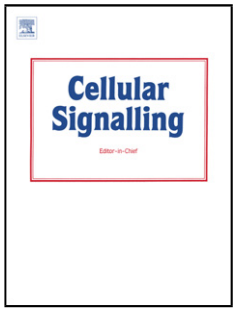

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Betacellulin enhances ovarian cancer cell migration by up-regulating Connexin43 via MEK-ERK signaling

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Running title: BTC enhances ovarian cancer cell migration

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Highlights

- Betacellulin enhances ovarian cancer cell migration.
- Betacellulin up-regulate the expression of Connexin43 by activating MEK-ERK signaling and U0126 reverses MEK-ERK signaling.
- AG1478 totally blocked the activation of MEK-ERK signaling and partially inhibited betacellulin-induced cell migration.
- Knockdown of Connexin43 attenuates betacellulin-induced cell migration.
- Gap junction inhibitor carbenoxolone did not alter betacellulin-induced cell migration.

ABSTRACT

Epithelial ovarian cancer is the fifth common cause of cancer death in women and the most lethal gynecological malignancies. Our previous studies have shown that up-regulation of Connexin43, a gap-junction subunit crucial for cell-cell communication, enhances ovarian cancer cell migration. Betacellulin is a member of the epidermal growth factor (EGF) family which can bind

to multiple EGF family receptors. Overexpression of betacellulin is found in a variety of cancers and is associated with reduced survival. However, the specific roles and molecular mechanisms of betacellulin in ovarian cancer progression are poorly understood. In the current study, we tested the hypothesis that betacellulin induces ovarian cancer cell migration by up-regulating Connexin43. Our results showed that treatment with betacellulin significantly increased Connexin43 expression and cell migration in both OVCAR4 and SKOV3 ovarian cancer cell lines. Moreover, betacellulin induced the activation of MEK-ERK signaling, and its effects on Connexin43 were inhibited by pre-treatment with U0126. Pre-treatment with AG1478 totally blocked the activation of MEK-ERK signaling but only partially inhibited betacellulin-induced Connexin43 expression and cell migration. Most importantly, betacellulin-induced cell migration was attenuated by knockdown of Connexin43, and co-treatment with gap junction inhibitor carbenoxolone did not alter this effect. Our results suggest a bilateral role of Connexin43 in ovarian cancer migration, and also demonstrate a gap junction-independent mechanism of betacellulin.

Keywords: Betacellulin; Ovarian cancer; Connexin43; Cell migration; MEK-ERK

1 Introduction

Ovarian cancer is the fifth leading cause of cancer-related death in women in developed countries [1]. Despite the progression in conventional therapies such as surgery and chemotherapy in the past decades, the 5-year survival of ovarian cancer remains lower than 50%. The main reasons for this situation are lack of accurate screening test for early diagnosis and the

high metastasis ability of ovarian cancer cells. The 5-year survival of ovarian cancer patients diagnosed at a distant stage is only 28%, compared to the 92% in localized stage group [2].

Gap junctions, composed of more than 20 connexin (Cx) subunit proteins, are specialized channel structures between adjacent cells [3]. They are essential for numerous physiological activities such as embryonic development, cell proliferation and differentiation, and are also involved in many pathological processes [4, 5]. Among all the connexin family members, connexin43 (Cx43) is the most studied and widely expressed gap junction protein [6, 7]. Cx43 was initially considered to be a tumor-suppressor since reduced expression of Cx43 has been observed in various types of cancer [8]. The expression of Cx43 was barely detectable in ovarian tumors compared to moderate expression in normal ovarian epithelial cells [9]. Our previous study also showed that epidermal growth factor (EGF)-induced Cx43 negatively regulated ovarian cancer cell proliferation [10]. However, increasing evidence suggests the involvement of Cx43 in oncogenesis and cancer progression [11-13]. Elevated Cx43 mRNA expression was shown to be associated with reduced overall survival in the TCGA database of 489 high-grade serous ovarian cancer cases [10], and we have also found that Cx43 mediates the stimulatory effects of TGF-β on ovarian cancer cell migration [14]. Taken together, the roles of Cx43 in ovarian cancer development and progression may be more complex than previously thought and are worth further investigating.

Betacellulin (BTC) is a ligand for the ERBB receptor family which contains 4 members: ERBB1 (EGFR), ERBB2 (HER2), ERBB3 (HER3) and ERBB4 (HER4). BTC can bind to both ERBB1 and ERBB4, and also activates all possible homodimeric and heterodimeric combinations of ERBB receptors [15]. These binding properties may give BTC unique biological functions which are distinct from other ligands in this family. The overexpression of BTC has been detected in

several types of human malignancies [16, 17], and up-regulation of BTC was shown to be associated with poor clinical outcome in breast cancer [18]. Our previous study has found that BTC induced ovarian cancer cell migration by down-regulating E-cadherin [19]. However, the specific functions of BTC in ovarian cancer progression and their underlying mechanisms are still largely unknown.

Given the importance of Cx43 in ovarian cancer progression and known links between Cx43 and EGF signaling [10], we hypothesize that Cx43 is involved in BTC-induced ovarian cancer cell migration. Our results show that BTC up-regulates Cx43 expression and cell migration in two ovarian cancer cell lines (OVCAR4 and SKOV3), and these effects are primarily mediated by MEK-ERK signaling via activation of EGFR. Furthermore, knockdown of endogenous Cx43 attenuates BTC-induced ovarian cancer cell migration likely in a gap junction-independent manner.

2 Materials and methods

2.1 Cell culture

OVCAR4 and SKOV3 human epithelial ovarian cancer cell lines were obtained from American Type Culture Collection. Cells were incubated in a 1:1 (vol/vol) mixture of M199/MCDB105 medium (Sigma-Aldrich) supplemented with 10% (vol/vol) fetal bovine serum (FBS; HyClone Laboratories) and 1% (vol/vol) penicillin/streptomycin (Gibco). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air, and were serum starved for 24 hours prior to treatment.

