c-Rel Promotes Invasion of Choriocarcinoma Cells via PI3K/Akt Signaling

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Short title

The role of c-Rel in choriocarcinoma

Key words

choriocarcinoma, c-Rel, invasion, migration, p65

Abstract

Objective: Choriocarcinoma is the most common epithelial cancer among gestational trophoblastic diseases (GTDs) and the mechanism of trophoblastic carcinogenesis is unknown. This study aimed to examine the expression of NF-κB family proteins in GTDs and placenta tissues, and the roles of c-Rel in choriocarcinoma. **Methods:** We examined the expression of NF-κB family proteins in normal placenta and hydatidiform mole tissues, and extravillous trophoblast (EVT) and choriocarcinoma cell lines by western blot and immunohistochemistry. Immunoprecipitation was performed to determine which proteins can bind with c-Rel in choriocarcinoma cells. To investigate the roles of c-Rel in choriocarcinoma, we examined the effects of c-Rel knockdown and overexpression on cell proliferation, migration, and invasion using small interfering RNAs and gene activation plasmid. **Results:** The expression of c-Rel was strong in choriocarcinoma and EVTs but very weak in villi of normal placenta and hydatidiform mole. Immunoprecipitation suggested that c-Rel heterodimerizes with p65 in choriocarcinoma. c-Rel knockdown reduced invasion, migration, and Akt phosphorylation in choriocarcinoma cells. c-Rel overexpression in choriocarcinoma increased migratory and invasive abilities, and the effects on invasion was inhibited by a PI3K inhibitor. **Conclusion:** These findings suggest that c-Rel might play a role in promoting the invasion of choriocarcinoma cells through PI3K/Akt signaling.

Introduction

Gestational trophoblastic disease (GTD) is a designated disease group comprising abnormal cellular proliferations of atypical placental trophoblasts, including hydatidiform mole, invasive mole, choriocarcinoma, placental site trophoblastic tumor (PSTT), and epithelial trophoblastic tumor (ETT). Hydatidiform mole is an abnormal conceptus caused by genetic fertilization disorders but it is not a tumor. The other four diseases are tumors that need chemotherapy and/or an operation for complete remission. Choriocarcinoma is the most common epithelial cancer in GTD and arises after any kind of pregnancy or invasive mole. The survival rate of choriocarcinoma is approximately 90% because chemotherapy for this disease is very effective, whereas in patients with multiple metastases or metastasis to the organs except the lung, it is difficult to achieve complete remission. Invasive mole is a benign tumor and in recent years, all patients have been cured by chemotherapy. However, invasive mole is considered as a pre-malignancy because 15–40% patients have metastases to the lungs and the vagina [1, 2] and 1–3% develop choriocarcinoma after having invasive moles [3].

The mechanism of carcinogenesis of choriocarcinoma has not been revealed, although some oncogenes, tumor suppressor genes, and molecular regulators were reported to be up-regulated or down-regulated in choriocarcinoma tissue compared with normal placenta or hydatidiform mole tissues [4-7]. *KRAS* mutations, which are common in most cancers, have not been observed in any studies using samples of choriocarcinoma tissue [8]. In normal human placenta, cytotrophoblasts are trophoblast stem cells and can differentiate into syncytiotrophoblasts and extravillous trophoblasts (EVTs). Syncytiotrophoblasts and cytotrophoblasts form villi, which play an important role in exchanging oxygen and nutrition between mothers and fetuses. In normal placentation, EVTs invade into the myometrium and replace the endothelial cells of uterine arteries to maintain sufficient blood flow from mothers to fetuses. Although the invasion of EVTs is contained within one-third of the myometrium in normal placentation, EVTs have some similar characteristics to those of cancer cells, especially regarding their invasive ability. Studying the mechanism of EVT invasion may lead us to discover key factors underlying the development of choriocarcinoma.

NF-κB is a transcription factor with five protein subunits: RelA (p65), RelB, c-Rel, NF-κB1, and NF-κB2. The subunits of NF-κB form homo- or heterodimers in various

combinations and act on their target genes. The N-terminal of each member has a conserved Rel homology domain for dimerization and DNA binding [9]. Although NF-κB complexes exist in all cells, they are generally inactivated by binding to another protein called inhibitor of NF-κB (IκB). To release active NF-κB dimers, activation of the IκB kinase (IKK) complex and subsequent IκB phosphorylation and degradation are necessary. Signals that can induce NF-κB by activating IKK include inflammatory cytokines, immune receptor engagement, bacterial products, viral proteins, and cell stresses such as DNA damage. The main role of NF-κB is to regulate the inflammatory response. NF-κB target genes encode proteins and microRNAs that regulate a wide range of target genes, including those encoding cytokines, chemokines, and their receptors, together with those that regulate cell survival, proliferation, adhesion, and the cellular microenvironment [10]. It has become clear that NF-κB signaling also has a critical role in the development and progression of cancers, such as hepatocellular carcinoma, colorectal cancer, breast cancer, prostate cancer, and squamous cell carcinomas of the head and neck [11].

c-Rel is encoded by *REL* and has been reported to play roles in the development of human diseases as well as the functions of normal cells, such as lymphoid cells. c-Rel is involved in inflammatory bowel disease, rheumatoid arthritis, cardiac and skin fibrosis, and the malignant progression of solid tumors, such as breast cancer, colitis-associated adenoma, gastric cancer, head and neck cancer, and pancreatic cancer [12]. Boucraut *et al.* showed that c-Rel might heterodimerize with NF-κB1 and bind DNA in the nuclei of EVTs and Jar, which is a choriocarcinoma cell line, but not in those of syncytiotrophoblasts [13]. Their study used isolated trophoblasts from human placentas of the first trimester and suggested that trophoblasts having an invasive phenotype expressed c-Rel as a DNA-binding factor. However, the aim of that study was to investigate the molecular mechanism that down-regulates the classical MHC class I genes and up-regulates *HLA-G* expression in trophoblasts, and no previous study has examined the expression and functions of NF-κB family proteins in choriocarcinoma.

