

Integrative Networks by BRWD3 Regulates Cytoskeleton Organization and Cell Motility

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Abstract

Background

Eukaryotic cytoskeleton forms and keeps cell shape, transports intracellular particles and organelles, determines cell motility and other important cellular events. A large number of regulators of cytoskeleton organization have been identified, but the detailed regulatory mechanism still remains obscure. Previous reports suggest that BRWD3 may be a regulator of cytoskeleton organization in *Drosophila melanogaster*, and influences cell shape. Therefore, we investigated the molecular network of BRWD3 regulating cytoskeleton organization.

Results

In this study, we observed the alteration of cell shape, cell motility, and proliferation when BRWD3 was knocked down in MCF-7 and MDA-MB-231 cell lines. The cells were rounded, cell motility decreased when BRWD3 was knocked down. Using chromatin immunoprecipitation combining with sequencing, we found that BRWD3 influenced the cytoskeleton organization, cell shape, and cell motility through regulating expression of the cytoskeleton associative genes including ARF1, ABI2, ARPC3, ARPC1A, RHOC, MEF2C, and VIM.

Conclusions

A molecular network by BRWD3 is sketched to elucidate that BRWD3 may not only regulate actin filament but also regulate microtubule and intermediate filament-based cytoskeleton organization. These efforts provide an overview of a BRWD3 network regulating cytoskeleton organization, cell shape and motility, and allow a better understanding of cytoskeleton (re)organization and pathogenesis of mental retardation X-linked 93 and relative carcinomas.

Background

The dynamic organization of the eukaryotic cytoskeleton determines cell shape, transportation of intracellular particles and organelles, cell motility, and plays critical roles in embryonic development, homeostasis, and disorders [1]. Regulating cytoskeleton organizations have been focused on in the models including *C. elegans* [2,3] and *Drosophila* [4,5]. A large number of regulators have been identified, such as FAM40A, FAM40B, Rac2, and BRWD3 [5]. Both FAM40A and FAM40B regulate stress fibers and angiogenic loop formation [6]. Rac2 encoding small GTPase regulates the actin cytoskeleton and controls salivary gland migration [7]. We still don't know about the role of BRWD3 in the cytoskeleton organization.

BRWD3 (Bromodomain and WD Repeat Domain Containing 3), also is known as FLJ38658 or BRODL. Mutant *dBRWD3* (also named as ramshackle in *Drosophila*) leads to dying earlier during the development [8,9] or to disrupt dendritic morphogenesis and sensory organ differentiation [10]. In human, abnormal

BRWD3 expression causes intellectual disability [11-19], leukemia [20], breast cancer [21] and head & neck cancer [22]. The human BRWD3 containing several WD repeats and 2 bromodomain (BRD) repeats, belongs to bromodomain-containing proteins (BCPs) family [12]. The BCPs are involved in the formation of protein complex anchoring to and remodeling chromatin, histone modification, and transcription regulation [23,24]. BRWD3 is speculated as a chromatin modification factor [8] and transcription regulator [11,12,25]. In this study, we present a comprehensive analysis of how BRWD3 regulation on cytoskeleton organization, cell shape, and motility.

Results

Previously, the alterations of cell morphology and cytoskeleton organization are observed in PC3, S2R+, and HeLa cell lines when *BRWD3* was depleted [4, 5]. With the method of cell immunofluorescence, we observed that number of protrusion decreased, cellular shape rounded and smaller, and spread area decreased clearly when BRWD3 was knocked down in the MCF-7 and the MDA-MB-231 cell lines (Figure 1, supplementary Figure 1).

Alteration of cellular morphology, cytoskeleton, and adhesion state turnover contribute to cell motility. In the MCF-7 cell line, the scratch area healing rate was 75.88 ± 1.10 (Blank), 78.18 ± 0.64 (siRNA-control), 33.12 ± 2.16 (siRNA-*BRWD3*) (Figure 2). While in MDA-MB-231 cell lines, the scratch area healing rate was 93.69 ± 1.88 (Blank), 94.85 ± 1.30 (siRNA-control), 68.17 ± 2.64 (siRNA-*BRWD3*) (Figure 3). Consistent with the results of the scratch wound assay, the number of invaded cells has decreased significantly in both MCF-7 and MDA-MB-231 cell lines, comparing to untreated cells or siRNA-Control (Figure 4). To confirm the result above-mentioned and monitor cell growth, a real-time x-Celligence RTCA system was used. The cell motility decreased significantly in the si-*BRWD3* group in two cell lines (Figure 5A, B). However, no significant difference in cell growth was observed between the control and si-*BRWD3* groups (Figure 5C, D).

After the cytoskeleton alteration was observed in MDA-MB-231 cell lines when BRWD3 was knocked down, we resolved the regulation mechanism of BRWD3 on cytoskeleton organization and cell motility. ChIP-Seq was used to explore an integrative molecular network of BRWD3. The quality of the raw data was checked, and the clean data was matched to the Ensemble GRCh38.81. We identified 5491 ChIP-seq peaks ($-10 \times \log_{10} (p\text{-value}) > 50$). The 2939 unique genes were annotated and enriched with the GENEONTOLOGY (<http://geneontology.org/>). With the Fisher's exact test and the Bonferroni correction for multiple testing, the uniquely mapped genes were annotated with the biological process (GO: 0008150) and enriched. 264 genes were enriched with the [cytoskeleton organization](#) (GO: 0007010, $p = 3.4E-07$), and 131 genes were enriched with the [regulation of cytoskeleton organization](#) (GO: 0051493, $p = 0.00505$). 194 genes were enriched with the regulation of cell motility (GO: 2000145, $p = 0.0207$), and 156 genes were enriched with cell morphogenesis (GO: 0000902, $p = 0.0386$) (Figure 6). Six genes, including *NRP1*, *PTK2*, *ABL1*, *CDK5*, *EVL*, and *ARHGEF2*, were identified among the four groups, including regulation of cell morphogenesis, cytoskeleton organization, cell motility, and regulation of cytoskeleton organization.

From a ChIP-seq experiment, the identification of sites was enriched, and not be purified. To measure effective genes whose functions are enriched with the regulation of cytoskeleton organization, cell morphogenesis, and cell motility, and whose promoters are affinity with BRWD3, we analyzed the regulation relationship between BRWD3 and its binding genes using real-time quantities PCR. We excluded *ABL1* and *EVL* because they were bound to BRWD3, not through promoters. From the results of gene annotation, several critical genes related to cytoskeleton organization were also selected, including *ARF1*, *ABI2*, *ARPC3*, *ARPC1A*, *RHOC*, *MEF2C*, and *VIM*. When BRWD3 was knocked down in MCF-7 and MDA-MB-231 cell lines (Figure 7 A), the transcription level of most of the selected genes, including *PTK2*, *ARF1*, *ABI2*, *ARPC3*, *ARPC1A*, *RHOC*, *VIM*, *CDK5*, *MEF2C*, and *ARHGEF2*, were influenced significantly ($p < 0.05$) (Figure 7 B, C). These 10 genes might be important nodes with a close relationship between BRWD3 and cell shape, cytoskeleton organization, and cell motility.