2.2 Antibodies and reagents

The monoclonal antibodies used in this study were: anti-porcine α-tubulin (B-5-1-2, Santa Cruz Biotechnology), anti-human phospho-ERK1/2 (Thr202/Tyr204) (E10, Cell Signaling Technology). The polyclonal antibodies used were: anti-human Cx43 (3512, Cell Signaling Technology), anti-rat ERK1/2 (9102, Cell Signaling Technology), anti-human phospho-Akt (9271, Cell Signaling Technology), anti-mouse Akt (9272, Cell Signaling Technology). The horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were obtained from Bio-Rad Laboratories. *E. coli*-derived recombinant human betacellulin (Asp32-Tyr111) was obtained from R&D Systems. Human recombinant epidermal growth factor (E9644), AG1478 and LY294002 were obtained from Sigma-Aldrich. U0126 was obtained from Calbiochem and AST1306 (S2185) was obtained from Selleckchem. Carbenoxolone (C4790) was obtained from Sigma-Aldrich.

2.3 Small interfering RNA (siRNA) transfection

To knock down endogenous Cx43, cells were plated at low density, allowed to recover for 24 hrs, and then transfected with 50 nM ON-TARGET*plus*SMARTpool siRNA (Dharmacon) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. ON-TARGET*plus* non-targeting control pool siRNA (50 nM; Dharmacon) was used as a transfection control in all experiments.

2.4 Reverse transcription-quantitative real-time PCR (RT-qPCR)

Total RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed with 3 μg of RNA, random primers, and

Moloney murine leukemia virus reverse transcriptase (Promega). SYBR Green RT-qPCR was performed on Applied Biosystems 7300 Real-Time PCR System equipped with 96-well optical reaction plates. Each 20 µl RT-qPCR reaction contained 1×SYBR Green PCR Master Mix (Applied Biosystems), 20 ng cDNA and 150 nM of each specific primer. The primers used were: Cx43, 5'-TAC CAA ACA GCA GCGGAG TT-3' (sense) and 5'-TGG GCA CCA CTC TTT TGC TT-3'(antisense); and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 5'-GAG TCA ACGGAT TTG GTC GT-3' (sense) and 5'-GAC AAG CTT CCC GTTCTC AG-3' (antisense). The amplification parameters were 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. At least three separate experiments were performed on different cultures and each sample was assayed in triplicate. A mean value was used for the determination of mRNA levels by the comparative Cq method ($2^{-\Delta\Delta Cq}$) with GAPDH as the reference gene.

2.5 Western blot analysis

Cells were lysed in lysis buffer (Cell Signaling Technology) containing protease inhibitor cocktail (Sigma-Aldrich). Lysates were centrifuged at 20,000 ×*g* for 10 minutes at 4°C and supernatant protein concentrations were quantified using the DC Protein Assay (Bio-Rad Laboratories) with BSA (A4503, Sigma-Aldrich) as the standard. Equal amounts of protein were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. After blocking with Tris-buffered saline containing 5% non-fat dry milk for 1 hour, membranes were incubated overnight at 4°C with primary antibodies Cx43 (1:1000), α-Tubulin (1:3000), phospho-ERK1/2 (1:3000), ERK1/2 (1:3000), phospho-Akt (1:3000) or Akt (1:3000), followed by incubation with the peroxidase-conjugated secondary antibody (1:5000). Immunoreactive bands were detected

with enhanced chemiluminescent or SuperSignal West Femto substrate (Pierce). Membranes were stripped with stripping buffer (50 mM Tris-HCl pH 7.6, 10 mM β-mercaptoethanol and 1% SDS) at 50°C for 30 minutes and reprobed with anti-α-Tubulin, anti-ERK1/2 or anti-Akt as a loading control. Immunoreactive band intensities were quantified by densitometry, normalized to those of the relevant loading control, and the results are expressed as fold change relative to the respective control.

2.6 Transwell migration assays

Migration assays were performed in Boyden chambers with minor modifications [20]. Transwell cell culture inserts (24-well, pore size 8 μm; BD Biosciences) were seeded with $1x10^5$ cells in 250 μL of medium with 0.1% FBS. Medium with 10% FBS (750 μl) was added to the lower chamber and served as a chemotactic agent. After 24 hrs incubation, non-migrating cells were wiped from the upper side of the membrane and cells on the lower side were fixed in cold methanol (-20°C) and air dried. Cells were stained with Crystal Violet and counted using a light microscope (10× objective) equipped with a digital camera (QImaging) and Northern Eclipse 6.0 software. Five microscopic fields were counted per insert, triplicate inserts were used for each individual experiment, and each experiment was repeated at least three times.

2.7 Wound healing assay

Wound healing assay was used to visualize the effect of BTC on cell motility. Briefly, cells were grown to full confluence on 24-well plates and serum starved for 24 h prior to treatment. Subsequently, cells were wounded by pipette tips and washed twice with DPBS to remove detached cells. Cells were observed for 24 h by Molecular Devices ImageXpress Micro Confocal

High-Content Imaging System (at 40× magnification), which maintained the temperature at 37°C and atmosphere with 5% of CO_2. Wound area measurement was performed using the ImageJ software. The migration distance was calculated the difference of relative residual wound at 24 h and the initial wound area.

2.8 Statistical analysis

Results are presented as the mean ± SEM of at least three independent experiments. PRISM software (GraphPad Software Inc.) was used to perform one-way ANOVA followed by Tukey's multiple comparison test. Means were considered significantly different if $P<0.05$ and are indicated by different letters.

3 Results

3.1 BTC induces Cx43 expression in ovarian cancer cells

To investigate whether BTC regulates Cx43 expression, we first treated two ovarian cancer cell lines (OVCAR4 and SKOV3) with BTC (50 ng/ml) for different amounts of time (1-24 hrs). As shown in Figure 1A-B, BTC treatment up-regulated Cx43 mRNA and protein levels in a time-dependent manner in both cell lines, with maximum effects at 24 hrs. Next, cells were treated with varying concentrations of BTC for 24 hrs and the RT-qPCR results showed that BTC induced Cx43 expression in a concentration-dependent manner, with more than a 3-fold increase at a concentration of 50 ng/ml (Figure 1C). The up-regulation of Cx43 protein levels (approximately 2.5-fold increase) was confirmed by Western blot following BTC treatment for 24 hrs (Figure 1D).

