, we performed RNA-seq analyses of Oct4-overexpressing and control MDA-MB-231 cells. By comparing the gene expression in Oct4-overexpressing MDA-MB-231 cells and control cells, we that found 97 and 25 genes were upregulated and downregulated by more than 4-fold, respectively (Fig. 2A). Among these genes, the expression of Rnd1, which encodes a cell adhesion and cytoskeleton remolding-related protein, was decreased by about 11-fold in cells overexpressing Oct4. RT-PCR and Western blotting confirmed the suppression of Rnd1 in Oct4-

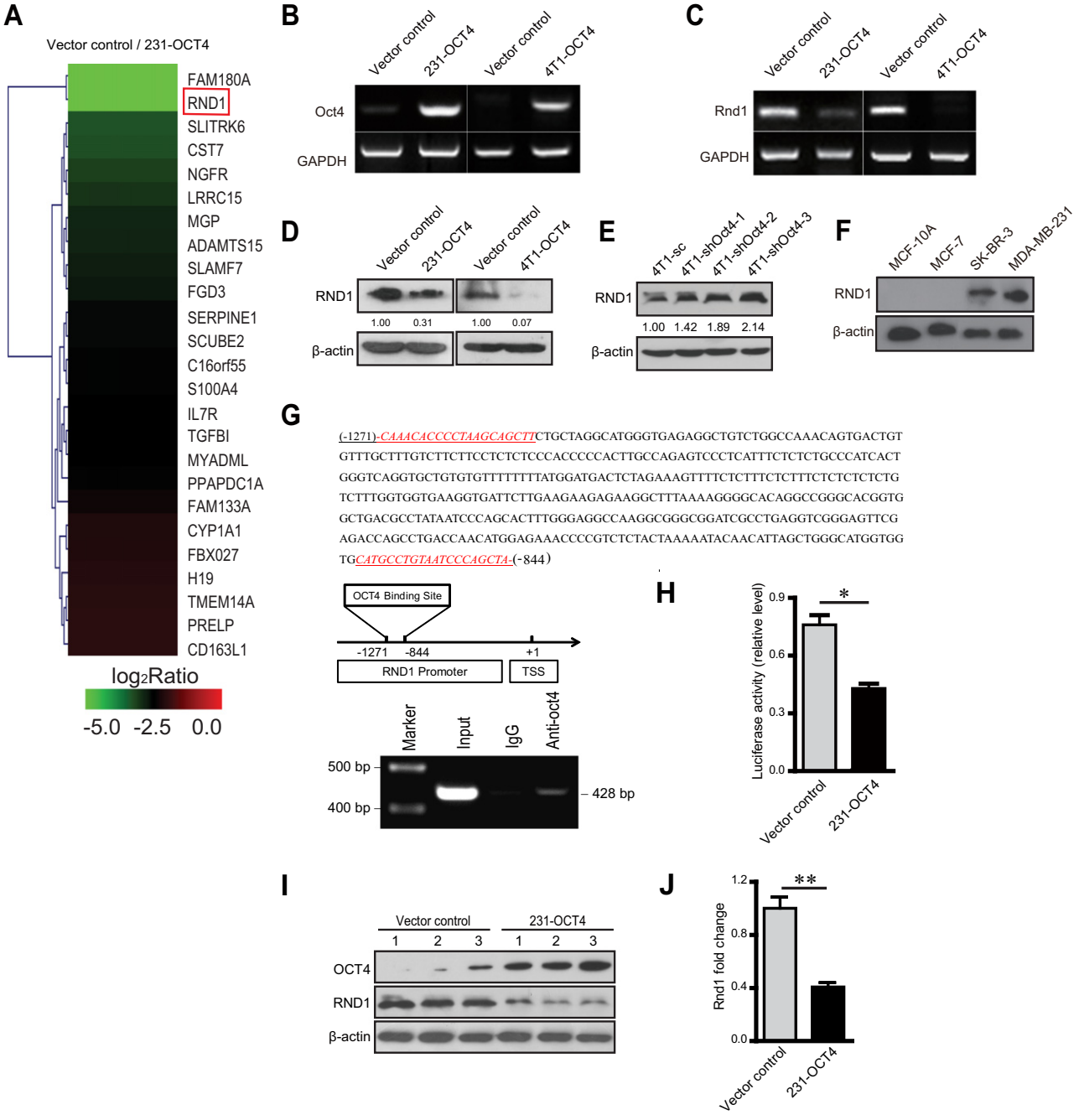

Fig. 2. Ectopic expression of Oct4 induces its binding to the Rnd1 promoter and downregulates Rnd1 expression. mRNA from stable Oct4-overexpressing or control MDA-MB-231 cells were collected and underwent RNA-seq analysis. (A) Heat map depiction of genes with significant differential expression in Oct4-overexpressing MDA-MB-231 cells. (B & C) mRNA expression of Oct4 and Rnd1 in 231-vector control/231-OCT4 cells and 4T1-vector control/4T1-Oct4 cells. (D) Western blot analysis of RND1 levels in 231-vector control/231-OCT4 cells and 4T1-vector control/4T1-Oct4 cells. (E) Detection of Rnd1 in 4T1-sc and Oct4-silenced 4T1 cells by Western blotting. (F) RND1 expression level in different human breast cancer cell lines detected by using Western blot assay. (G) Sequence of Oct4 binding site in the promoter of RND1 and schematic diagram of the human RND1 gene promoter region. PCR primers were labeled in red font. Box indicates the predicted OCT4 binding site in this region. ChIP assay demonstrated that OCT4 was bound to its cognate *cis*-acting element in the RND1 promoter. (H) Dual luciferase reporter assays demonstrated that OCT4 was bound to the promoter of RND1 and repressed gene transcription (n = 3). Data represent the mean ± S.E.M. $^{*}P < 0.05$. (I) Representative images of Western blotting by using antibody against OCT4 and RND1 in primary tumor tissues from the breast cancer mouse model with stable Oct4-overexpressing or control MDA-MB-231 cells (n = 5). (J) Densitometric analysis of RND1 expression. Data represent the mean ± S.E.M. $^{**}P < 0.01$.

overexpressing-MDA-MB-231 and 4T1 cells (Fig. 2C & D). Accordingly, the downregulation of Oct4 increased Rnd1 expression in 4T1 cells (Fig. 2E). Furthermore, Rnd1 expression levels were higher in SK-BR-3 and MDA-MB-231 cells compared with that in the cell lines with a relatively low metastatic potential (Fig. 2F). The results of ChIP assays indicated a binding site for Oct4 in the promoter region of Rnd1 (Fig. 2G). Upon the overexpression of Oct4, luciferase activity was impaired in cells transfected with a plasmid containing the Rnd1 promoter (Fig. 2H). *In vivo*, Western blotting showed that the Rnd1 level in tumor tissue homogenates of the Oct4-overexpressing group was significantly lower than that in the control group (Fig. 2I and J). All these data indicate that the overexpression of Oct4 exerts an inhibitory effect on the expression of Rnd1 in breast cancer cells.

3.3. Suppression of Rnd1 partially contributes to the inhibitory effect of Oct4 overexpression on the metastasis of MDA-MB-231 cells

Rnd1 is a member of the Rho GTPase family and involved in the regulation of cell motility. Considering the significant inhibitory effect of Oct4 on Rnd1 expression as well as the essential role of cell motility regulation in the metastatic process, we hypothesized that Rnd1 might mediate the effects of Oct4 on metastasis. First, we examined the effects of Rnd1 in MDA-MB-231 cells. Transwell assays revealed