Discussion

Previous reports suggest that BRWD3 be a regulator of actin filament and microtubules, based upon cell shape and actin phenotype alteration when *dBRWD3* was knocked down [4,5]. In this study, we have provided a novel insight into the molecular network of BRWD3, which influences actin filaments- and microtubule-based organization, cell shape and motility, and may lay the foundation for understanding the pathogenesis of complex disorders including mental retardation X-linked 93 and relative carcinoma phenotype.

Previously, *dBRWD3* encoding protein is identified as a dominant suppressor of hedgehog loss-of-function in the developing eye. Partial loss-of-function for the dBRWD3 leads to small rhabdomeres in photoreceptor cells. It is the first article to report that dBRWD3 function is involved in cell shape regulation in *Drosophila* [8]. The dBRWD3, clustered together with ZMYM6, Cdc42, WTIP, BRWD1, HYPC, and EPB41I4A, influences peripheral actin, microtubule process, and cell shape in *Drosophila* cell lines [4, 5]. However, how to regulate cytoskeleton organization remains obscure. In this study, we observed that alteration of protrusion structures, cell shape, and cell movements in either MCF-7 or MDA-MB-231 cell lines, when *BRWD3* was knocked down. Subsequently, the BRWD3 network regulating the cytoskeleton organization was screened with ChIP-seq. The genes selected in this study were enriched with the regulation of cytoskeleton organization and bound to BRWD3 through their promoters, including *PTK2*, *ARF1*, *ABI2*, *ARPC3*, *ARPC1A*, *RHOC*, *VIM*, *CDK5*, *MEF2C*, and *ARHGEF2*. The transcription level of these 10 genes was changed significantly when *BRWD3* was knocked down. During the cytoskeleton organization process, the proteins of ARPC3 and ARPC1A are involved in the formation of the actin-related protein 2/3 (Arp2/3) protein complex, and the proteins of VIM, ARHGEF2, CDK5, ARPC3 are involved in the process of polymeric cytoskeletal fiber. All of these molecules are involved in the regulation of actin polymerization and mediates the formation of branched actin networks. The collective results provided a potential possibility that BRWD3 was a modulator of cytoskeleton organization through regulating on actin filaments, microtubule, and intermediate filaments, and engaged extensive cross-talk among their organization.

Enriched with the term of cellular component, the proteins located at cell leading edge include VIM, ARHGEF2, CDK5, ARPC3, PTK2B, ARF1, and ABI2, and located in plasma membrane bounded region include VIM, ARHGEF2, CDK5, ARPC3, PTK2B, ARF1, ABI2, and RHOC. The proteins of CDK5, ARPC3, PTK2B, and ABI2 are located in the lamellipodium, and CDK5, ABI2 are located in filopodium. ABI2, belonging to Abelson interacting protein, which exists in a pentameric complex with WAVE1-3/PIR121/NAP1/HSPC300 in lamellipodia, regulates actin cytoskeleton dynamics underlying cell motility and cell adhesion.

RHOC regulates cell shape, attachment, and cell motility through promoting reorganization of the actin cytoskeleton. MEF2C is enriched in the regulation of microtubule-based cytoskeleton organization (GO: 0051493 and GO: 0001667), cell morphogenesis (GO: 0000904), and the ameboidal-type cell migration (GO: 0001667) of muscle cells and neuron. ARHGEF2 belongs to guanine nucleotide exchange factors and plays a role as a molecular switch through exchanging GDP for GTP to induce GTPase activation. Rho GTPases are critical molecules involved in the regulation of cytoskeleton organization and cell adhesions. VIM, a type III intermediate filament protein, maintains cell shape and integrity of the cytoplasm through stabilizing cytoskeletal interactions. With these results, we can speculate that BRWD3 maintains cell shape and motility as a platform to regulate organization of microtubule, actin filament, and intermediate filament.

Conclusions

In summary, the molecular network by BRWD3 may underlay the molecular mechanism on alteration of cytoskeleton reorganization, cell shape and motility. The cell shape changes due to microtubules contact the grid and tensile actomyosin cortex rounded-up underneath the plasm membrane [26]. Our observations support the view that the cytoskeleton should be considered as a unified system to exert its functions in the development and pathogenesis of complex disorders [27]. Although the view is supported by microscopic observation and cell motility assay in cultured living cells, the underlying precise mechanism still needs to study furthermore in cytoskeleton biology.

Methods

Cell culture and cell treatment

Human breast cancer cell lines (MCF-7 and MDA-MB-231) were purchased from the Cell Bank of China Academy of Sciences Type Culture Collection. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) plus 10% fetal bovine serum (Gibco, Vienna, Austria), 100 µg/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA). The cells were cultured at 37 °C in a humidified 5% CO₂ incubator. All transfection assays were carried out with Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific) referring to protocol. Opti-MEM (Invitrogen) was used as the transfection medium. RNA interference was carried out by transfecting 50 nM control and si-*BRWD3* respectively, from GenePharma Company (China). Silencer si-*BRWD3* sequence was followed as: GCAAACGUCAGCAUACUUATT. Scrambled siRNA

(UUCUCCGAACGUGUCACGUTT) was used as control. All these assays were carried out at least in triplicate.

Cell immunofluorescence

The cells were plated on slides. The next day, cells were infected to silence *BRWD3*. After 48 h of transfection, cells were fixed with 4% paraformaldehyde (PFA) for 10 min, permeabilized with 0.05% Triton-X for 3 min, and then blocked with 2% bovine serum albumin for 1 h. The antibodies including FITC-labeled Phalloidin (#40735ES75, YIASSEN Biotech Co.Ltd, China), β -tubulin (ab6046, USA) were used to show microfilament and microtubules in the cells. After sections were washed with phosphate-buffered saline (PBS), 3×5 min, the cells were then counter-stained with 4',6-diamidino-2- phenylindole (DAPI) for nuclei labeling. Images were collected by an Olympus fluorescence microscope at ×40 magnification.