In the current study, we investigated the expression of NF-κB family proteins in trophoblastic cells and tissues of GTDs and normal placenta, and we examined the roles of c-Rel in choriocarcinoma.

Material and methods

Cell lines and culture

Human choriocarcinoma cell lines (Jar and Bewo cells) were purchased from the American Type Culture Collection (Manassas, VA, USA). The human EVT cell line HTR-8/SVneo was kindly provided by Dr. Charles H. Graham (Queen's University, Kingston, ON, Canada) [14]. The human choriocarcinoma cell lines NaUCC (CC)-1 and CC-4 were previously established in our laboratory [15]. All cell lines were cultured in PRMI1640 (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C and 5% CO_2.

Sample collection

Placentas of first trimester (8-13 weeks, n=3) were obtained from women undergoing elective pregnancy termination. Placentas of late second trimester and third trimester (26-40w, n=4) were collected from women undergoing selective cesarean section before the onset of labor pain. The GTD tissues, including hydtidiform mole (n=3) and choriocarcinoma (n=3), were obtained from patients who underwent surgical treatment and the specimens were classified based on their histopathological characteristics. Informed consent was obtained from individual patients for the use of their placental samples and molar tissue. These tissue samples were washed with phosphate-buffered saline (PBS), frozen in liquid nitrogen immediately after removal, and then stored at −80 °C until protein extraction. This study was approved by the ethics committee of Nagoya University Graduate School of Medicine.

Antibodies

Rabbit monoclonal antibodies against p65 (#8242), RelB (#4922), NF-κB1 (p105/p50, #12540), NF-κB2 (p100/p52, #3017), phospho-Akt (Ser473, #4060), phospho-ERK1/2 (Thr202/Tyr204, #4376) phospho-Bcl-2 (Ser70, #2827), cleaved PARP (Asp214, #5625), and cleaved caspase-3 (Asp175, #9664), rabbit polyclonal antibodies against c-Rel (#4727), ERK1/2 (#9102), and Bcl-xL (#2762), and a mouse monoclonal antibody against Akt (#2966) were purchased from Cell Signaling Technology (Danvers, MA, USA). A mouse monoclonal antibody against PTEN (A2B1, sc-7974) was purchased from Santa

Cruz Biotechnology (Dallas, TX, USA). A mouse monoclonal antibody against Bcl-2 (610538) was purchased from BD Biosciences (Franklin Lakes, NJ, USA).

Western blot analysis

Cells and tissue samples were homogenized in radioimmunoprecipitation assay lysis buffer (Millipore, Bedford, MA, USA). After centrifugation at 15,000 × *g* for 20 min, the supernatant was obtained. Thirty micrograms of protein extract was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel, transferred onto a nitrocellulose membrane, and immunoblotted with antibodies. Immunoreactive proteins were stained using an enhanced chemiluminescence (ECL) detection system (Amersham, Arlington Heights, IL, USA). The blots were probed with an antibody against β-actin (AC-15, Sigma-Aldrich) as the loading control.

Immunoprecipitation

For immunoprecipitation, 1 mg of protein was extracted from each sample tissue. After incubation with 4 μl of c-Rel antibody overnight at 4 °C, immune complexes were collected with 100 μl of protein G-Sepharose 4EF beads (GE Healthcare, Buckinghamshire, UK). The complexes were released by boiling in sampling buffer without a detergent, separated by SDS-PAGE on a 10% polyacrylamide gel, transferred onto a nitrocellulose membrane, and immunoblotted with antibodies. Immunoreactive proteins were visualized using ECL.

Immunohistochemistry

For immunohistochemical studies, formaldehyde-fixed and paraffin-embedded tumor tissue sections were deparaffinized and boiled in target retrieval solution at pH 9 (Dako, Glostrup, Denmark) for 30 min. Subsequently, tissue sections were washed with PBS, blocked with blocking reagent (Dako), and incubated overnight at 4 °C with appropriate primary antibodies diluted in antibody diluents (Dako). Tissue sections were then washed, treated with 5% hydrogen peroxide/ethanol solution for 15 min at room temperature, and incubated at room temperature for 30 min with horseradish peroxidase–conjugated anti-rabbit IgG secondary antibodies (EnVision System; Dako), followed by signal detection using 3,3′-diaminobenzidine solution (Dako).