3.2 BTC up-regulates Cx43 via EGFR

To investigate the receptor requirements of BTC-induced Cx43 production, we pre-treated OVCAR4 and SKOV3 cells with the EGFR-specific inhibitor AG1478 for 1 hr prior to BTC treatment for 24 hrs. As shown in Figure 2A, pre-treatment with AG1478 partially attenuated the up-regulation of Cx43 mRNA by BTC in both cell lines. Western blot results showed a similar suppressive effect of AG1478 on BTC-induced Cx43 protein levels (Figure 2B).

3.3 MEK-ERK signaling mediates BTC-induced Cx43 expression

To investigate the signaling pathways involved in BTC-induced Cx43 expression, we first treated SKOV3 and OVCAR4 cells with BTC (50 ng/ml) for 10, 30 or 60 mins and used Western blot to measure the phosphorylation/activation of ERK1/2 and Akt, two common signaling mediators for EGF-like growth factors. As shown in Figure 3A, the phosphorylation of both ERK1/2 and Akt was increased at all time-points in BTC-treated SKOV3 cells. Likewise, 30 min BTC treatment increased the levels of phosphorylated ERK1/2 and Akt in OVCAR4 cells, activation of Akt was much lower than that of EGF-treated positive control cells. In addition, pre-treatment with AG1478 totally blocked the stimulatory effects of BTC on ERK1/2 and Akt phosphorylation in both cell lines (Figure 3B).

Next, we used the MEK inhibitor U0126 and the PI3K inhibitor LY294002 to confirm the involvement of these two pathways in BTC-induced Cx43 expression. Pre-treatment of OVCAR4 and SKOV3 cells with U0126 totally blocked the up-regulation of Cx43 mRNA and protein levels by BTC. In contrast, pre-treatment with LY294002 only partially attenuated the

effects of BTC in SKOV3 cells whereas it did not alter the effects of BTC in OVCAR4 cells (Figures 3C and 3D).

3.4 Connexin43 is involved in BTC-induced ovarian cancer cell migration

We have previously shown that treatment with BTC enhances SKOV3 and OVCAR5 ovarian cancer cell migration (12 hrs)[19]. To confirm this effect in our current cell models, BTC-treated OVCAR4 and SKOV3 cells were cultured in transwell migration assays for 24 hrs. As shown in Figures 4A and 4B, treatment with BTC (50 ng/ml) enhanced the migration of both cell lines, and these effects were partially abolished by AG1478 pre-treatment.

Previous studies have shown that knockdown of Connexin43 attenuates TGF-β-stimulated OVCAR4 and SKOV3 cell migration [14]. To determine whether Cx43 is required for BTC-induced cell migration, we transfected OVCAR4 and SKOV3 cells with Cx43 siRNA for 48 hrs prior to BTC-treatment and subsequent analysis of transwell migration. Pre-treatment with Cx43 siRNA significantly reduced Cx43 protein levels and partially reversed BTC-induced transwell cell migration (Figures 4C and 4D). EGF was used as a positive control and knockdown of Cx43 had similar inhibitory effects on migration induced by this ligand (Figures 4C and 4D).

3.5 BTC induces ovarian cancer cell migration in a gap junction-independent manner

Cx43 has been shown to modulate cell migration via both gap junction-dependent and -independent mechanisms [21]. To investigate if gap junction intercellular communication (GJIC) is required for the effects of BTC on OVCAR4 and SKOV3 cell migration, we examined its effects following pre-treatment with or without Cx43 siRNA in the presence or absence of the

well-known gap junction inhibitor carbenoxolone [22]. Western blot results showed that carbenoxolone treatment had no effect on Cx43 protein levels (Figure 5A). In control siRNA-treated cells, pre-treatment with carbenoxolone did not alter the stimulatory effects of BTC on OVCAR4 or SKOV3 cell migration (Figure 5B). Similarly, although Cx43 siRNA treatment reduced BTC-induced cell migration, combined treatment with carbenoxolone did not result in any further reductions in OVCAR4 or SKOV3 cell migration (Figure 5B). In addition, wound healing assays showed that the pro-migratory effects of BTC in OVCAR4 or SKOV3 cells were inhibited by Cx43 siRNA treatment, while combined carbenoxilone treatment did not further affect the cell motility. Taken together, these results suggest that BTC promotes ovarian cancer cell migration in a gap junction-independent manner.

4 Discussion

Previous studies have shown that overexpression of Cx43 attenuated EGF-induced ovarian cancer cell proliferation [10]. However, such a finding appears to conflict with the negative correlation between Cx43 mRNA expression and overall survival of ovarian cancer patients in the TCGA dataset [10]. In the current study, we found that Cx43 positively regulated BTC-induced ovarian cancer cell migration, which may be indicative of cellular functions that could explain the above mentioned correlation. Peritoneal implantation of ovarian cancer cells in later-stages is one of the reasons why ovarian cancer patients have high mortality. 5-year survival rates of patients who have disseminated disease is only 28% compared to 92% for patients whose tumor are still localized [1]. Considering this, the high motility and metastatic capability of ovarian cancer cells could be a vital factor for cancer development and prognosis. Our results suggest that the roles of Cx43 in ovarian cancer progression are multifaceted and Cx43 may act

to promote ovarian cancer progression or metastasis by enhancing cell migration. However, this effect needs to be further confirmed and the roles of Cx43 in other cell functions related to the metastatic process (e.g. invasion and adhesion) require further investigation.