Detection of protein expression with western blot

The cells were washed with cold PBS and then were harvested with RIPA buffer containing proteinase inhibitors. The protein was separated by 10% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, MA). After block for 1 h with 5% nonfat milk powder in PBS, the proteins were incubated o/n with anti-GFP antibody (#AG281, Beyotime Biotechnology, China) or anti-GAPDH (Sigma-Aldrich, USA). The membrane was washed with PBS with Tween detergent (PBST), 3×10 min. After incubation with horseradish peroxidase-coupled secondary antibodies for 1 h at room temperature, the membrane was washed with PBST again, 3×10 min. Then, the proteins were detected and exhibited with ECL reagents (Pierce, USA).

Cell motility

To measure the motility ability of cells, scratch wound assay and transwell assays were carried out. For scratch wound assay, breast cancer cells were cultured into a 6-well plate. After 24 hours of RNAi, the wound was scraped with a sterile pipette tip. The 6-well plate was washed twice to clean the cells fallen off. The cells were cultured with media as above-mentioned, except with 5% FBS. The images were obtained at 0 h, 24 h, and 48 h, and analyzed with Image J. The cell motility ability was measured and evaluated with the scratch wound healing area.

To do transwell assays, according to a previous report with modification [28], the mixture of serum-free media and matrigel (Corning, NY, USA) was put into the insert chamber (BD Falcon, Franklin Lakes, NJ, USA) overnight. The 5×10^4 of control cells (siRNA-Control) and treated (si-*BRWD3*) of MCF-7 or MDA-MB-231 were digested and re-suspended in serum-free DMEM and seeded into the insert. The bottom chamber was filled with 600 μ l of DMEM plus 10% FBS. After migration for 48 h, non-migration cells on the upper side of the filter were scraped off with a cotton swab, and cells on the bottom were fixed with 4% PFA for 10 min, and then stained with 0.2% crystal violet in 20% methanol for 15 min at room temperature, and washed twice with PBS, 2×5 min. The migration ability of cells was evaluated by

counting the number of invasive cells on the bottom of the inserts with a light microscope. The assay was performed in triplicate and was repeated at least three times.

Cell proliferation and invasion detected with the xCELLigence™ viability assay

The xCELLigence® RTCA DP system (Roche Applied Science, Penzberg, Germany) was used to automatically and continuously monitor live cell proliferation, growth, viability, migration, and spreading with characters of non-invasive, close to physiological status, a higher degree of accuracy, and lab free [29, 30]. The cells transfected with siRNA-Control or si-*BRWD3* were seeded on an E-plate-16 (ACEA Biosciences, San Diego, CA) at the optimal cell density (4×10^4 cells) with 100 μ l DMEM media without FBS for proliferation assay. Cells' growth was recorded in real-time every 30 min. The cell growth status was exhibited with normalized cell index values. The assays were repeated three times.

As previously reported [29], the mixture of serum-free media and matrigel (Corning, NY, USA) was put into the upper chamber of CIM (cellular invasion/migration)-Plate 16. After 4 h, the cells transfected with siRNA-Control or si-*BRWD3*, 4×10^4 cells of MCF-7 or MDA-MB-231 were digested and re-suspended in serum-free DMEM and seeded into the insert chamber with FBS- free DMEM. The bottom chamber contained 165 μ l of DMEM plus 10% FBS. The xCELLigence® RTCA DP system was used to monitor cell migration in real-time every 30 min. The difference between siRNA-Control and si-*BRWD3* was shown with a normalized cell index. All data (including from the growth monitor assay) were expressed as mean $\pm$ SD. Statistical analysis was performed with two-way ANOVA. Statistically significant was set as $p < 0.05$.

Chromatin immunoprecipitation combining with Sequencing (ChIP-seq) assay

Construction of plasmids and Cell transfection

No antibody was available for ChIP or even for immunoprecipitation for BRWD3, thus ChIP assay was performed with anti-GFP antibody (#AG281, Beyotime Biotechnology), which had been used in ChIP or immunoprecipitation assays [31, 32]. The EGFP from pEGFP-N1 (Clontech) was amplified with PCR, and then the amplicon was inserted into the pcDNA3.1 (+) vector (Invitrogen, Thermo Fisher Scientific) to construct pcDNA3.1-EGFP. Because of the large molecular weight of BRWD3 (203.5KDa), it's very difficult to introduce the *BRWD3* into cell lines. So the sequences (3691-5815 bp) encoding two BRD repeats of BRWD3 was amplified with PCR and inserted into pcDNA3.1-EGFP to construct pcDNA3.1-2BRD (BRWD3)-EGFP. The primers and reaction conditions were all listed in supplementary table 1. Both the plasmid sequences were confirmed with the Sanger sequencing. Both pcDNA3.1-EGFP and pcDNA3.1-2BRD (BRWD3)-EGFP were introduced into MDA-MB-231 cells when the cell was grown to 50-60% confluence respectively. Transfection was confirmed by the assays of cell immunofluorescence and western blot. The plasmid of pcDNA3.1-2BRD (BRWD3)-EGFP was used as bait to immunoprecipitate specific DNA fragments binding to BRWD3 through 2BRD, and the pcDNA3.1-EGFP was used as the matching control.

ChIP assay The ChIP was carried out referenced to a previous report [33]. Briefly, when the confluence of the cell transfected with target plasmids reached 80-90%, the cells were incubated with 1% formaldehyde in 1×PBS (final concentration of 0.75%) at room temperature for 10 min to chemically cross-link DNA to 2BRD(BRWD3). Glycine (final concentration of 125 mM) was added to quench the cross-link reaction. Washed twice with 1×ice-cold PBS, and harvested in cold lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.0, 1 µg/ml aprotinin, 10 µg/ml leupeptin, 50 µM iodoacetamide) containing 1 mM fresh phenylmethylsulfonyl fluoride (PMSF). The cell lysate was plated on ice and sonicated to 200-1,000 bp. The supernatant was obtained from the mixture with centrifuge for 10 min at 10,000 rpm at 4°C and was diluted to 2 A₂₆₀ units/ml in 1×RIPA buffer containing proteinase inhibitors. 200 µL mixture was put aside as input. The lysates were pre-cleared for 2 h at 4°C by the protein A/G-Sepharose-salmon sperm competitor DNA. Then the supernatant was incubated with anti-GFP (#AG281, Beyotime Biotechnology, China) and 50 µL protein G sepharose (GE Healthcare, Japan) overnight at 4°C. The resin was centrifuged, 3000 rpm for 1 min and washed three times with Low Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), High Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 500mM NaCl), LiCl Immune Complex Wash Buffer (0.25 M LiCl, 1% IGEPAL CA630, 1% deoxycholic acid sodium salt, 1mM EDTA, 10 mM Tris, pH 8.1). The beads were washed at 4°C for 5 min at every step above-mentioned. The complexes were eluted twice from beads with 250 µl elution buffer (1% SDS, 0.1 M NaHCO₃). 20 µl NaCl (5 M) was added into the supernatant (~500 µl) to reverse cross-link overnight at 65°C.