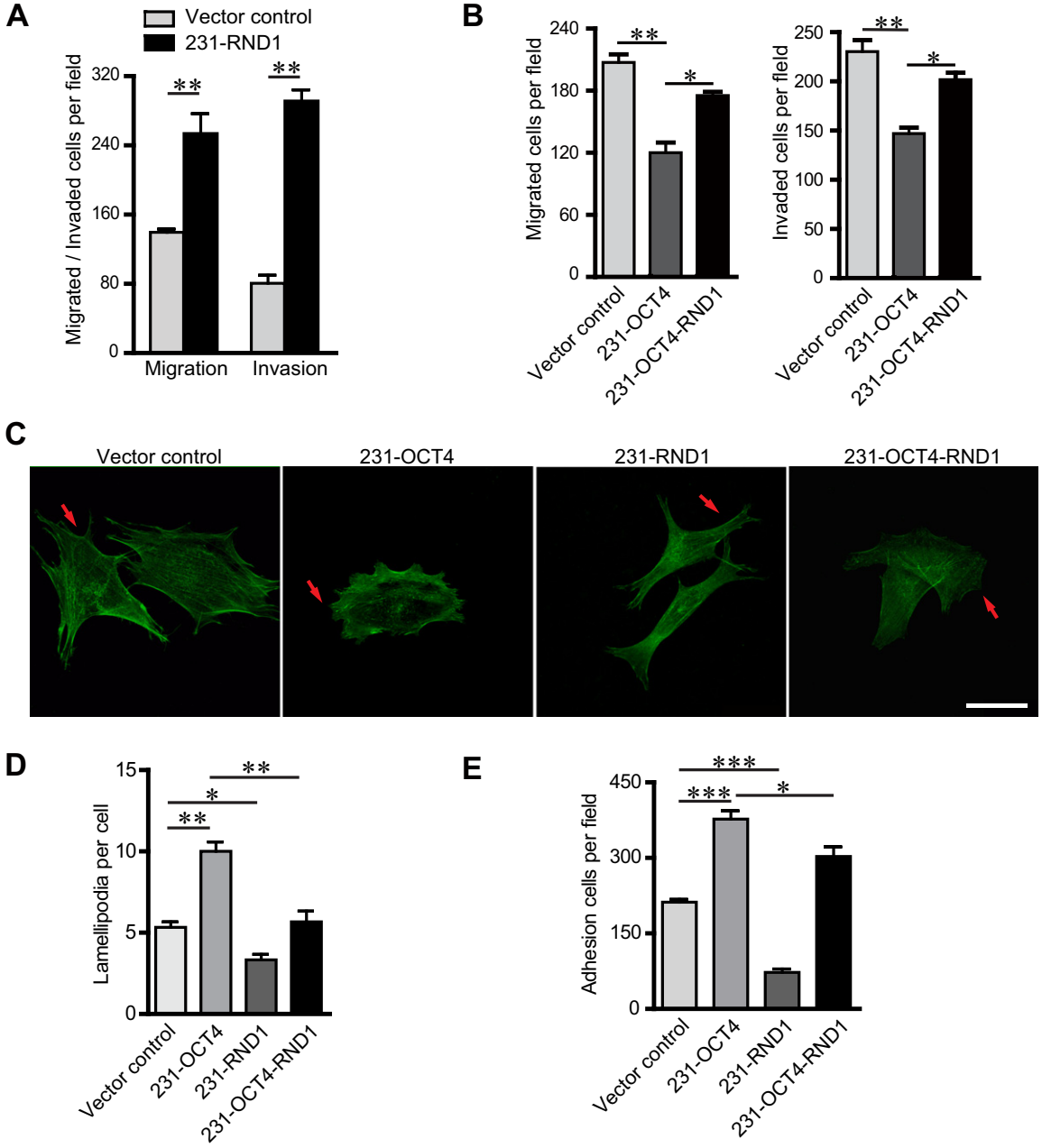

Fig. 3. Rnd1 partly rescues the inhibitory effect of Oct4 overexpression on the metastasis of MDA-MB-231 cells. For RNA-seq analysis, 1×10^7 Oct4-overexpressing or control MDA-MB-231 cells were collected to extract total RNA. mRNA was purified by Oligo(dT) magnetic bead adsorption. (A) Results of transwell assays. The migration and invasion capacities of RND1-overexpressing MDA-MB-231 cells were markedly increased compared with those of control cells. Data represent the mean ± S.E.M. of triplicate wells. Experiments were repeated at least three times. $^{**}P < 0.01$. (B) Stably transfected MDA-MB-231 cells overexpressing OCT4 were transiently transfected with control or RND1 vectors. The migrated and invaded cell counts of 231-vector control, 231-OCT4-control, and 231-OCT4-RND1 cells are shown. $^{**}P < 0.01$, $^{*}P < 0.05$. (C) To detect F-actin rearrangement in variously transfected MDA-MB-231 cells, the cytoskeleton was stained with FITC-phalloidin. Scale bar = 10 μm. (D) Lamellipodia per cell. $^{*}P < 0.05$, $^{**}P < 0.01$. (E) Adhesion assays were performed to detect the cell adhesion capacity of MDA-MB-231 cells. Adhesive cell counts are shown. $^{*}P < 0.05$, $^{***}P < 0.001$.

that ectopic expression of Rnd1 significantly increased the migration and invasion capacities of MDA-MB-231 cells (Fig. 3A). Furthermore, Rnd1 overexpression partially rescued the migration and invasion defects induced by Oct4 (Fig. 3B). Immunofluorescence staining showed that overexpression of Oct4 increased the formation of lamellipodia in MDA-MB-231 cells, whereas this effect was partially reversed by the overexpression of Rnd1 (Fig. 3C and D). In addition, increased adhesion to the extracellular matrix protein collagen I, which was induced by the overexpression of Oct4, was also partially rescued by Rnd1 overexpression (Fig. 3E). These data suggest that Rnd1 mediates the effect of Oct4 on metastasis by influencing the lamellipodia formation as well as cell adhesion.