Gene silencing

To investigate the roles of c-Rel in choriocarcinoma, we used predesigned small interfering RNAs (siRNAs) (SC-29857, Santa Cruz Biotechnology) together with non-targeting siRNAs (Nippon Gene Material, Toyama, Japan) as a control (control siRNAs: 5′-GGAUUAUUACGCAGUUAAATT-3′ and 5′-UUUAACUGCGUAAUAAUCCTT-3′). Another siRNAs of c-Rel (si-Rel1 and si-Rel6) (FlexiTube GeneSolution GS5966 for REL), and non-targeting siRNAs (SI03650325) were purchased from QIAGEN (Venlo, Netherlands). Jar and Bewo cells were grown in 35 mm plates to 60% confluency and then transfected with the siRNAs using Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. At 48 h after exposure to the siRNAs, whole-cell lysates and total RNA extracts were prepared for western blotting analysis and reverse transcription polymerase chain reaction (RT-PCR), respectively.

RNA extraction and quantitative RT-PCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen) and an RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. cDNA was synthesized from total RNA using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). Quantitative RT-PCR was performed using a SYBR Premix Ex Taq II kit (TaKaRa Bio, Otsu, Japan). We used the following primers for *REL*: forward primer, 5′-TGACCTCAATGTGGTGAGACTGTGT-3′; reverse primer, 5′-CATCTCCTCCTCTGACACTTCCACA-3′ and the following primers for *GAPDH*: forward primer, 5′-CGGGAAACTGTGGCGTGAT-3′; reverse primer, 5′-ATGCCAGTGAGCTTCCCGT-3′. The PCR profile comprised an initial incubation at 95 °C for 10 s, followed by 45 cycles of denaturation at 95 °C for 5 s and annealing at 58 °C for 30 s, and a final extraction step at 72 °C for 30 s.

In vitro cell proliferation assay

The proliferation assay was performed as previously reported [16]. Jar and Bewo cells were transfected with siRNA 24 h before seeding for the proliferation assay, and cells (5×10^3) were plated in 100 μl of medium in 96-well plates and incubated for 72 h at 37 °C.

Cell viability was determined using the modified tetrazolium salt (MTS) assay using the Cell Titer 96 AQueous One Solution Proliferation Assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The mean values of three independent experiments, each performed in eight replicate wells, were used for statistical analyses and figure construction.

Transwell migration and invasion assay

Migration and invasion assays were performed as previously reported [17]. Jar and Bewo cells were transfected with siRNAs 24 h before seeding for the migration and invasion assays, and both assays were performed after 24 h incubation. The number of cells was counted under a microscope at ×40 magnification. Data were obtained from three individual experiments performed in triplicate.

Wound healing assay

Jar and Bewo cells were seeded in 35-mm tissue culture dishes, and confluent monolayers after siRNAs transfection were scratched with a 200-μl disposable plastic pipette tip and allowed to migrate toward the wound. Migration distances were measured using a microscope at 0 and 24 h after scratching.

Zymography

Cells were incubated with serum-free medium for 24 h after siRNAs transfection. The conditioned medium was mixed with the same volume of 2× non-reducing sample buffer (0.125 M Tris-HCl, pH 6.8; 20% glycerol; 4% sodium dodecyl sulfate; and 0.005% bromophenol blue) and loaded onto 10% polyacrylamide gels containing 0.1% gelatin. The proteins were separated by electrophoresis at a constant voltage of 100 V for 150 min. Gels were washed to remove sodium dodecyl sulfate and incubated overnight at 37 °C in a solution of 50 mM Tris and 10 mM $CaCl_2$ to allow the enzymes to digest the gelatin. Gels were stained with 0.5% Coomassie Brilliant Blue and destained in 10% acetic acid/30% methanol.

CRISPR activation plasmid transfection

Control CRISPR activation plasmid (sc-437275), and c-Rel CRISPR activation plasmid (sc-400478-ACT) were purchased from Santa Cruz Biotechnology. Transfection was performed according to the manufacturer's instructions. In brief, Bewo cells were grown in 35 mm plates to 80% confluency and then transfected with these plasmid using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. After 48 h, cells were selected with puromycin for a week and then analyzed for efficiency of activation. MTS assay, migration assay and invasion assay were performed as the same as transfection of siRNAs.

Inhibition of phosphorylation of Akt

To examine the effect of suppression of phosphorylation of Akt, LY294002 (Wako Pure Chemical Industries, Ltd., Osaka, Japan), a PI3K inhibitor, was added to cells at 10μM. Whole cell lysate was collected from the cells after culture with LY294002 for 30 min. In invasion assays, LY294002 was added to the medium of the upper chambers at 10μM and the assay was performed after 6 h incubation. Data were obtained from three individual experiments performed in triplicate.

Statistical analysis

Data were expressed as the mean ± standard deviation. For data obtained from *in vitro* experiments, statistical comparisons between groups were performed using Student's *t*-test for two independent samples and the one-way ANOVA with Bonferroni corrections for multiple comparisons. Differences were considered significant when $p < 0.05$.

Results

Expression levels of NF-κB family proteins in trophoblasts and heterodimerization of c-Rel with p65 in choriocarcinoma

To evaluate the expression levels of NF-κB family proteins in various kinds of trophoblasts, we first performed western blot analyses in normal placenta and hydatidiform mole tissues, and in EVT and choriocarcinoma cell lines. The expression of p65 and NF-κB1 was strong in all kinds of trophoblasts, and that of RelB and NF-κB2 (p52) was stronger in normal trophoblasts and hydatidiform mole tissues than in choriocarcinoma cell lines (fig. 1a and suppl. fig. 1). On the other hand, the expression of

c-Rel was strong in all choriocarcinoma cell lines and an EVT cell line but very weak in normal placenta and hydatidiform mole tissues (fig. 1a and suppl. fig. 1), and this expression pattern was different from that of the other four NF-κB family proteins. These results suggest that c-Rel may express strongly in cells with invasion ability. Next, we performed immunoprecipitation experiments using Jar and Bewo cells to determine which proteins can bind with c-Rel, and our results showed that c-Rel heterodimerized with p65 in choriocarcinoma cells (fig. 1b–e).