Both immunoprecipitated DNA and input DNA were purified with the QIAquick® PCR purification kit (#28104, QIAGEN, Germany). The DNA concentration was measured with QUBIT dsDNA HS ASSAY KIT (Q32851, ThermoFisher, USA), and total DNA is required to be more than 10 ng.

HiSeq 2500 Illumina sequencing All samples were performed a conventional procedure, including end repair, adaptor ligation, size selection, amplification with PCR, enriched with AMPure XP beads, to construct library expressing 2BRD (BRWD3)-EGFP and control. The quality of libraries was evaluated with a 2100 Bioanalyzer High Sensitivity DNA chip, and the quantity was evaluated with the KAPA kit (#KK4602, illumina). Finally, the samples were subjected to HiSeq 2500 Illumina sequencing in CapitalBio Technology (China).

The quality of the raw data was checked with the FastQC software. Clean data was matched to the human genome (Ensemble GRCh38.81) with the bowtie1 [34]. Peak calling of unique mapped reads was conducted with MACS14 [35]. Annotation of ChIP peak, comparison, and visualization were conducted with ChIPseeker [36]. All the sites binding to pcDNA3.1-2BRD (BRWD3)-EGFP were selected through eliminating the sites binding to pcDNA3.1-EGFP. All sequencing data including will be deposited in NCBI's Gene Expression Omnibus when the article is exhibited.

Annotation and enrichment of gene functions All Ensemble gene IDs of sites were converted into gene symbols, then performed gene function annotation and enrichment by utilizing the Gene Ontology[37]. The unique genes were submitted and analyzed in the database (Released 20200407). Fisher's Exact test

and the correction of Bonferroni for multiple testing were set and only items with Bonferroni-corrected for $P < 0.05$ were displayed and listed.

Quantitative Real-time Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

After the selection of the core genes with ChIP-seq, the transcription level was measured with qRT-PCR, when *BRWD3* was knocked down in MDA-MB-231 and MCF-7, to determine the interaction among *BRWD3* and its influencing genes. Total RNA was isolated with RNAsimple Total RNA kit (Tiangen, China), according to the manufacturer's protocol. Total RNA was reverse transcribed using PrimeScript™ RT Master Mix-Perfect Real Time kit (TaKaRa, Japan), following the instruction. QRT-PCR was carried out with SuperReal Color Premix (Tiangen, China) on LightCycler® 480 (Roche, German). *GAPDH* was selected as the reference gene for the normalized of all other gene expression results. All primers and reaction conditions were listed in the supplementary table 1. The relative expression levels were calculated by $2^{-\Delta\Delta Ct}$ ($\Delta Ct = Ct_{mRNA} - Ct_{GAPDH}$ RNA). The experiments were carried out at least in triplicate. The average of independent analyses for each gene was calculated. The data were shown as mean $\pm$ standard deviation (SD) of independent samples. SPSS 20.0 program was used to test significant differences through one-way analysis of variance (one-way ANOVA) and multiple comparisons. The value of a statistically significant difference was set as $p < 0.05$.

Abbreviations

BCPs: bromodomain-contain proteins

BRD: bromodomain

BRWD3: Bromodomain and WD Repeat Domain Containing 3

ChIP-Seq: chromatin immunoprecipitation followed by massively parallel DNA sequencing

GO: Gene Ontology

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not Applicable.

Availability of data and materials

All sequencing data including will be deposited in NCBI's Gene Expression Omnibus when the article is exhibited. The relative method are available when in need.

Competing interests

No conflict of interesting.

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Authors' contributions

YY, YW, HL and KF performed the cell culture and treatments, supervised by JH. YY and YW prepared the original paper. KJ and XC analyzed and interpreted the data. JL designed the project, funded and completed the manuscript.

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A few researches on molecular function associated with cytoskeleton organization were mentioned in this paper, but we have to summarize without list the contributors.