3.4. Expression levels of Oct4 affect E-cadherin expression in breast cancer cells

Our previous study showed that the downregulation of Oct4 suppresses the expression of E-cadherin in MCF7 cells. To further confirm the influence of Oct4 on E-cadherin expression, we examined the expression level of E-cadherin in stable Oct-overexpressing MDA-MB-231 and 4T1 cell lines. Western blotting showed that the ectopic overexpression of Oct4 induced E-cadherin expression in both cell lines compared with that in their control cells (Fig. 4A and B). E-cadherin expression at cell–cell junctions was significantly enhanced in Oct4-overexpressing cells compared with that in control cells (Fig. 4C). Interestingly, even in 4T1 cells, which have a relatively high basal level of E-cadherin, the expression of E-cadherin was still elevated by 55.8% in cells overexpressing Oct4 (Fig. 4D). The expression of E-cadherin was compared in tumor tissues from mice injected with Oct4-overexpressing or control MDA-MB-231 cells. RT-PCR and Western blotting showed higher E-cadherin expression in the Oct4-overexpressing group at both mRNA and protein levels (Fig. 4E). Accordingly, the downregulation of Oct4 led to the suppression of E-cadherin expression in not only cultured 4T1 cells but also in tumor tissues from mice injected with Oct4-silenced 4T1 cells (Fig. 4F and G).

3.5. Rnd1 mediates the suppression of E-cadherin induced by Oct4 in MDA-MB-231 cells

To determine whether the influence of Oct4 on Rnd1 plays a role in the regulation of E-cadherin expression by Oct4, we first detected the expression level of E-cadherin in Rnd1-silenced MDA-MB-231 cells. It is clear that 231-sh2 can effectively knockdown the expression of Rnd1 in MDA-MB-231 cells. The results showed the significantly suppressed expression of E-cadherin (Fig. 5A). Moreover, Western blotting and immunofluorescence showed that Rnd1 rescued the increase of E-cadherin caused by the overexpression of Oct4 (Fig. 5B and C). These data suggest that Rnd1 mediates the regulatory effect of Oct4 on E-cadherin in MDA-MB-231 cells.