Immunohistochemical expression of c-Rel and p65 in choriocarcinoma, hydatifirom mole and normal placenta

We examined the expression of c-Rel and p65 in choriocarcinoma tissue, hydatidiform mole and normal placenta by immunohistochemistry. Cytoplasmic and focal nuclear staining of both proteins was found in choriocarcinoma tissues (fig. 2a, b) and in metastases of choriocarcinoma in the spleen (fig. 2c, d). The tissue of intraplacental choriocarcinoma in 38 gestational weeks showed that the staining of c-Rel in choriocarcinoma was strong and weak in normal villi (fig. 2a, b). Immunohistochemistry showed that c-Rel was expressed strongly in choriocarcinoma (fig. 2a, c) but very weakly in normal villi (fig. 2a, g, k) and hydatidiform mole (fig. 2e); on the other hand, the expression of p65 in choriocarcinoma (fig. 2b, d) was the same as that in normal villi (fig. 2b, h, l) and hydatidiform mole (fig. 2f). The expression of c-Rel and p65 was strong in EVTs of the placenta in the first trimester (fig. 2i, j). These results were consistent with the results of our western blot experiments.

Knockdown of c-Rel expression in Jar and Bewo cells

To investigate the functions of c-Rel in choriocarcinoma, we used siRNA (SC-29857) to establish a c-Rel knockdown model using Jar and Bewo cells. We confirmed that the expression of c-Rel protein was effectively decreased in c-Rel siRNA transfectants (si c-Rel) compared with that in control siRNA transfectants (mock) at 48 h after transfection (fig. 3a). Quantitative RT-PCR analysis also indicated that treatment with c-Rel siRNAs in Jar and Bewo cells suppressed the mRNA expression of *REL* to 14.8% and 27.4%, respectively, compared with mock (fig. 3b). Morphologically, there were no significant changes between mock and cells treated with si c-Rel (fig. 3c).

Effects of c-Rel knockdown on cell proliferation, migration, and invasion

We assessed the effects of c-Rel knockdown on cell proliferation by MTS assay. The proliferation of si c-Rel cells of Jar and Bewo was lower than that of the mock by 16.0% and 8.9% at 48 h, and 28.2% and 18.3% at 72 h after seeding the siRNA-transfected cells, respectively (fig. 3d). However, a significant change in proliferation between si c-Rel and mock was found only in Jar cells ($p < 0.01$). Migration and invasion assays revealed that c-Rel knockdown significantly reduced the migratory and invasive abilities of both Jar and Bewo cells (fig. 4a, b, $p < 0.01$). The attenuating effect of c-Rel knockdown on cell migration was stronger in Jar cells than in Bewo cells (15.6% and 40.4%, respectively), and a similar result was seen for cell invasion (13.4% and 58.3%, respectively). In addition, we found that si c-Rel treatment reduced the degree of directional cell migration in culture dishes as assessed by wound healing assay (fig. 4c).

We used another siRNAs of c-Rel (si-Rel1 and si-Rel6) to confirm the effects by c-Rel suppression in choriocarcinoma after confirming the suppression of c-Rel expression by western blot and RT-PCR (suppl. fig. 2a, b). Migration and invasion assays revealed that c-Rel knockdown significantly reduced the migratory and invasive abilities of both Jar and Bewo cells (suppl. fig. 2c, d, $p < 0.01$). These results showed that c-Rel knockdown led to decreased proliferation, migration, and invasion in choriocarcinoma cells.

Effects of suppressing c-Rel expression on the activities of MMP-2 and MMP-9

Multiple processes are involved in the invasion of trophoblasts and cancer cells, including the attachment of cells to the basement membrane and extracellular matrix (ECM) components, degradation of the ECM components, and subsequent migration through the degraded ECM components. MMP-2 and MMP-9 are type IV collagenases that are thought to be primary mediators of not only trophoblast invasion but also cancer invasion. We performed gelatin zymography and the results showed that Jar and Bewo cells predominantly secreted MMP-2. Knockdown of c-Rel did not significantly influence the expression of either the pro (72 kDa) or active (62 kDa) forms of this enzyme (fig. 5a).

c-Rel down-regulation led to inactivation of Akt and activation of PARP and caspase-3

