

Figure 1. — Increased ROR expression is associated with the poor BC prognosis. (A) ROR expression is compared by qRT-PCR from biopsies between BC tissues and adjacent tissues ( $n=37$ ,  $p=0.0035$ ). (B) Kaplan-Meier survival curves stratified a total of 37 BC patients according to ROR expression.  $P=0.019$  between low ROR ( $n=16$ ) and high ROR ( $n=21$ ) groups.

OEH/9" cpf" OFC/OD/453" ygtg" ewnwtgf" kp" FOGO" uwrrng/ o gpygf" ykvj"32 " HDU."322" Wl on"qh" r gplekmp"cpf"322" o il on"qh" uwtgrvqo{ekp} All the cells were grown at 37°C in a humidified incubator with 5% CO<sub>2</sub>.

Cells in each group were harvested in TRIzol reagent and total RNA was extracted. The qPCR analysis followed the manufacturer's instructions strictly as mentioned in previous study [12]. The primers used was shown below: ROR, F: 5'-TCAGTTCCC-TAAAGTCACCC-3', R: 5'-TCGTCCTTCTAAGCCTCTGT-3'. GAPDH, F: 5'-ACAACCTTGGTATCGTGGAAGG-3', R: 5'-GCCATCACGCCACAGTTTC-3'.

The ROR overexpression plasmid and the empty vector were purchased. Plasmids were transfected into cells by Lipofectamine 2000 using a DNA/Lipofectamine 2000 ratio of 1:3 for 48 hours.

Cells were lysed in RIPA buffer supplemented with complete protease inhibitors. Aliquots of proteins were separated by 12% SDS-PAGE and transferred onto polyvinylidene fluoride membrane, which was later blocked with 5% (w/v) blotting grade milk for one hour. Membrane was then incubated with the primary antibodies overnight at 4°C. Sources of the primary antibodies were: anti-ALDH1 and anti-GAPDH. After three times of wash with TBST, the membrane was further incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies for one hour. Proteins of interest were visualized with the Pierce's ECL Plus substrate according to the manufacturer's protocols.

Cells were seeded at a density of  $5 \times 10^3$  cells/well in 96-well culture plate and maintained overnight. Then, cells were exposed to increasing concentrations of ADR or VCR for 48 hours. Following treatment, 20  $\mu$ L of MTT solution (5 mg/mL in PBS) was added to each well and the plates were incubated for four hours at 37°C. At the end of incubation, the supernatants were aspirated and 150  $\mu$ L of DMSO was added into each well for dissolving the formazan crystals. Absorbance at 570 nm was measured using a microplate reader. Each assay was performed in triplicate with three independent replicates.

A cell invasion assay was performed using a 24-well Matrigel invasion chamber in accordance with the manufacturer's instructions. Cells ( $5 \times 10^4$  per well) were seeded into the upper compartment of the invasion chamber in DMEM without serum, while the lower well contained DMEM with serum to stimulate cell invasion. After in-

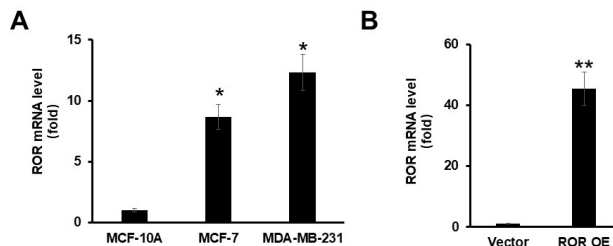


Figure 2. — ROR expression is upregulated in BC cells. (A) ROR expression is compared between MCF-10A, MCF-7, and MDA-MB-231 cell lines.  $*P < 0.05$ . MCF-10A was used as control. (B) MCF-10A cells were transfected with ROR overexpression (OE) plasmid, and ROR expression is compared between Vector group and ROR OE group.  $**p < 0.01$ .

cubation for 24 hours, non-invading cells on the upper side of the membrane were removed, while the cells on the bottom side were fixed with 3% paraformaldehyde and stained with 0.1% crystal violet. Cells were then extracted with 33% acetic acid and quantitatively detected using a standard microplate reader at 570 nm. Three independent experiments were performed in triplicate.