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Figures

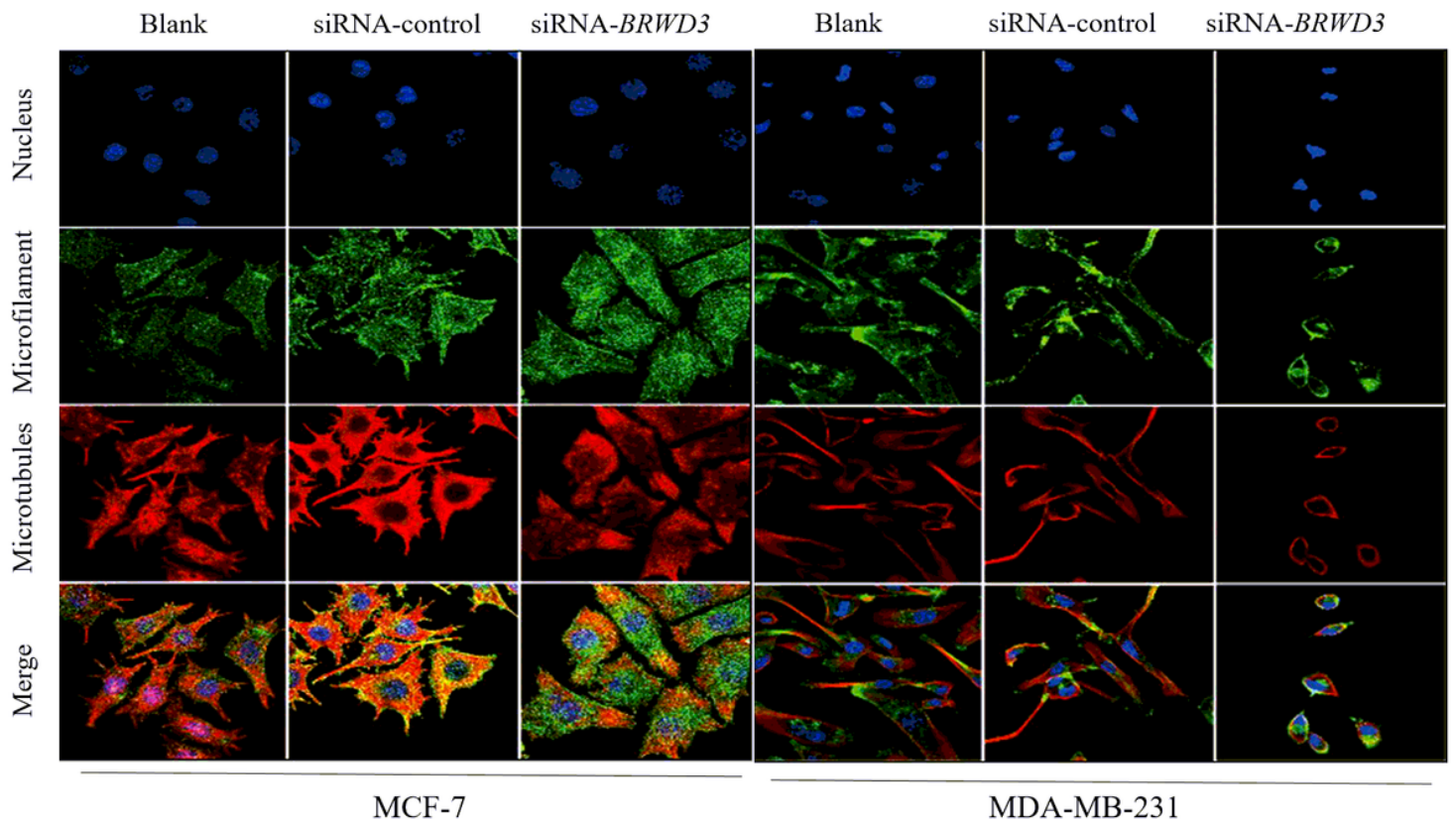


Figure 1

Alteration of cell shape observed with cell immunofluorescence (×60 magnification), when *BRWD3* is knocked down in MCF-7 and MDA-MB-231.

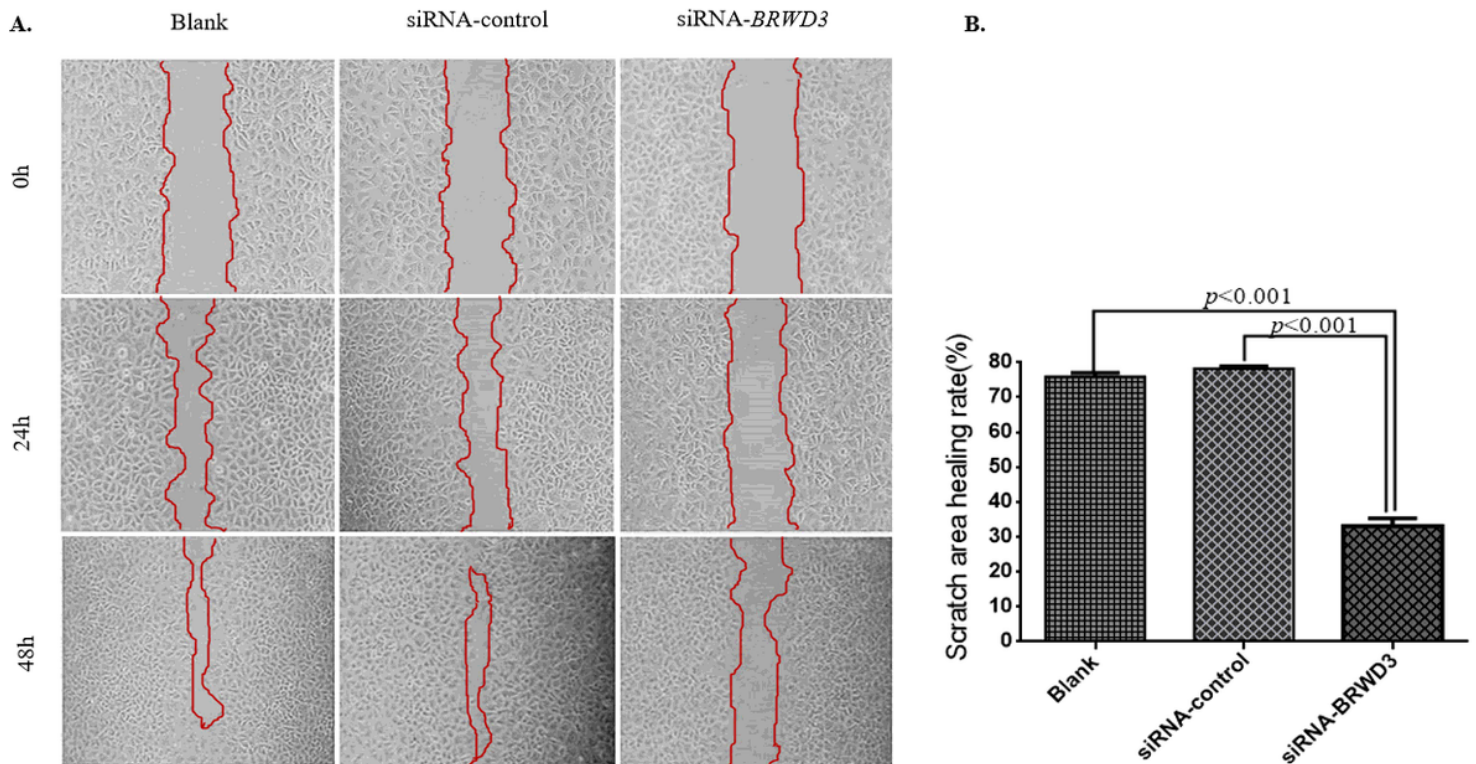


Figure 2

Alteration of cell motility detected with scratch healing, when BRWD3 is knocked down in MCF-7×10 magnification. (A). Movement of cells into wound is shown. The red lines indicate the boundary lines of scratch. (B). Alteration of cell motility is assessed by recover of the scratch. Data are shown as the mean±SD, from three independent experiments.