In trophoblast migration and invasion, activation of the MAPK and PI3K signaling pathways plays an important role [18-20]. Therefore, we investigated whether the suppression of c-Rel expression induces the inactivation of these pathways. Western blot experiments showed that c-Rel knockdown reduced the expression of the phosphorylated form of Akt (phosphorylated at Ser 473) compared with mock in both Jar and Bewo cells (fig. 5b). However, the expression of the phosphorylated form of ERK (pERK) was not changed by c-Rel knockdown (fig. 5b). These results suggest that c-Rel increases proliferation, migration, and invasion via PI3K-AKT signaling in choriocarcinoma cells. PTEN is a tumor suppressor that plays a role in regulating cell survival via negatively regulating the PI3K/Akt pathway, and p65 down-regulates PTEN expression [21]. To examine whether c-Rel knockdown causes apoptosis in choriocarcinoma, western blot analysis was performed using antibodies of cleaved PARP and cleaved caspase-3. The expression levels of cleaved PARP and cleaved caspase-3 were increased (fig. 5c), suggesting that c-Rel knockdown leads choriocarcinoma cells to apoptosis. Our results suggested that c-Rel forms a heterodimer with p65. Taken together, these observations suggest that the knockdown of c-Rel may increase PTEN expression by either suppressing p65 or decreasing Akt phosphorylation. Bcl-2 and its mammalian homologs, such as Bcl-xL, are apoptosis inhibitors and c-Rel regulates the expression of Bcl-xL, but not that of Bcl-2 [22]. However, the expression levels of PTEN, Bcl-xL, and Bcl-2 were not changed by the down-regulation of c-Rel (fig. 5b), although apoptosis was increased.

c-Rel overexpression promoted migration and invasion abilities

To confirm the effects by c-Rel suppression, we transfected c-Rel CRISPR activation plasmid into Bewo cells (c-Rel act) as well as control CRISPR activation plasmid (mock). Western blot showed that c-Rel act increased the expression level of c-Rel compared to mock (fig. 6a). The proliferation of c-Rel act cells was higher than that of the mock by 16.7% at 72 h compared to mock cells, but the difference was not significant (fig. 6b). Migration and invasion assays revealed that c-Rel overexpression significantly promoted the migratory and invasive abilities of Bewo cells (fig. 5c, $p < 0.01$). The migrated cells and the invaded cells of c-Rel act were increased to 242.6% and 184.0% compared to those of mock, respectively.

Inhibition of phosphorylation Akt decreased invasion ability of c-Rel act

The results of exams using siRNAs of c-Rel suggested that c-Rel may increase migration and invasion abilities via activation of Akt. To determine whether activation of Akt is involved in promoting invasion in choriocarcinoma, we examined the effects of LY294002 on invasion in c-Rel act and mock cells. Treatment with LY294002 reduced the level of phosphorylation of Akt in both c-Rel act and mock, but did not influence on c-Rel expression (fig. 6d). Invasion assay showed that inactivation of Akt by LY294002 significantly reduced invasion abilities (fig. 6e, $p < 0.01$).

Discussion

This is the first report to analyze the expression and function of c-Rel in choriocarcinoma cells. Western blot and immunohistochemistry experiments showed that c-Rel was expressed strongly in choriocarcinoma and EVTs, but it was expressed weakly in villi of normal placenta and hydatidiform mole tissues. These results suggest that c-Rel may play a role in promoting the invasive ability of choriocarcinoma cells. EVTs have invasive ability as the same as cancer cells and invade into myometrium and decidua during the first trimester [23]. Although most studies on c-Rel have been related to immune cells and inflammation, some studies have reported a relationship between c-Rel and malignant characteristics in epithelial cell cancers. Transgenic mice with c-Rel expression driven by a mammary cell-specific promoter were demonstrated to develop mammary tumors [24]. In head and neck squamous cell cancer, it was suggested that c-Rel transports an alternative form of tumor suppressor p53 (ΔNp63) into the nucleus by heterodimerizing with it and thereby promotes cell proliferation and migration through p63 and c-Rel binding sites [25, 26]. These previous studies support that c-Rel might be one factor that can promote the malignant potential of choriocarcinoma cells.

Our results showed that c-Rel promotes migration and invasion in choriocarcinoma cells. Suppression of c-Rel expression significantly reduced the migration and invasion abilities of both Jar and Bewo cells and the exams using c-Rel act showed the supportive results. The assays for migration and invasion were performed at 24 h after siRNA transfection, and cell proliferation did not show a significant difference between the si c-Rel and the mock at 24 h in either cell line (fig. 3d). The differences in migration and

invasion were statistically significant and large enough to suggest that c-Rel knockdown led to decreases in the migration and invasion abilities of the cells. The results of the wound healing assay were consistent with those of the migration assay. Western blot experiments showed that the expression level of phosphorylated Akt was decreased by c-Rel suppression through siRNA treatment. These results suggest that c-Rel might promote migration and invasion via PI3K-Akt signaling in choriocarcinoma cells.

In terms of proliferation, c-Rel knockdown decreased the proliferation and induced apoptosis in Jar and Bewo. However, MTS assay using c-Rel act cells did not show a significant difference on cell proliferation during 72 h. The expression levels of Bcl-xL and Bcl-2 were not changed by c-Rel knockdown in this study, although a previous study reported that Bcl-xL was significantly up-regulated by c-Rel and p65 [22]. An inhibitor of NF-κB activity might antagonize this effect, but our study did not study the effects of using an inhibitor of NF-κB. Apoptosis by c-Rel knockdown in choriocarcinoma may be induced by tumor necrosis factor alpha (TNF-α)-triggered apoptosis pathway [27], because previous studies reported that c-Rel increased the basal rate of apoptosis, inhibited cell proliferation and protected cells against apoptosis induced by tumor necrosis factor alpha (TNF-alpha) [28, 29]. Further studies are needed to confirm the role of c-Rel on cell proliferation and the pathway of apoptosis.