Cells were cultured on chamber slides then fixed with 4% paraformaldehyde solution for 10 minutes at room temperature (RT), washed three times with PBST, and then permeabilized with 0.1% Triton X-100 for 10 minutes. The slides were blocked with 5% BSA and 10% horse serum in PBST for one hour at room temperature, and incubated with antibodies against CD44 (1:200) at 4°C overnight. After being rinsed with PBST three times, cells were incubated with a secondary antibody Alexa Fluor 633 (1:200) for one hour at room temperature. After being washed twice, cells were stained with 5  $\mu$ g/mL DAPI, followed by imaging with confocal microscope. Data are expressed as the mean  $\pm$  standard deviation. Continuous variables were compared using the Student's *t*-test. Statistical analyses were performed using JMP statistical software, version 9.0.

This study was approved by the Research Ethics Committee of the affiliated hospital of Qingdao University, (Qingdao, Qingdao, PR China). Written informed consent was obtained from all patients. The authors confirm that all experiments were performed in accordance with relevant guidelines and regulations.

## Results

A total of 37 pairs of BC biopsies were involved in the current study. As shown in Figure 1A, the expression of ROR was increased in BC group compared with the control. Then the biopsies were allotted to two groups according to the ROR mRNA level, Kaplan-Meier survival curve analysis have shown that the overall survival rate of BC patients ( $n=21$ ) markedly decreased in the high ROR expression group compared with the low ROR expression ( $n=16$ ) group (Figure 1B), indicating that ROR might play a key role in tumorigenesis of BC.

As ROR expression is associated with the poor BC prognosis. Next, the authors detected the level of ROR mRNA in MCF-10A, MCF-7 and MDA-MB-231 cells with qPCR analysis. As shown in Figure 2A, they found that the ex-

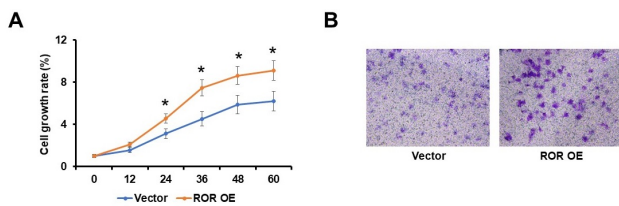


Figure 3. — Overexpression of ROR promotes proliferation and invasion of MCF-10A cells. (A) MTT assay detected fold change of cell growth rate between Vector group and ROR OE group MCF-10A in cell lines. (B) Transwell assay detected cell invasive capability between Vector group and ROR OE group MCF-10A in cell lines, and the representative images are shown.

pression of ROR was significantly increased in MCF-7 and MDA-MB-231 cancer cells compared to the MCF-10A cells which is a non-tumorigenic epithelial cell. To explore the biological function of ROR during the progression of BC, the authors increased the ROR expression via plasmid transfection, and confirmed the function via qPCR analysis in MCF-10A cells (Figure 2B). Next, the authors examined whether ROR overexpression affected the growth and invasion of MCF-10A cells. They found that the MCF-10A cells with ROR overexpression exhibited significantly higher growth rate compared with the control group (Figure 3A). In addition, more invading cells appeared in the ROR overexpression group compared to control group (Figure 3B). Taken together, overexpression of ROR with plasmid transfection could promote the capability of proliferation and invasion in MCF-10A cells.

As mentioned above, the appearance of CD44 and ALDH1 could be used as the BCSC properties. In the MCF-10A cells, the authors found that overexpression of ROR with plasmid transfection resulted could increase the expression of CD44 on the surface of cells with immunofluorescence assay (Figure 4A), as well as lead to induce expression of ALDH1 with western blot assay (Figure 4B). These data suggest that ROR play a key role in initiation of BCSC stemness through regulating the CD44 and ALDH1 expression possibly.