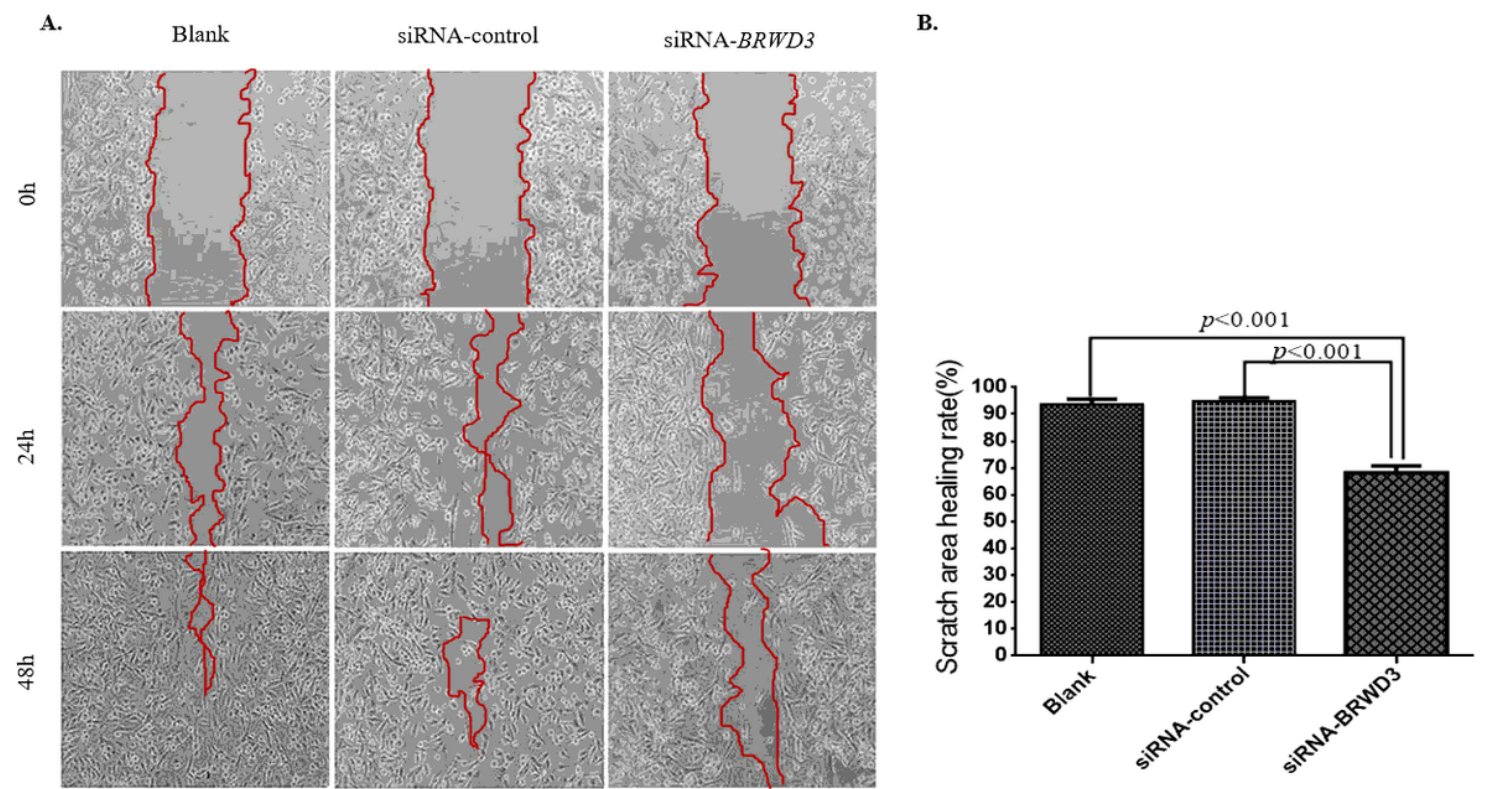


Figure 3

Alteration of cell motility detected with scratch healing, when BRWD3 is knocked down in MDA-MB-231 ×10 magnification. (A). Movement of cells into wound is shown. The red lines indicate the boundary lines of scratch. (B). Alteration of cell motility is assessed by recover of the scratch. Data are shown as the mean±SD, from three independent experiments.

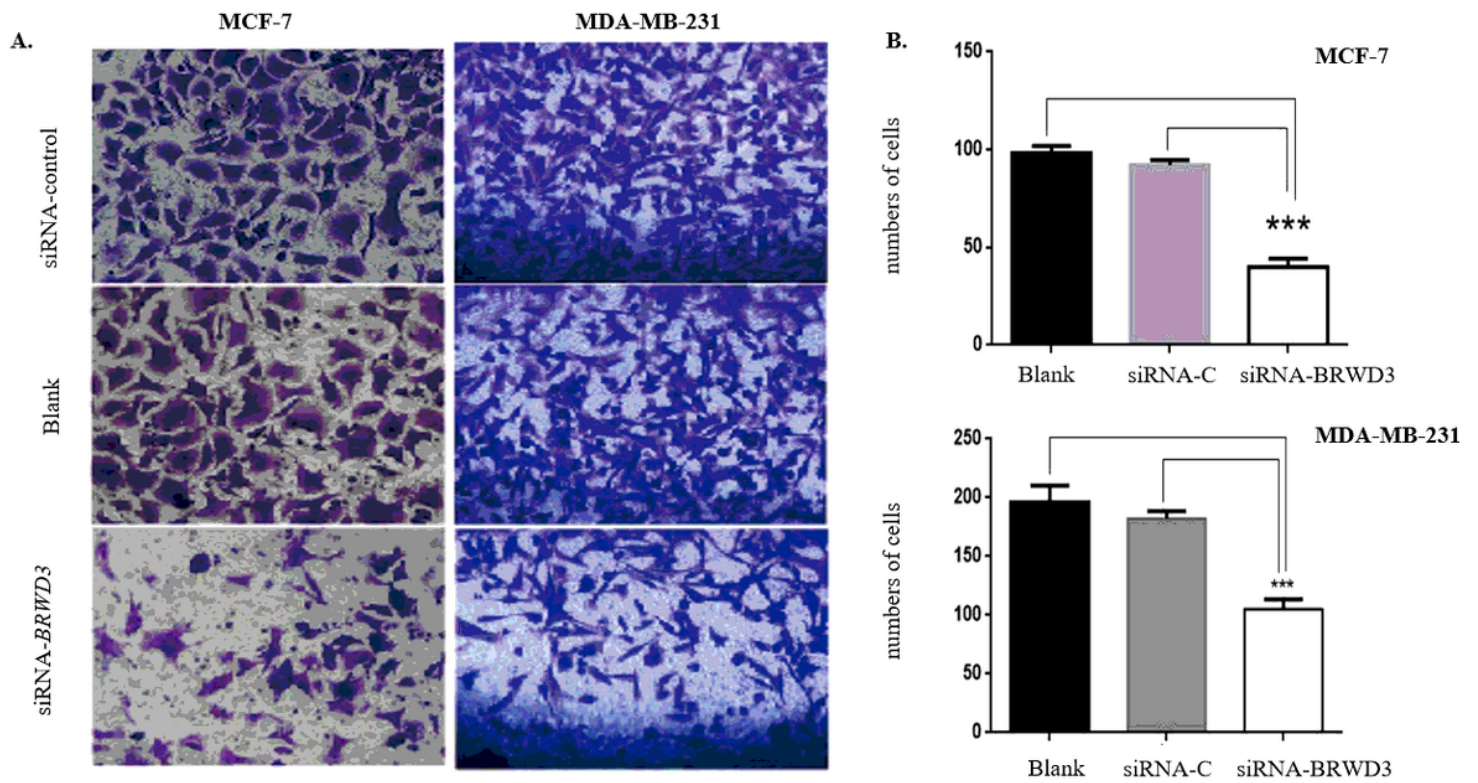


Figure 4

Transwell assay for cell migration (×40 magnification). (A) . Microscopic images of the cell migration at 48 h, following si-Control or si-BRWD3. (B) Migration assay data are shown as the mean±SD, from three independent experiments. $P^{***}<0.001$.