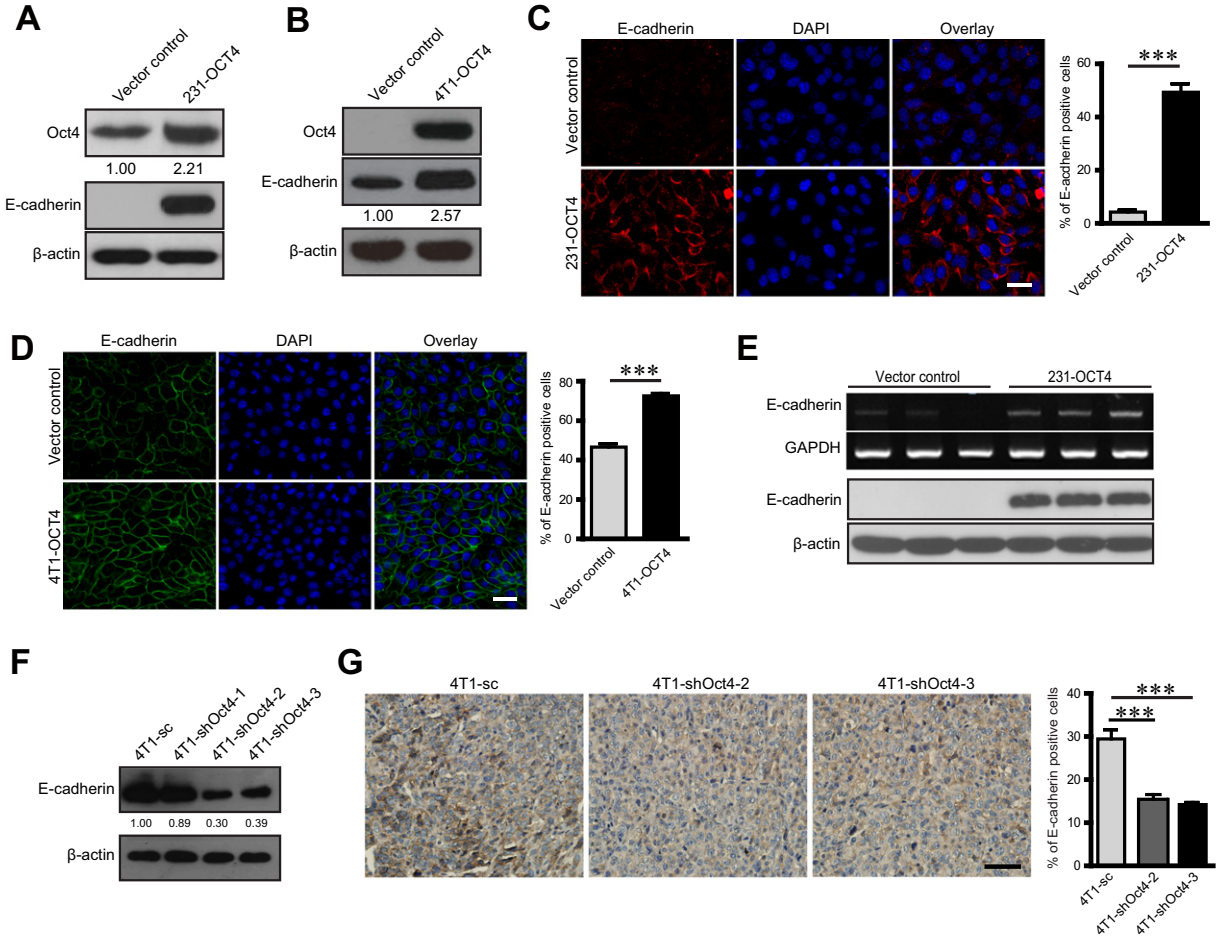

Fig. 4. Oct4 expression levels affect the expression of E-cadherin in breast cancer cells. (A & B) Western blotting was used to detect the expression of Oct4 and E-cadherin in Oct4-overexpressing MDA-MB-231 and 4T1 cell lines as well as their respective vector control-overexpressing control cell lines. Representative images are shown. (C & D) Expression of E-cadherin was detected by immunofluorescence staining of the cell lines described above. Representative images and the percentages of E-cadherin-positive cells are shown. Scale bar = 20 μm. $^{***}P < 0.001$. (E) RT-PCR and Western blotting were performed to detect the expression level of E-cadherin in primary tumor tissues. GAPDH was used as the loading control. (F) Western blotting was used to detect the expression of E-cadherin in Oct4-silenced 4T1 cells. (G) Tumors were established as described in Fig. 1. Representative images and the percentages of E-cadherin-positive cells are shown. Scale bar = 40 μm. $^{***}P < 0.001$.