As a member of the EGF-like growth factor family, BTC works similarly to EGF in many respects. However, BTC's ability to bind with ERBB4 as well as EGFR may give BTC unique functions. In present study, the EGFR-specific inhibitor AG1478 only partially attenuated BTC-induced Connexin43 expression and cell migration, suggesting a potential role for ERBB4. Several research groups have observed a high incidence of ERBB4 expression in ovarian cancer [23-25]; and ERBB4 has also been shown to correlate with serous histological subtype and cisplatin resistance [24]. However, no specific study has investigated how ERBB4 contributes to the functions of BTC in cancer or its potential mechanisms. Future studies will be required to further confirm the contributions of ERBB4 signaling to BTC-regulated cellular functions in ovarian cancer, and to clarify the signaling pathways involved.

GJIC is a double-edge sword in tumorigenesis. Gap junctions were initially implicated in tumor suppression since they played crucial roles in suppressing cell proliferation and maintaining tissue homeostasis. However, increasing evidence has implicated GJIC in cell adhesion, migration and invasion, mostly in a manner that would promote cancer development and progression [21, 26, 27]. In our study, pre-treatment with the gap junction inhibitor carbenoxolone altered neither BTC-induced ovarian cancer cell migration nor the partial inhibition of BTC-induced cell migration by Cx43 siRNA, suggesting that the contributions of Cx43 to BTC-induced ovarian cancer migration are independent of GJIC. The mechanisms by which Cx43 enhances cell migration independent of GJIC have been studied by several groups.

In general, the extracellular domains of Cx43 increase cell-cell adhesion and the intracellular domains interact with cytoskeletal molecules, junctional proteins and signaling enzymes [27]. One study showed that the adherens junction protein E-cadherin could modulate the transportation of Cx43 from the cytoplasm to points of cell-cell contact [28]. Interestingly, we have recently reported that BTC-induced ovarian cancer migration involves the down-regulation of E-cadherin [19]. Future studies will be required to clarify the precise mechanisms by which Cx43 contributes to BTC-induced ovarian cancer cell migration.

In summary, our study suggests a dual role of Cx43 in ovarian cancer progression and further elucidates how BTC enhances ovarian cancer migration. We show that BTC induces Cx43 expression and ovarian cancer cell migration mainly through EGFR via MEK/ERK signaling. Furthermore, Cx43 positively regulates BTC-induced cell migration and this effect is likely gap junction-independent.

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Conflicts of interest

The authors declare no conflict of interest.

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Figure legends

Figure 1. BTC induces Cx43 expression in ovarian cancer cells. A, OVCAR4 and SKOV3 cells were treated without (Ctrl) or with 50 ng/ml BTC for 1, 3, 6, 12 and 24 hrs, and Cx43 mRNA levels were examined by RT-qPCR. **B**, Cells were treated without (Ctrl) or with 50 ng/ml BTC for 3, 6, 12 or 24 hrs, and Cx43 protein levels were examined by Western blot. **C-D**, Cells were treated for 24 hrs without (Ctrl) or with increasing concentrations of BTC (1, 10, 20 or 50

ng/ml), and Cx43 mRNA (**C**) and protein (**D**) levels were examined by RT-qPCR and Western blot, respectively. Results are expressed as the mean ± SEM of at least three independent experiments and values without common letters are significantly different (P<0.05).

Figure 2. BTC up-regulates Cx43 via EGFR

OVCAR4 and SKOV3 cells were pre-treated for 1 hr with vehicle control (DMSO) or 10 μM AG1478 prior to treatment with or without 50 ng/ml BTC for 24 hours. Cx43 mRNA (**A**) and protein (**B**) levels were examined by RT-qPCR and Western blot, respectively. Results are expressed as the mean ± SEM of at least three independent experiments and values without common letters are significantly different (P<0.05).

Figure 3. MEK-ERK signaling mediates the enhance effects of BTC on Cx43 expression

A, Cells were treated without (Ctrl) or with 50 ng/ml BTC for different time durations (SKOV3: 10, 30 or 60 minutes; OVCAR4, 30 minutes), and Western blot was used to measure the levels of phosphorylated Akt (p-Akt) and ERK1/2 (p-ERK1/2) in relation to their total levels (Akt and ERK1/2, respectively). **B**, OVCAR4 and SKOV3 cells were pre-treated for 1 hr with vehicle control (DMSO) or 10 μM AG1478 prior to treatment with or without 50 ng/ml BTC for 30 minutes. Western blot was used to measure the Akt and ERK1/2 phosphorylation/activation. **C-D**, OVCAR4 and SKOV3 cells were pre-treated for 1 hr with vehicle control (DMSO), 5 μM U0126 (MEK inhibitor) or 5 μM LY294002 (PI3K inhibitor) prior to treatment with or without 50 ng/ml BTC for 24 hrs. Cx43 mRNA (**C**) and protein (**D**) levels were examined by RT-qPCR and Western blot, respectively. Results are expressed as the mean ± SEM of at least three independent experiments and values without common letters are significantly different (P<0.05).

Figure 4. Cx43 positively regulated BTC-induced ovarian cancer cell migration

A-B, OVCAR4 (**A**) and SKOV3 (**B**) cells were pre-treated for 1 hr with vehicle control (DMSO) or 10 μM AG1478 prior to treatment without (Ctrl) or with 50 ng/ml BTC, and cell migration was examined by transwell assay (24 hrs). **C-D**, OVCAR4 (**C**) and SKOV3 (**D**) cells were transfected for 48 hrs with 50 nM non-targeting control siRNA (si-Ctrl) or 50 nM siRNA targeting Cx43 (si-Cx43) prior to treatment without (Ctrl) or with 50 ng/ml BTC, and cell migration was examined by transwell assay (24 hrs). Results are expressed as the mean ± SEM of at least three independent experiments and values without common letters are significantly different (P<0.05).

Figure 5. BTC induced ovarian cancer cell migration in a gap junction-independent manner.

A, OVCAR4 and SKOV3 cells were treated with vehicle control (H_2O) or carbenoxolone (CBX; 25μM) for 48 hrs and Cx43 protein levels were measured by Western blot. **B**, Cells were transfected for 24 hrs with 50 nM non-targeting control siRNA (si-Ctrl) or 50 nM siRNA targeting Cx43 (si-Cx43), then pre-treated with CBX (25μM) for 1 hr prior to treatment without (Ctrl) or with 50 ng/ml BTC, and cell migration was examined by transwell assay (24 hrs). Results are expressed as the mean ± SEM of at least three independent experiments and values without common letters are significantly different (P<0.05).