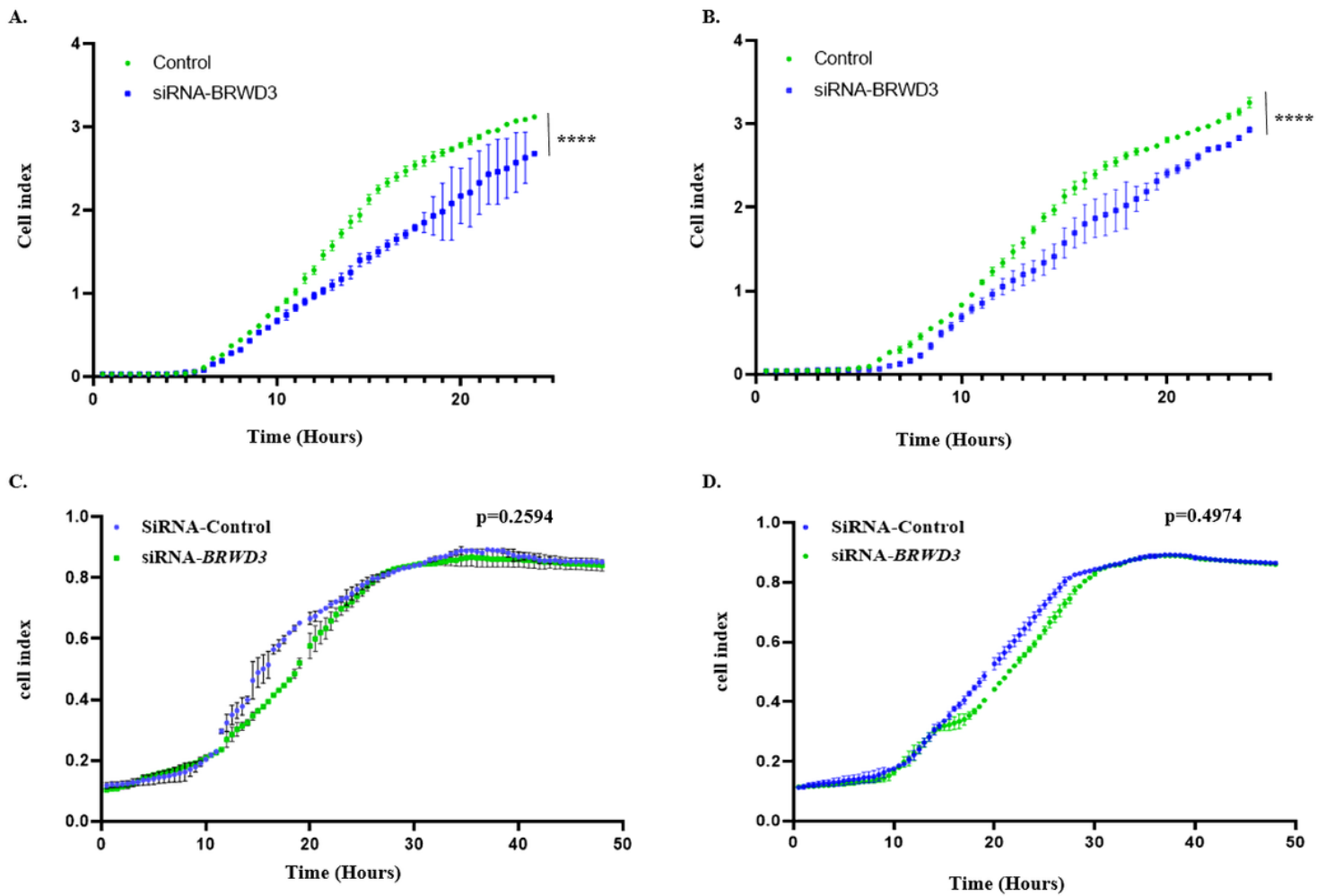


Figure 5

Real-time monitoring of cell migration and growth. Real-time analysis of MCF-7 (A) and MDA-MB-231 cell lines migration, and MCF-7 (C) and MDA-MB-231 (D) cell lines growth, when BRWD3 is knocked down. Cell index (A and B) correlates linearly with the number of cells migration through the pores of the CIM-Plate to chemo attractant FBS in the lower chamber after seeding. Cell index (C and D) shows time-dependent proliferation and growth during two day incubation. All graphs represent results from three independent experiments.