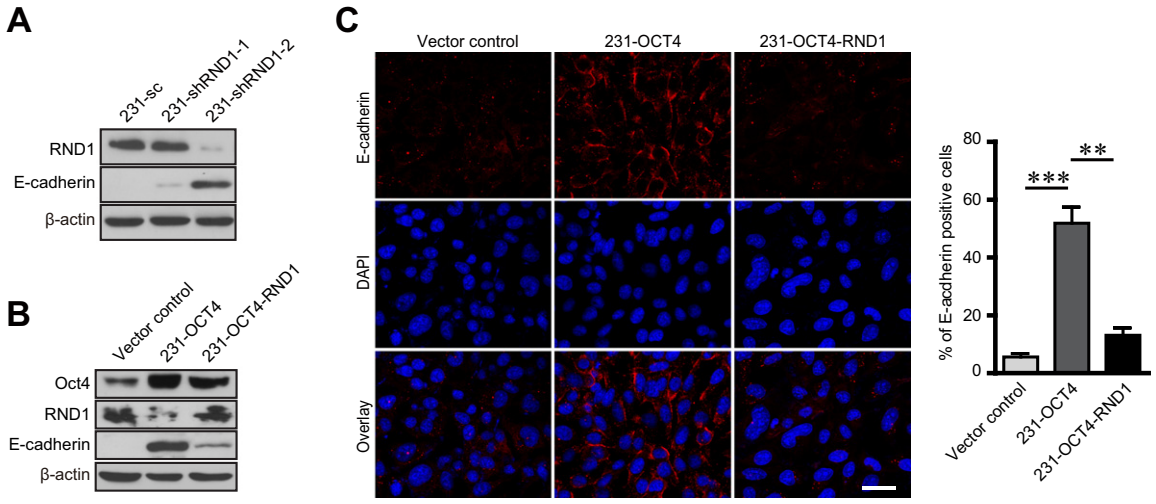

Fig. 5. Rnd1 mediates the decrease of E-cadherin induced by Oct4 in MDA-MB-231 cells. (A) MDA-MB-231 cells were stably transfected with scrambled shRNA (sc) or Rnd1 shRNA (sh1 or sh2). Western blotting was performed to detect the knockdown efficiency of Rnd1 and the expression of E-cadherin in Rnd1-silenced MDA-MB-231 cells. (B) Western blotting of Oct4, Rnd1 and E-cadherin in Oct4-overexpressing MDA-MB-231 cells and 231-OCT4 transient transfected with Rnd1 as well as vector control–overexpressing MDA-MB-231 cells. (C) Immunofluorescence staining of E-cadherin (red) in MDA-MB-231 cells. Nuclei were counterstained with DAPI (blue). Representative images and the statistical results of percentages of E-cadherin-positive cells are shown. Scale bar = 20 μm, $^{**}P < 0.01$, $^{***}P < 0.001$.

4. Discussion

Oct4 is well known for its key role in the maintenance of self-renewal and pluripotency, the central features of embryonic stem cells. Recent studies indicate that some tumor cells also possess stem cell-like properties [26]. Moreover, Oct4 is regarded as a marker of these stem-like cells in cancers. Although several studies have tried to establish a link between Oct4 and the malignant potential of cancer cells [27], the exact role of Oct4 in tumor metastasis and its underlying mechanism are still unclear. Our previous study demonstrated that the downregulation of Oct4 in MCF7 cells suppresses E-cadherin expression, thus promoting their migration and invasion. However, considering the heterogeneity of breast cancer cells, especially in terms of metastatic characteristics, a more thorough and detailed study appears to be necessary. To further evaluate the role of Oct4 in the metastasis of breast cancer, we chose two breast cancer cell lines with high metastatic potentials, MDA-MB-231 and 4T1. Our data showed that basal levels of Oct4 expression in MDA-MB-231 and 4T1 cells were relatively lower than that in the breast cancer cells with a low potential for metastasis. The ectopic expression of Oct4 in MDA-MB-231 and 4T1 breast cancer cells suppressed cell migration and invasion *in vitro* as well as metastatic lung nodules *in vivo*. No significant differences were found in the tumor volume and weight by altering the Oct4 expression levels. Therefore, our data suggest that the expression of Oct4 affects the metastatic potential of breast cancer cells.