Figure 6. BTC promoted ovarian cancer cell motility in a gap junction-independent manner.

OVCAR4 (**A**) and SKOV3 (**B**) cells were transfected for 24 h with 50 nM non-targeting control siRNA (si-Ctrl) or 50 nM siRNA targeting Cx43 (si-Cx43), then pre-treated with vehicle control (H_2O) or 25μM CBX for 1 h prior to treatment without (Ctrl) or with 50 ng/ml BTC, and cell motility was examined by wound healing assay (24 h). Results are expressed as the mean ± SEM of at least three independent experiments and values without common letters are significantly different ($P<0.05$).

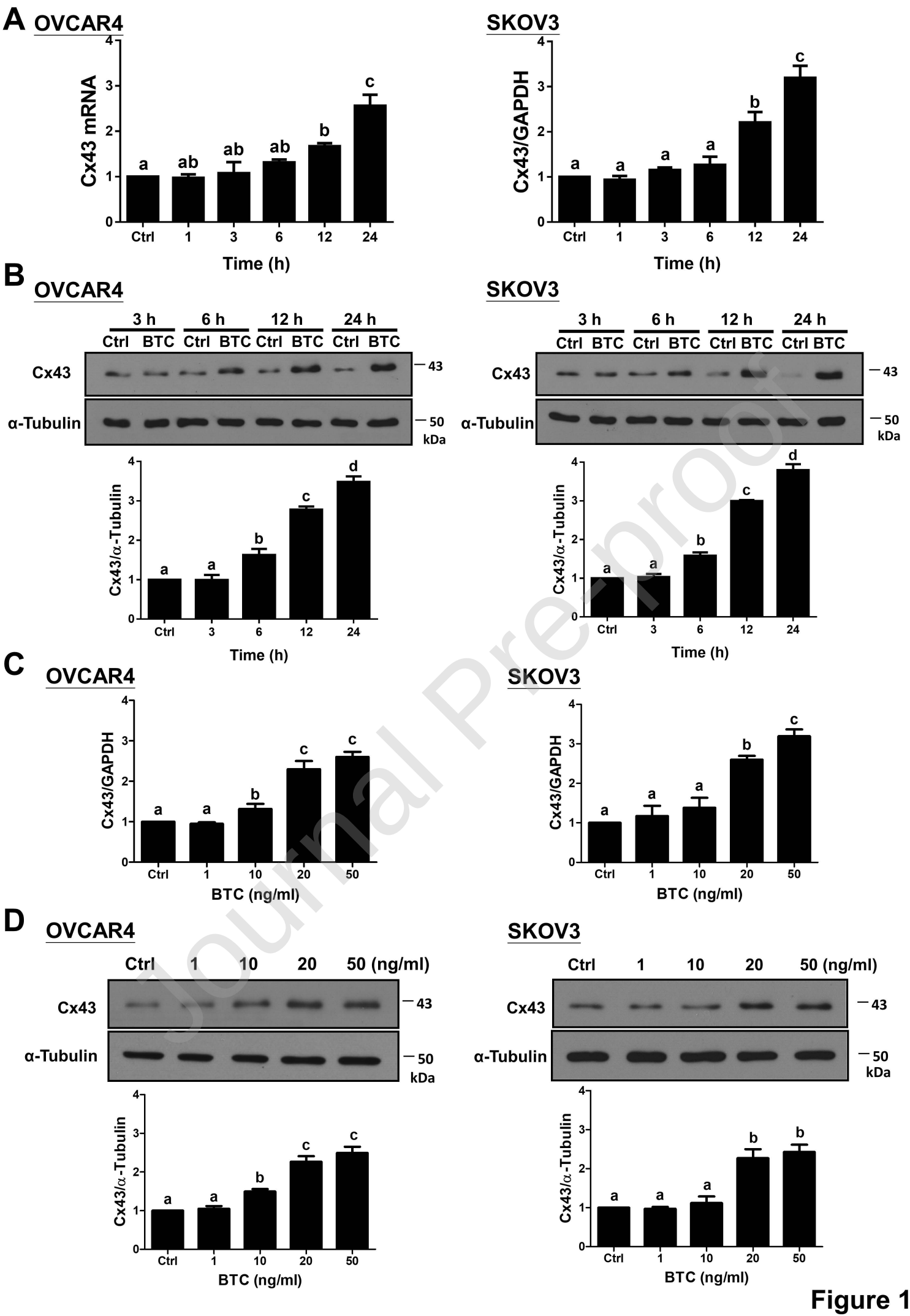

Figure 1

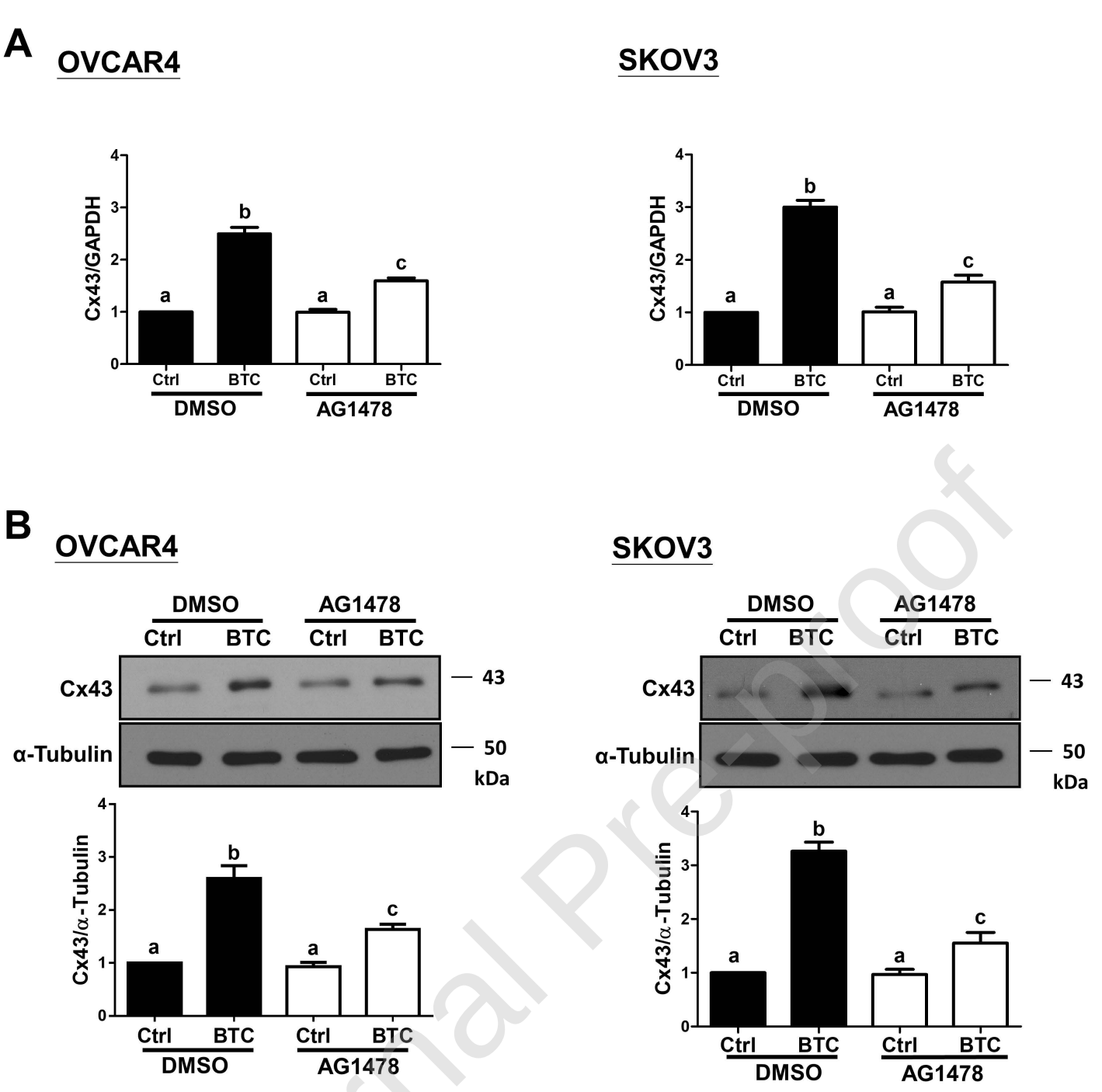