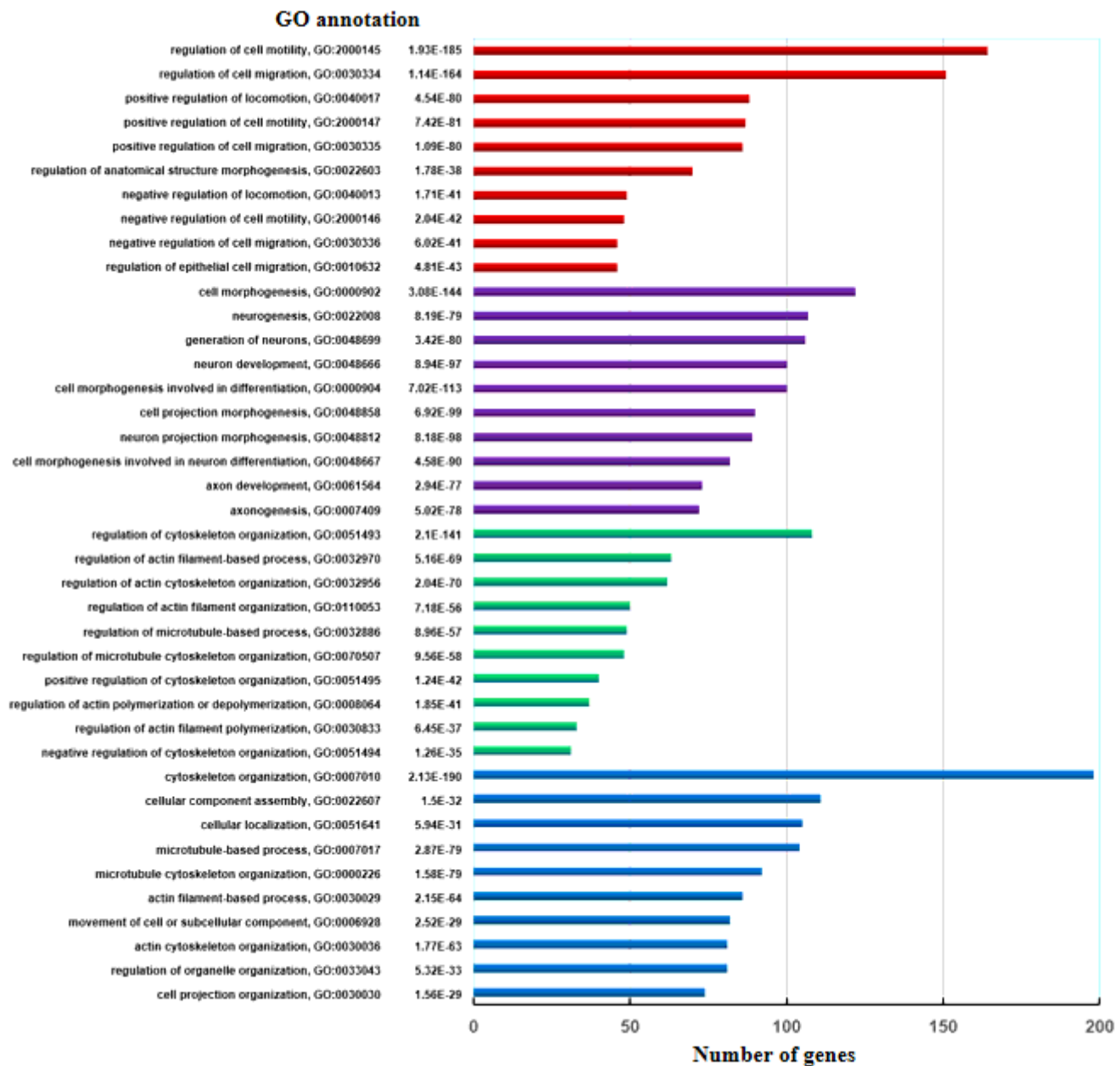


Figure 6

Enrichment with cytoskeleton organization, regulation of cytoskeleton organization, cell motility, cell morphogenesis. The directly relative items are selected from at top 20 in every group respectively. Red columns represent regulation of cell motility. Purple columns represent regulation of cell morphogenesis. Green columns represent regulation of cytoskeleton organization. Blue columns represent association with cytoskeleton organization.

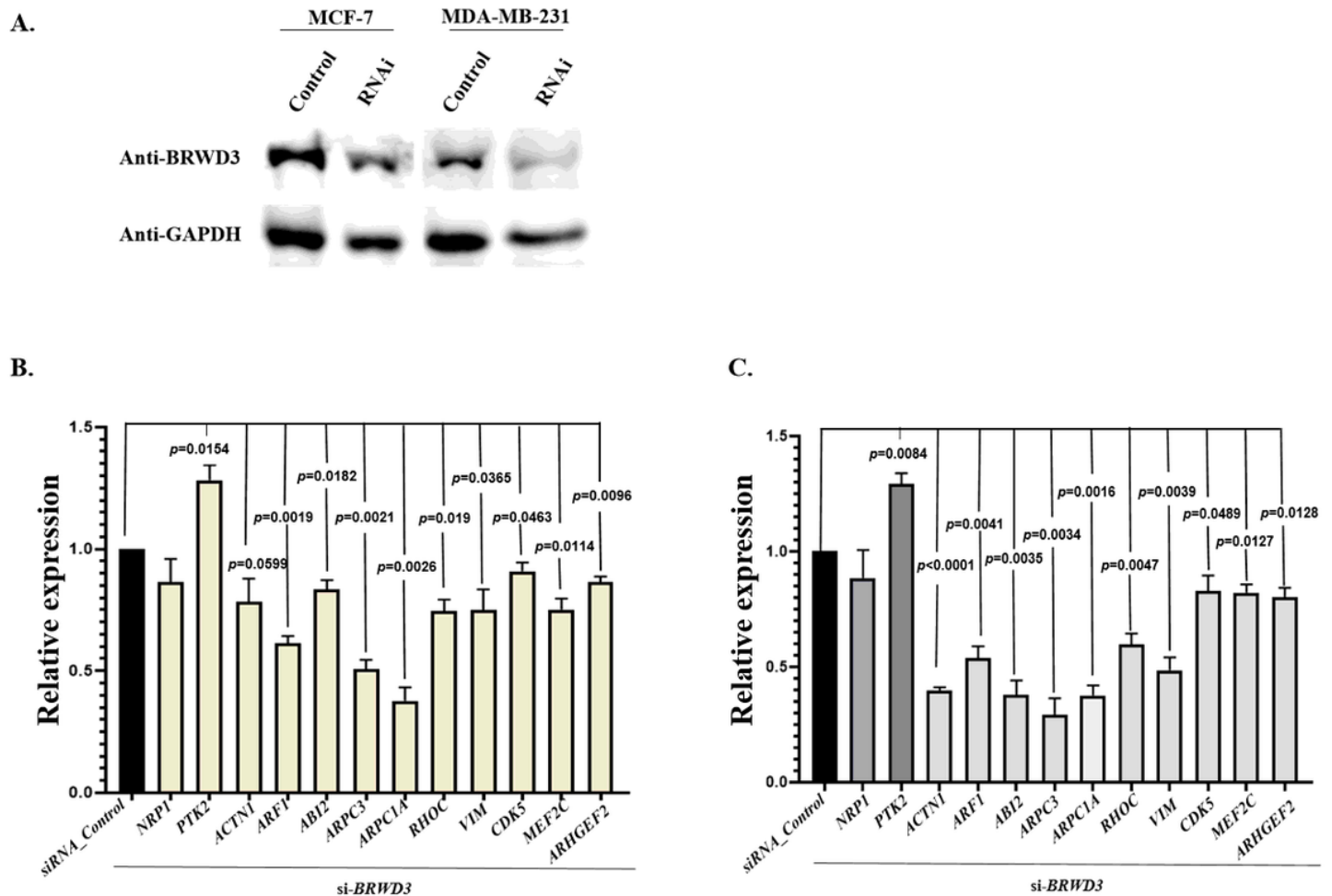


Figure 7

Relative expression of genes when BRWD3 was knocked down in MCF-7 and MDA-MB-231. (A). Protein expression detected with western blot, when BRWD3 is knocked down. (B). Transcription level alteration detected with Q-PCR, when BRWD3 is knocked down in MCF-7. (C). Transcription level alteration detected with Q-PCR, when BRWD3 is knocked down in MDA-MB-231.

Supplementary Files

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