In this study, RNA-seq analysis revealed that the ectopic expression of Oct4 led to a dramatic decrease of Rnd1 expression. ChIP assay showed that Oct4 directly bound to the promoter of Rnd1, thus inducing the suppression of Rnd1 expression in MDA-MB-231 cells. Ectopic Rnd1 overexpression increased the migration and invasion capacities of MDA-MB-231 cells. In addition, Rnd1 partially compensated for the impaired migration and invasion caused by Oct4 overexpression. As a member of the RhoGTPase family, Rnd1 has been previously reported to participate in the regulation of cellular motility. Recent studies have revealed that Rnd1 binds to and activates p190RhoGAP, and then antagonizes RhoA/ROCK-induced myosin light chain phosphorylation and actomyosin contractility to regulate cell motility [28]. On the other hand, Rnd1 also binds to plexins and activates their R-Ras GAP domain, resulting in actin rearrangement that reduces integrin-mediated cell adhesion. Therefore, by regulating the actin cytoskeleton, Rnd1 promotes the loss of actin stress fibers and the formation of focal adhesions, resulting in an increase of cell migration. Our data showed that ectopic Rnd1 suppressed the adhesion capacity of MDA-MB-231 cells. Also, it partially reduced the increased adhesion in the stable Oct4-overexpressing cells. To our surprise, the overexpression of Oct4 prompted the increased formation of lamellipodia, which was partially counteracted by the overexpression of Rnd1. The results seem likely at odd with the anti-metastatic effect of ectopic Oct4 expression since the formation of lamellipodia is conventionally thought as a facilitator of cell locomotion [29]. We consider that it might be a reflection of the complexity of RhoGTPase regulation. It has been reported that cell adhesion and the formation of lamellipodia were regulated via distinct RhoGTPase signals [30]. RhoA regulates cytoskeletal changes affecting cell adhesion while Rac1 majorly mediates lamellipodia formation. Nevertheless, there exists a crosslink between the two signaling pathways. Sander et al. showed that Rac1 exerts an inhibitory function on RhoA activity by inducing the reactive oxygen species [31]. Considering the sophistication of tumor metastasis, it is not hard to speculate that Oct4 influences the metastasis via diverse mechanisms although the role of Rnd1 in mediating lamellipodia formation remains unsolved.

Loss of cell–cell adhesion is a requirement for the invasion of epithelial tumors whether it occurs in a single cell or a multicellular unit [32]. As an epithelial marker associated with cell–cell junctions and adhesion, the loss of E-cadherin is the initial step and a driving force for epithelial-to-mesenchymal transition (EMT) that plays a critical role in tumor progression. Our data demonstrated that ectopic overexpression of Oct4 upregulated the expression of E-cadherin, even in 4T1 cells which basally express E-cadherin at an extremely high level. This result is consistent with the observation that Oct4 is a potent inducer of mesenchymal-to-epithelial transition (MET) in the induced pluripotent stem cell-related study [13]. Interestingly, silencing Rnd1 increased the expression of E-cadherin and the overexpression of Rnd1 partly inhibited the upregulation of E-cadherin induced by Oct4 in MDA-MB-231 cells. Recent studies have indicated that E-cadherin expression is correlated with the activities of RhoGTPases. Reynolds et al. reported that E-cadherin affects RhoA activity through its binding partner p120ctn [33]. p120ctn binds directly to E-cadherin and also inhibits RhoA activity by a direct interaction with p190RhoGEF. Thus, E-cadherin competes with p190RhoGEF for interactions with p120ctn in a cell density-dependent manner [34]. Consequently, high E-cadherin expression leads to a potent activation of RhoA. Meanwhile, p120ctn also promotes Rac1 activity and induces the formation of a

motile, protrusive membrane structure, such as lamellipodia [35]. These might explain why Rnd1, via the suppression of E-cadherin, exerts influence on the formation of lamellipodia as we mentioned above. Obviously, more convincing evidence is still needed to prove this hypothesis. Our finding that Rnd1 mediates the effect of Oct4 on E-cadherin expression sheds light on the mechanism in tumor metastasis.

In conclusion, our results demonstrate that the Oct4 expression level exerts regulatory effects on the metastasis of breast cancer cells. The underlying mechanism may involve Oct4 regulating the expression of Rnd1, which in turn influences E-cadherin expression as well as cytoskeleton rearrangement. Therefore, our findings may provide a novel potential strategy for the treatment of breast cancer metastasis.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work was supported by a grant from the National Basic Research Program of China to R. X. (No. 2013CB967200). X. T. was supported by the National Natural Science Foundation of China (No. 81270774).