Figure 2

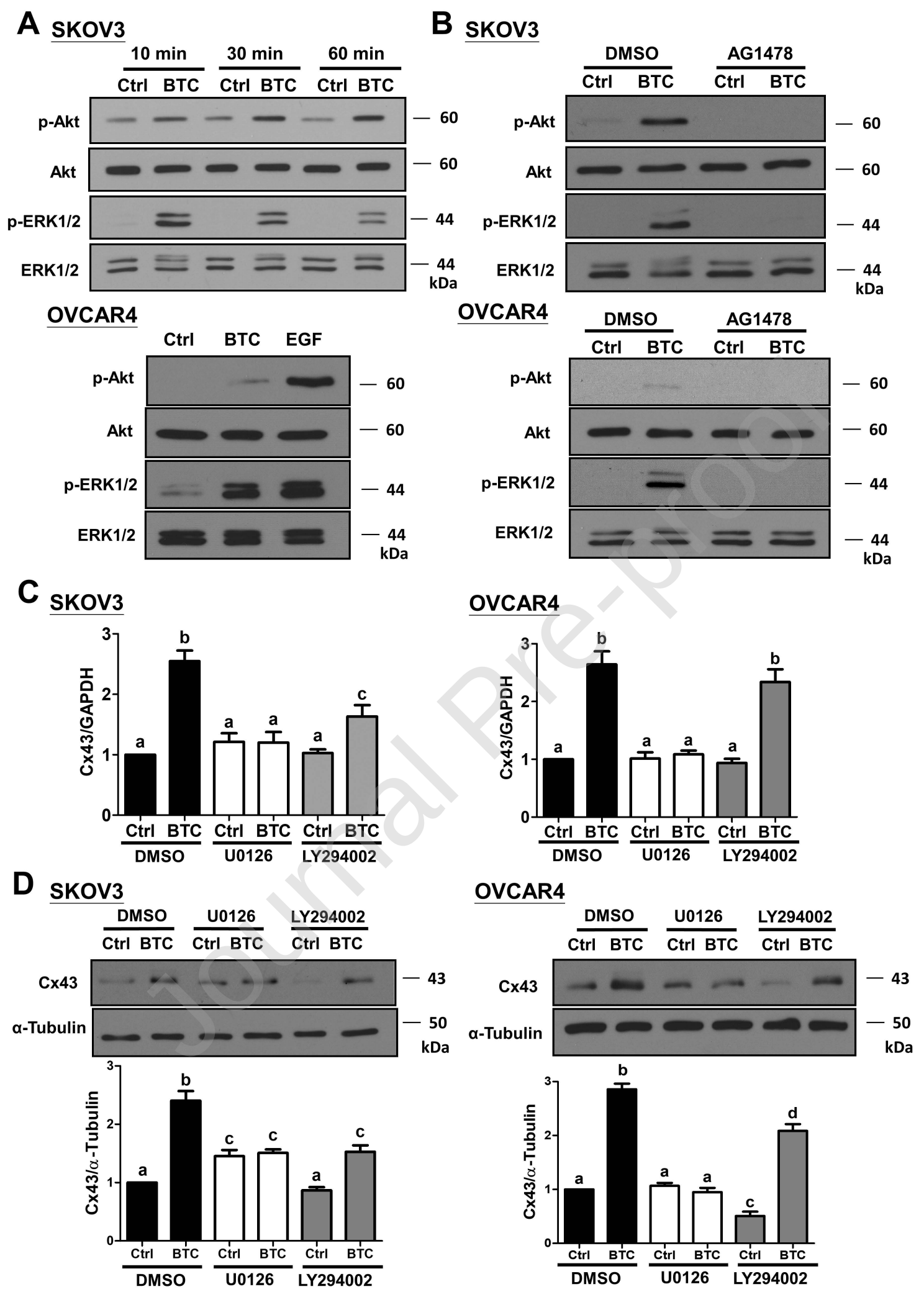

Figure 3

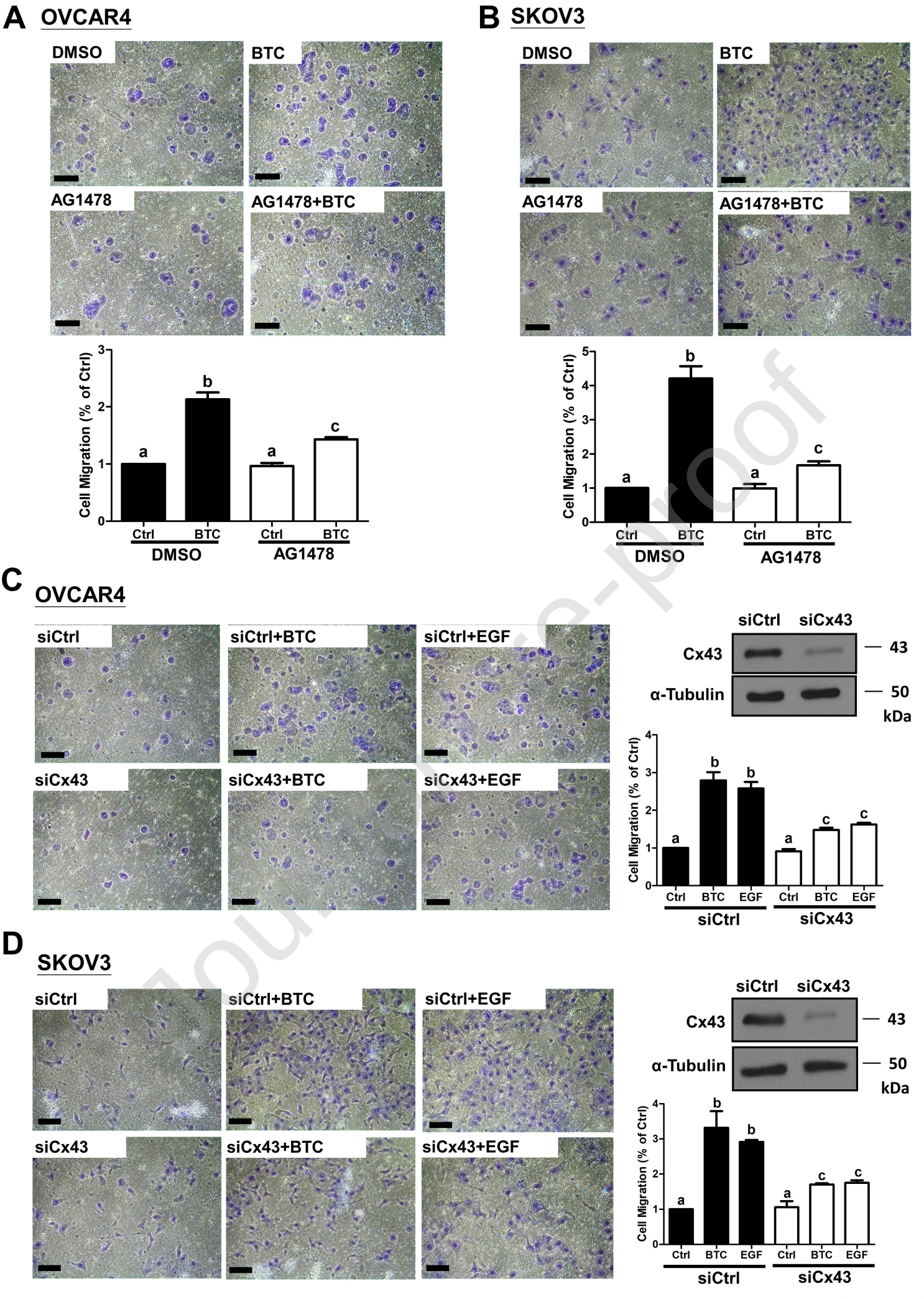

Figure 4

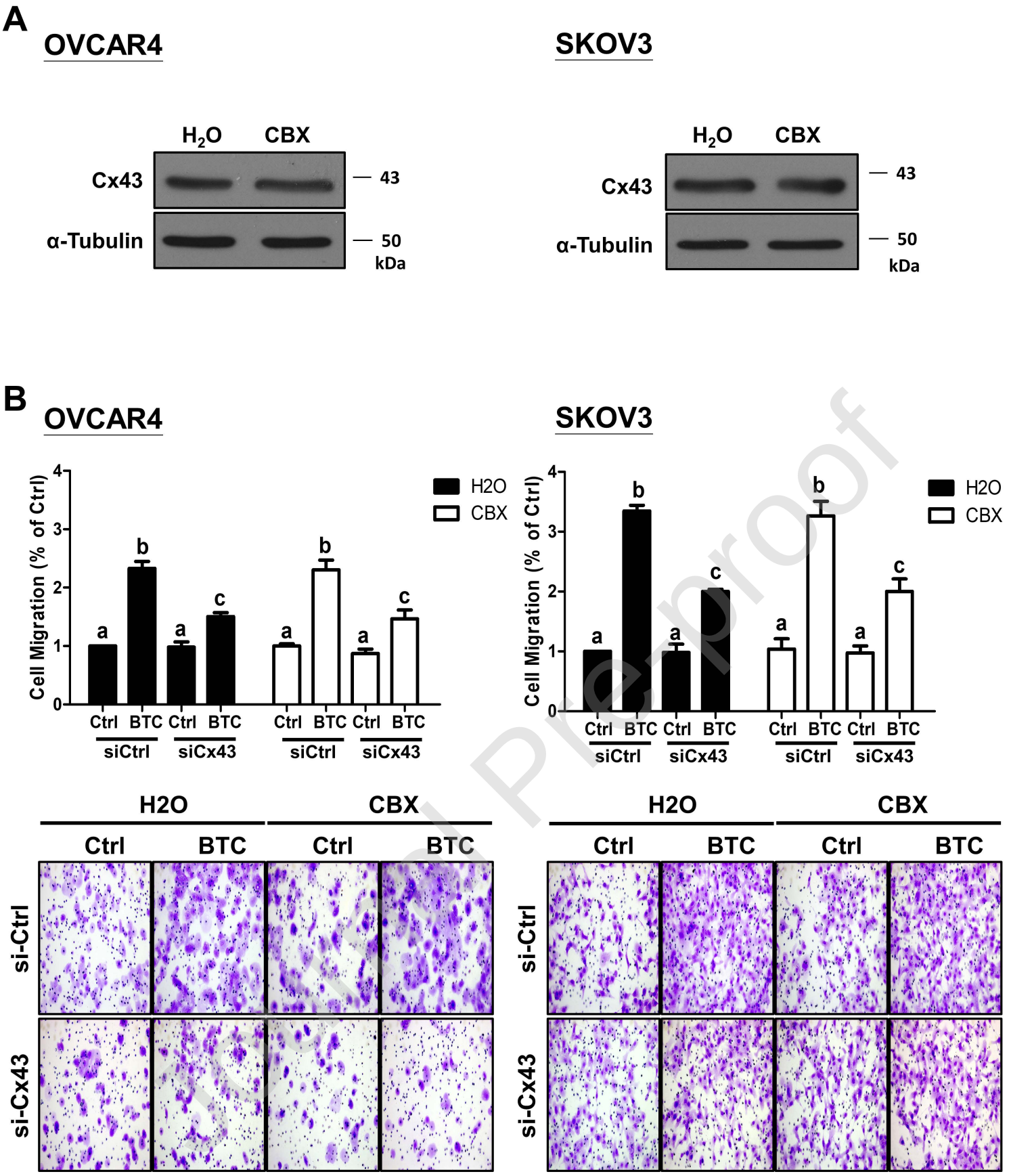

Figure 5

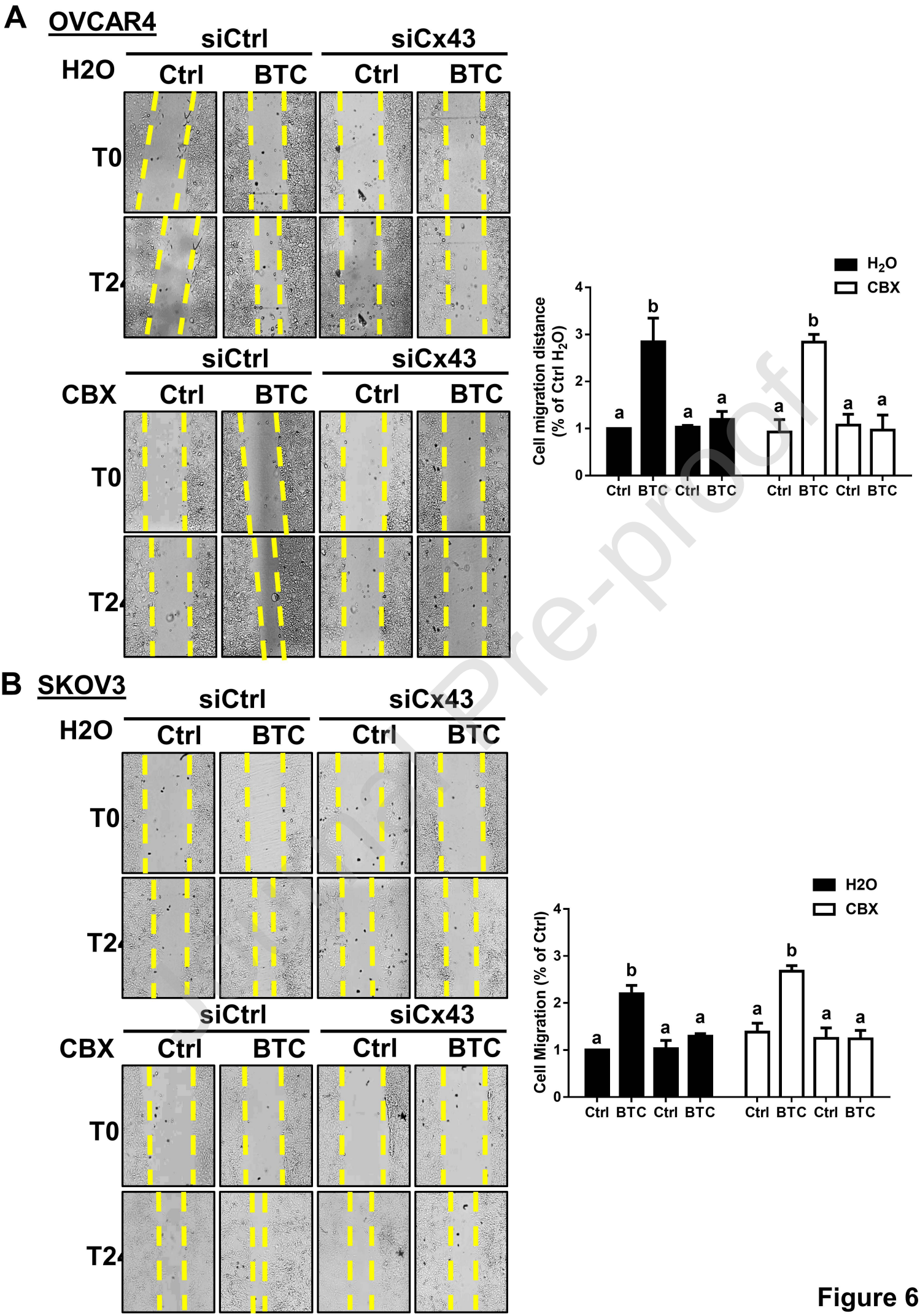

Figure 6