Immunoprecipitation experiments showed that c-Rel heterodimerizes with p65 in choriocarcinoma cells. As shown in fig. 2, we observed a strong expression of c-Rel and p65 in choriocarcinoma cells, mostly in the cytoplasm and slightly in the nuclei. NF-κB family members form homo- or hetero-dimers and are inactivated in the cytoplasm. Various kinds of NF-κB inducers can phosphorylate IκB and release active NF-κB dimers that translocate to the nuclei and bind to target genes. Boucraut *et al.* studied the expression and localization of NF-κB family proteins in EVTs and villous syncytiotrophoblasts that were isolated from first trimester human placenta [13]. Their study suggested that c-Rel might heterodimerize with NF-κB1 and bind DNA in the nuclei of EVTs, but not those of syncytiotrophoblasts. The aim of that study was to investigate the possible molecular mechanism that down-regulates the expression of classical MHC class I genes in both cytotrophoblasts and syncytiotrophoblasts, and up-regulates *HLA-G* expression in cytotrophoblasts. However, their findings suggested that trophoblasts having an invasive phenotype expressed c-Rel as a DNA-binding factor. Our experiments

showed that the expression of c-Rel in HTR-8/SVneo cells was as strong as that in choriocarcinoma and it was stronger compared with those in villi of normal placenta and hydatidiform mole tissues (fig. 1a). These results indicate that c-Rel might promote the malignant potential of trophoblasts.

Our results suggested that c-Rel might be involved in regulating PI3K/Akt signaling in choriocarcinoma cells. The activation of PI3K/Akt signaling plays an important role in the migration and invasion of trophoblasts, showing that inactivation of Akt by LY294002 decreased migration and invasion of trophoblasts and choriocarcinoma [18-20], and some studies have shown that it activates NF-κB in cancer cells [30, 31]. These findings indicate that NF-κB is downstream of PI3K/Akt signaling. On the other hand, it has been reported that p65 regulates PTEN, which is a negative regulator of PI3K/Akt signaling, and this observation would seem to indicate that NF-κB is upstream of PI3K/Akt signaling [21]. Our results suggest that c-Rel is a regulator of PI3K/Akt signaling, although it is unclear whether c-Rel is involved in regulating the phosphorylation of Akt directly or via other factors. Based on our finding that the expression level of PTEN was not changed with c-Rel knockdown, p65 expression might not be reduced by the suppression of c-Rel expression.

In conclusion, our study revealed that the transcription factor c-Rel was expressed strongly in choriocarcinoma cells but weakly in normal trophoblasts, and it formed a heterodimer with p65 in choriocarcinoma cells. The suppression of c-Rel expression reduced invasion, migration, proliferation, and Akt phosphorylation in choriocarcinoma cells. c-Rel overexpression in choriocarcinoma increased migratory and invasive abilities, and the effects on invasion was inhibited by a PI3K inhibitor. These findings suggest that c-Rel might play a role in promoting the invasion of choriocarcinoma cells through PI3K/Akt signaling.

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Disclosure statement

The authors have no conflicts of interests to disclose in relation to this study.

Figure legends

Fig. 1. The expression of NF-κB family proteins in various types of trophoblast cells and the heterodimerization of c-Rel with p65. **a** A representative western blot demonstrating c-Rel protein expression in all choriocarcinoma cell lines and weak c-Rel expression in normal placenta and hydatidiform mole tissues. **b-e** In Jar and Bewo cell lysates, total c-Rel protein was immunoprecipitated with an anti-c-Rel antibody, followed by immunoblot analyses using antibodies against (**b**) p65, (**c**) RelB, (**d**) NF-κB1 p105/p50, and (**e**) NF-κB2 p100/p52.

Fig. 2. Immunostaining of c-Rel and p65 in choriocarcinoma, hydatidiform mole and placenta. (**a** and **b**) Intraplacental choriocarcinoma in 38 gestational weeks, (**c** and **d**) spleen metastases of choriocarcinoma, (**e** and **f**) hydatidiform mole, (**g** and **h**) villi of 6 gestational weeks, (**i** and **j**) EVTs of 6 gestational weeks, and (**k** and **l**) placenta in 37 gestational weeks were stained with antibodies against (**a**, **c, e, g, i** and **k**) c-Rel and (**b, d, f, h, j** and **l**) p65. Magnification × 100; Scale bar = 100μm.

Fig. 3. The siRNA-mediated knockdown of c-Rel in Jar and Bewo cells decreased their proliferative abilities. **a** The expression of c-Rel protein in c-Rel siRNA transfectants (si c-Rel) was effectively down-regulated compared with that in control siRNA transfectants (mock) in Jar and Bewo cells at 48 h after transfection. **b** The mRNA levels of c-Rel in si c-Rel cells were determined using quantitative real-time PCR. The mRNA expression of c-Rel was decreased in si c-Rel cells compared with that in mock transfectants. **c** Mock transfectants and si c-Rel cells were analyzed for cell morphology by phase-contrast microscopy. Scale bar = 100 μm. **d** A graphical depiction of the relative absorbance readings obtained from modified tetrazolium salt (MTS) assay experiments demonstrating that c-Rel knockdown affected cell proliferation. The mean values of three independent experiments, each performed in eight replicate wells, are shown. Each bar represents the mean distance migrated as a percentage of the distance migrated by the mock transfectants ± standard deviation $^{**}p < 0.01$.