References

[1] S.L. Wood, J.A. Westbrook, J.E. Brown, Omic-profiling in breast cancer metastasis to bone: implications for mechanisms, biomarkers and treatment, Cancer Treat. Rev. 40 (2014) 139–152.
[2] R. Xiang, D. Liao, T. Cheng, H. Zhou, Q. Shi, T.H. Chuang, D. Markowitz, R.A. Reisfeld, Y. Luo, Downregulation of transcription factor SOX2 in cancer stem cells suppresses growth and metastasis of lung cancer, (vol 104, pg 1410, 2011) Br. J. Cancer 104 (2011) 1931.
[3] H. Liu, D.C. Radisky, D. Yang, R. Xu, E.S. Radisky, M.J. Bissell, J.M. Bishop, MYC suppresses cancer metastasis by direct transcriptional silencing of alphav and beta3 integrin subunits, Nat. Cell Biol. 14 (2012) 567–574.
[4] X. Dai, J. Ge, X. Wang, X. Qian, C. Zhang, X. Li, OCT4 regulates epithelial–mesenchymal transition and its knockdown inhibits colorectal cancer cell migration and invasion, Oncol. Rep. 29 (2013) 155–160.
[5] Z. Chen, W.R. Xu, H. Qian, W. Zhu, X.F. Bu, S. Wang, Y.M. Yan, F. Mao, H.B. Gu, H.L. Cao, X.J. Xu, Oct4, a novel marker for human gastric cancer, J. Surg. Oncol. 99 (2009) 414–419.
[6] Y. Lu, H. Zhu, H. Shan, J. Lu, X. Chang, X. Li, J. Lu, X. Fan, S. Zhu, Y. Wang, Q. Guo, L. Wang, Y. Huang, M. Zhu, Z. Wang, Knockdown of Oct4 and Nanog expression inhibits the stemness of pancreatic cancer cells, Cancer Lett. 340 (2013) 113–123.
[7] J. Hu, K. Qin, Y. Zhang, J. Gong, N. Li, D. Lv, R. Xiang, X. Tan, Downregulation of transcription factor Oct4 induces an epithelial-to-mesenchymal transition via enhancement of Ca^{2+} influx in breast cancer cells, Biochem. Biophys. Res. Commun. 411 (2011) 786–791.
[8] L. Ding, M.J. Ellis, S. Li, D.E. Larson, K. Chen, J.W. Wallis, C.C. Harris, M.D. McLellan, R.S. Fulton, L.L. Fulton, R.M. Abbott, J. Hoog, D.J. Dooling, D.C. Koboldt, H. Schmidt, J. Kalicki, Q. Zhang, L. Chen, L. Lin, M.C. Wendl, J.F. McMichael, V.J. Magrini, L. Cook, S.D. McGrath, T.L. Vickery, E. Appelbaum, K. Deschryver, S. Davies, T. Guintoli, R. Crowder, Y. Tao, J.E. Snider, S.M. Smith, A.F. Dukes, G.E. Sanderson, C.S. Pohl, K.D. Delehaunty, C.C. Fronick, K.A. Pape, J.S. Reed, J.S. Robinson, J.S. Hodges, W. Schierding, N.D. Dees, D. Shen, D.P. Locke, M.E. Wiechert, J.M. Eldred, J.B. Peck, B.J. Oberkfell, J.T. Lolofie, F. Du, A.E. Hawkins, M.D. O'Laughlin, K.E. Bernard, M. Cunningham, G. Elliott, M.D. Mason, D.M. Thompson Jr., J.L. Ivanovich, P.J. Goodfellow, C.M. Perou, G.M. Weinstock, R. Aft, M. Watson, T.J. Ley, R.K. Wilson, E. R. Mardis, Genome remodelling in a basal-like breast cancer metastasis and xenograft, Nature 464 (2010) 999–1005.
[9] M.M. Nijkamp, P.N. Span, I.J. Hoogsteen, A.J. van der Kogel, J.H. Kaanders, J. Bussink, Expression of E-cadherin and vimentin correlates with metastasis formation in head and neck squamous cell carcinoma patients, Radiother. Oncol. 99 (2011) 344–348.
[10] R.W. Mays, W.J. Nelson, J.A. Marrs, Generation of epithelial cell polarity: roles for protein trafficking, membrane-cytoskeleton, and E-cadherin-mediated cell adhesion, Cold Spring Harb. Symp. Quant. Biol. 60 (1995) 763–773.
[11] F. van Roy, G. Berx, The cell–cell adhesion molecule E-cadherin, Cell. Mol. Life Sci. 65 (2008) 3756–3788.
[12] L. Fan, H. Wang, X. Xia, Y. Rao, X. Ma, D. Ma, P. Wu, G. Chen, Loss of E-cadherin promotes prostate cancer metastasis via upregulation of metastasis-associated gene 1 expression, Oncol. Lett. 4 (2012) 1225–1233.
[13] R. Li, J. Liang, S. Ni, T. Zhou, X. Qing, H. Li, W. He, J. Chen, F. Li, Q. Zhuang, B. Qin, J. Xu, W. Li, J. Yang, Y. Gan, D. Qin, S. Feng, H. Song, D. Yang, B. Zhang, L. Zeng, L. Lai, M.A. Esteban, D. Pei, A mesenchymal-to-epithelial transition initiates and is required for the nuclear reprogramming of mouse fibroblasts, Cell Stem Cell 7 (2010) 51–63.
[14] J. Zhou, K. Li, Y. Gu, B. Feng, G. Ren, L. Zhang, Y. Wang, Y. Nie, D. Fan, Transcriptional up-regulation of RhoE by hypoxia-inducible factor (HIF)-1 promotes epithelial to mesenchymal transition of gastric cancer cells during hypoxia, Biochem. Biophys. Res. Commun. 415 (2011) 348–354.
[15] P. Riou, P. Villalonga, A.J. Ridley, Rnd proteins: multifunctional regulators of the cytoskeleton and cell cycle progression, Bioessays 32 (2010) 986–992.
[16] T. Goda, C. Takagi, N. Ueno, Xenopus Rnd1 and Rnd3 GTP-binding proteins are expressed under the control of segmentation clock and required for somite formation, Dev. Dyn. 238 (2009) 2867–2876.