## Discussion

LncRNAs are RNA polymerase II transcripts that are longer than 200 nucleotides and lack an open reading frame [14]. There are >10,000 types of lncRNAs that are thought to play crucial roles in development and differentiation of human disease, particularly in tumor development [15, 16]. Recently, numerous studies revealed that various lncRNAs could regulate cancer stem cell (CSC) in numerous types of cancer via different molecular mechanisms, which involve differentiation, proliferation and self-renewal, promotion of the metastasis, invasion and prediction of prognosis, and

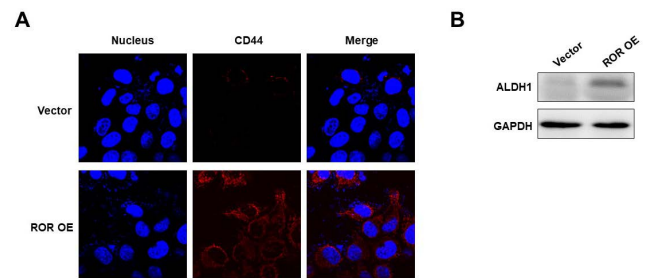


Figure 4. — BCSC properties appear in MCF-10A cells with ROR overexpression. (A) Immunofluorescence assay detected the CD44 expression on the surface of MCF-10A cells between Vector group and ROR OE group, and the representative images are shown. (B) ALDH1 protein level is detected between Vector group and ROR OE group in MCF-10A cells.

targeted therapies. To date, ROR, HOTAIR, H19, UCA1, and ARSR are the most highlighted lncRNAs in CSCs [17]. Therefore, lncRNAs could be used as a new master regulators of resistance to systemic treatments in BC [18].

The lncRNA ROR has been first described in iPSCs and have shown to have a role in embryonic stem cells generation [10]. In the field of BC, Hou *et al.* firstly reported that ROR could induce epithelial-to-mesenchymal transition and contribute to BC tumorigenesis and metastasis [19], and ROR could promote BC by regulating the TGF- $\beta$  pathway [20]. ROR and miR-145 were elaborated to regulate invasion in triple-negative BC via targeting ARF6 [11]. ROR could also promote estrogen-independent growth of BC via regulating MAPK/ERK signaling [21]. As known, multidrug resistance (MDR) in BC greatly hampers the therapeutic efficacy of chemotherapies [22]. ROR was shown to reverse gemcitabine-induced autophagy and apoptosis in BC cells [23], ROR could also enhance the sensibility of BC cells to tamoxifen by increasing miR-205 expression and suppressing the expressions of ZEB1 and ZEB2 [24]. Taken together, although ROR had multiple functions in the progression of BC tumorigenesis and drug-resistance, its role in the BCSC remains unclear.

In the authors/ previous study published in 2016, through fluorescence-activated cell sorting, they isolated gastric cancer stem cells (GCSC) from MKN-45 cells and demonstrated that ROR was highly expressed in CD133+ GCSCs. Overexpression of ROR significantly increased, but knock-down of ROR inhibited the proliferation and invasion of GCSCs. Most importantly, ROR led to upregulation of several key stemness transcriptional factors, such as OCT4, SOX2, and NANOG, as well as CD133 GCSC. In the current study, the data demonstrated for the first time that enhanced expression of ROR accelerates the progression of BC via promoting the expression of CD44 and ALDH1 to exhibit a BCSC stemness phenotype in MCF-10A cells.

In summary, further studies are necessary to improve the

understanding of the complex networks involved in ROR and BCSC, and ROR might be an efficacious and promising target in BC therapy.

### Acknowledgement

This study was supported by China Postdoctoral Application Project (2019M652331, 2018M642619), Qingdao Postdoctoral Application Project (2018121236, 2018121238), Department of Health of Shandong Province (2018WS068), Weifang City Science and Technology Bureau (2017YX053), and Department of Health of Weifang (2017wsjs122).

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