Fig. 4. The siRNA-mediated knockdown of c-Rel in Jar and Bewo cells decreased their migration and invasion abilities. Graphical depictions of data obtained from (**a**) migration

assay (n = 3) and (**b**) matrigel invasion assay (n = 3) after c-Rel knockdown, exhibiting the decreases in the relative mean distance migrated by si c-Rel cells compared with that of mock transfectants. **c** The directional cell migration of mock transfectants and si c-Rel cells was monitored in monolayers by scratch-wound assay. Dotted lines show the leading fronts of the migrating cells. Each bar represents the mean distance migrated as a percentage of the distance migrated by the mock transfectants ± standard deviation $**p < 0.01$.

Fig. 5. The siRNA-mediated knockdown of c-Rel regulated the phosphorylation of Akt. **a** Culture supernatants were separated on a gelatin-enbedded 10% polyacrylamide gel. There were no significant differences between mock transfectants and si c-Rel cells. **b** Western blot analysis of the phosphorylation status of various signaling proteins in mock transfectants and si c-Rel cells of the Jar and Bewo cell lines. **c** Western blot analysis of cleaved PARP and cleaved caspase-3 in mock transfectants and si c-Rel cells of the Jar and Bewo cell lines. The expression levels of cleaved PARP and cleaved caspase-3 were increased by c-Rel knockdown in Jar and Bewo.

Fig. 6. The effect of c-Rel overexpression and treatment of inhibitor of phosphorylation of Akt. **a** The expression of c-Rel protein in c-Rel CRISPR activation plasmid transfectants (c-Rel act) was effectively up-regulated compared with that in control CRISPR activation plasmid transfectants (mock) in Bewo cells. **b** A graphical depiction of the relative absorbance readings obtained from modified tetrazolium salt (MTS) assay experiments demonstrating that c-Rel overexpression did not affect cell proliferation. The mean values of three independent experiments, each performed in eight replicate wells, are shown. **c** Graphical depictions of data obtained from migration assay (n = 3) and matrigel invasion assay (n = 3) after c-Rel overexpression, exhibiting the increases in the relative mean distance migrated by c-Rel act cells compared with that of mock transfectants. **d** Western blot showed that treatment with a PIK3 inhibitor (LY294002) reduced the expression of the phosphorylated form of Akt in mock and c-Rel act cells, but did not affect on the expression of c-Rel. **e** Invasion assay showed that inactivation of Akt by LY294002 significantly reduced invasion abilities in mock and c-Rel act cells.

Each bar represents the mean distance migrated as a percentage of the distance migrated by the mock transfectants ± standard deviation $^{**}p < 0.01$.

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promotes migration and invasion of human trophoblast cells through activation of MAPK, PI3K and NOTCH signaling pathways. Mol Hum Reprod 2015;21:435-451.

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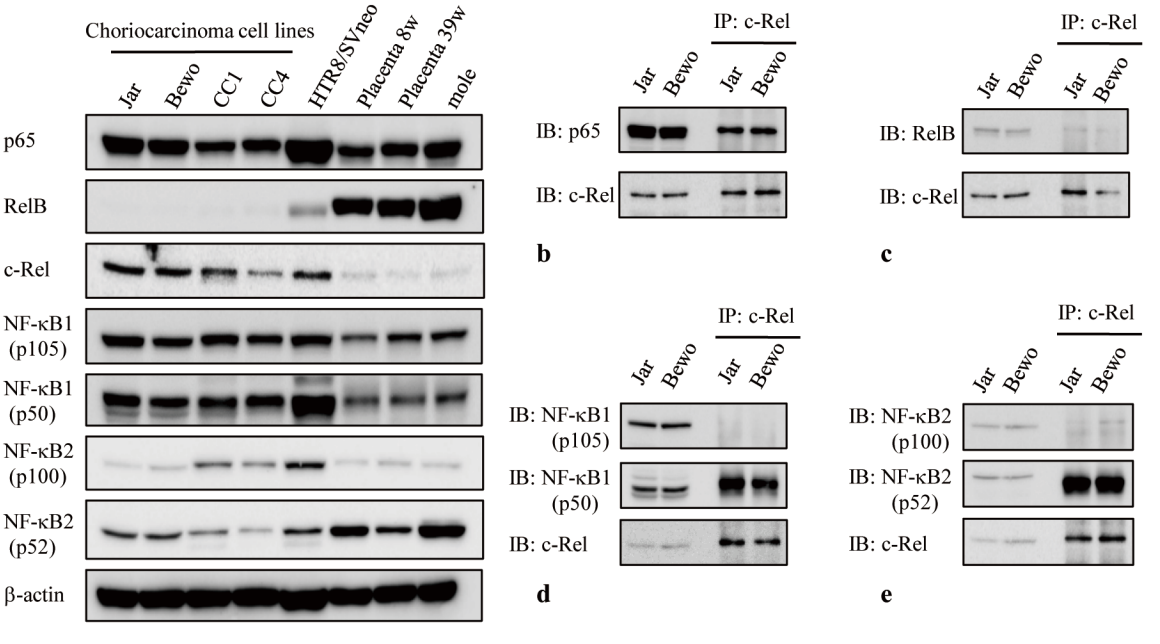
Fig. 1