[17] I. Oinuma, K. Kawada, K. Tsukagoshi, M. Negishi, Rnd1 and Rnd3 targeting to lipid raft is required for p190 RhoGAP activation, Mol. Biol. Cell 23 (2012) 1593–1604.
[18] Y. Zhou, S. Li, Q. Huang, L. Xie, X. Zhu, Nanog suppresses cell migration by down-regulating thymosin beta4 and Rnd3, J. Mol. Cell Biol. 5 (2013) 239–249.
[19] K. Wennerberg, M.A. Forget, S.M. Ellerbroek, W.T. Arthur, K. Burridge, J. Settleman, C. J. Der, S.H. Hansen, Rnd proteins function as RhoA antagonists by activating p190 RhoGAP, Curr. Biol. 13 (2003) 1106–1115.
[20] A. Harada, H. Katoh, M. Negishi, Direct interaction of Rnd1 with FRS2 beta regulates Rnd1-induced down-regulation of RhoA activity and is involved in fibroblast growth factor-induced neurite outgrowth in PC12 cells, J. Biol. Chem. 280 (2005) 18418–18424.
[21] M. Yang Zhang, R. Chen, W. Su, P. Li, S. Chen, Z. Chen, A. Chen, S. Li, C. Hu, IBP regulates epithelial-to-mesenchymal transition and the motility of breast cancer cells via Rac1, RhoA and Cdc42 signaling pathways, Oncogene 26 (2013) 3374–3382.
[22] M. Rousseau, M.H. Gaugler, A. Rodallec, S. Bonnaud, F. Paris, I. Corre, RhoA GTPase regulates radiation-induced alterations in endothelial cell adhesion and migration, Biochem. Biophys. Res. Commun. 414 (2011) 750–755.
[23] L. Wei, L. Wang, J.A. Carson, J.E. Agan, K. Imanaka-Yoshida, R.J. Schwartz, beta1 integrin and organized actin filaments facilitate cardiomyocyte-specific RhoA-dependent activation of the skeletal alpha-actin promoter, FASEB J. 15 (2001) 785–796.
[24] Q. Tao, S. Chen, Y. Xu, Y. Chen, X. Li, W. Mou, L. Wang, Y. Liu, R.A. Reisfeld, R. Xiang, D. Lv, N. Li, SOX2 gene regulates the transcriptional network of oncogenes and affects tumorigenesis of human lung cancer cells, PLoS One 7 (2012) e36326.
[25] X.P. Jia, X.F. Li, Y.X. Xu, S. Zhang, W.J. Mou, Y.H. Liu, Y. Liu, D. Lv, C.H. Liu, X.Y. Tan, R. Xiang, N. Li, SOX2 promotes tumorigenesis and increases the anti-apoptotic property of human prostate cancer cell, J. Mol. Cell Biol. 3 (2011) 230–238.
[26] W.D. Liu, T. Zhang, C.L. Wang, H.M. Meng, Y.W. Song, Z. Zhao, Z.M. Li, J.K. Liu, S.H. Pan, W.B. Wang, Sphere-forming tumor cells possess stem-like properties in human fibrosarcoma primary tumors and cell lines, Oncol. Lett. 4 (2012) 1315–1320.
[27] S.H. Chiou, M.L. Wang, Y.T. Chou, C.J. Chen, C.F. Hong, W.J. Hsieh, H.T. Chang, Y.S. Chen, T.W. Lin, H.S. Hsu, C.W. Wu, Coexpression of Oct4 and Nanog enhances malignancy in lung adenocarcinoma by inducing cancer stem cell-like properties and epithelial–mesenchymal transdifferentiation, Cancer Res. 70 (2010) 10433–10444.
[28] K. Oinuma, K. Kawada, M. Tsukagoshi, Negishi, Rnd1 and Rnd3 targeting to lipid raft is required for p190RhoGAP activation, Mol. Biol. of the Cell. 23 (2012) 1593–1604.
[29] H. Khoury, D.L. Dankort, S. Sadekova, M.A. Naujokas, W.J. Muller, M. Park, Distinct tyrosine autophosphorylation sites mediate induction of epithelial mesenchymal like transition by an activated ErbB-2/Neu receptor, Oncogene 20 (2001) 788–799.
[30] S. Hanna, M. El-Sibai, Signaling networks of Rho GTPases in cell motility, Cell. Signal. 25 (2013) 1955–1961.
[31] E.E. Sander, J.P. ten Klooster, S. van Delft, R.A. van der Kammen, J.G. Collard, Rac downregulates Rho activity: reciprocal balance between both GTPases determines cellular morphology and migratory behavior, J. Cell Biol. 147 (1999) 1009–1022.
[32] P. Radvak, M. Repic, E. Svastova, M. Takacova, L. Csaderova, H. Strnad, J. Pastorek, S. Pastorekova, J. Kopacek, Suppression of carbonic anhydrase IX leads to aberrant focal adhesion and decreased invasion of tumor cells, Oncol. Rep. 29 (2013) 1147–1153.
[33] A.B. Reynolds, A. Roczniak-Ferguson, Emerging roles for p120-catenin in cell adhesion and cancer, Oncogene 23 (2004) 7947–7956.
[34] H. Kim, M. Oh, Q. Lu, K. Kim, E-Cadherin negatively modulates delta-catenin-induced morphological changes and RhoA activity reduction by competing with p190RhoGEF for delta-catenin, Biochem. Biophys. Res. Commun. 377 (2008) 636–641.
[35] D.I. Bellovin, R.C. Bates, A. Muzikansky, D.L. Rimm, A.M. Mercurio, Altered localization of p120 catenin during epithelial to mesenchymal transition of colon carcinoma is prognostic for aggressive disease, Cancer Res. 65 (2005) 10938–10945.