Choriocarcinoma cell lines
Jar
Bewo
CC1
CC4
HTR8/SVneo
Placenta 8w
Placenta 39w
mole
p65
RelB
c-Rel
NF-κB1 (p105)
NF-κB1 (p50)
NF-κB2 (p100)
NF-κB2 (p52)
β-actin
a
IP: c-Rel
IB: p65
IB: c-Rel
b
IB: RelB
c
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d
IB: NF-κB2 (p100)
IB: NF-κB2 (p52)
e

Fig. 2

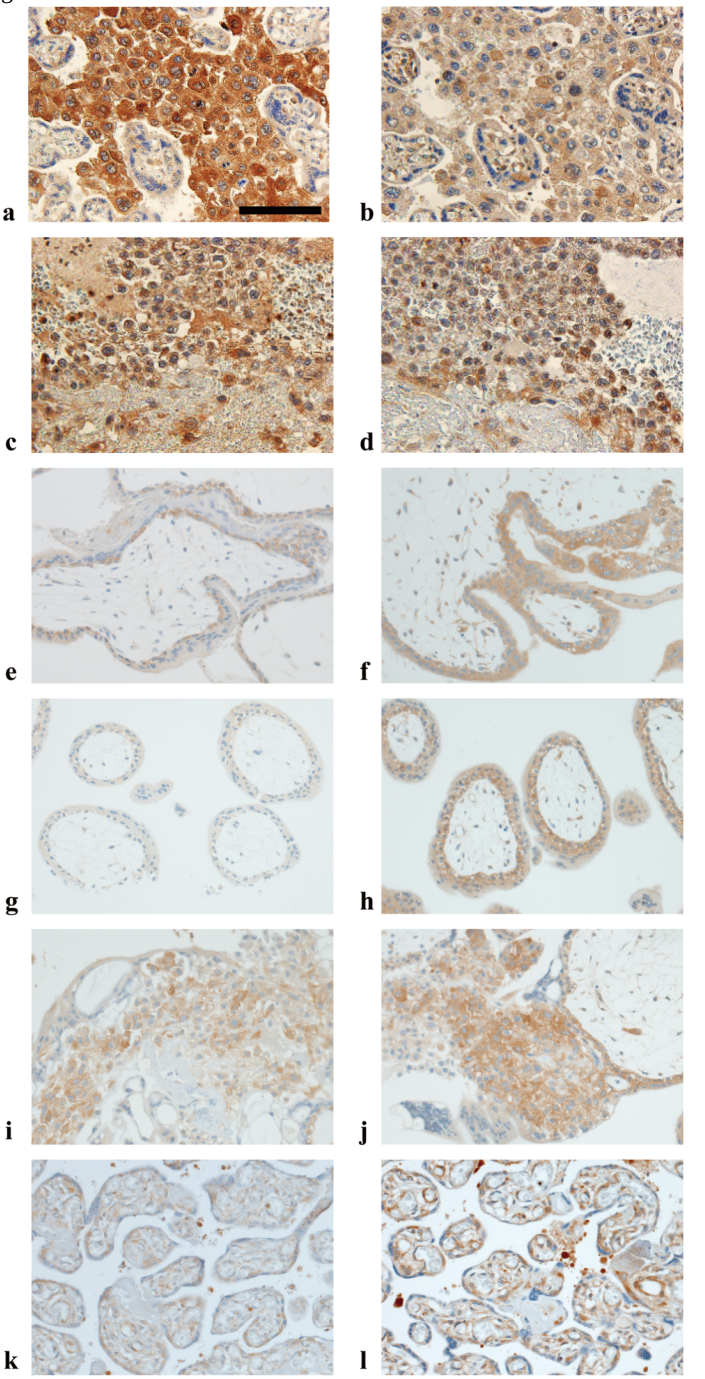
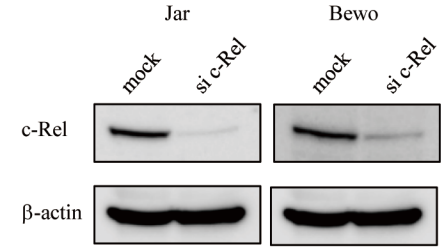
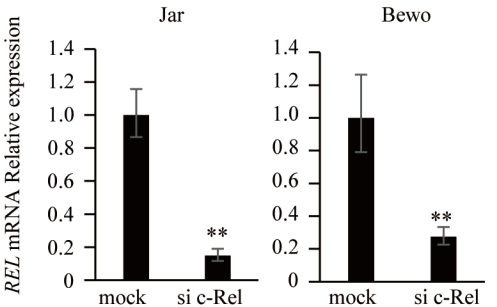


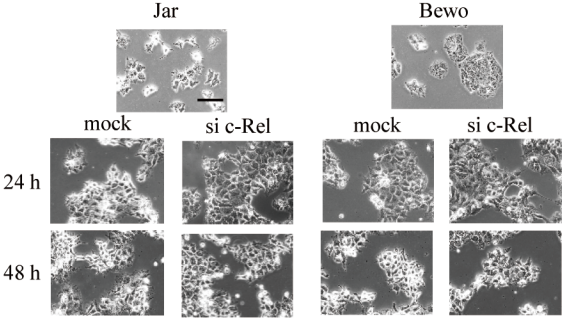
Fig. 3


Jar
Bewo
mock
si c-Rel
mock
si c-Rel
c-Rel
β-actin

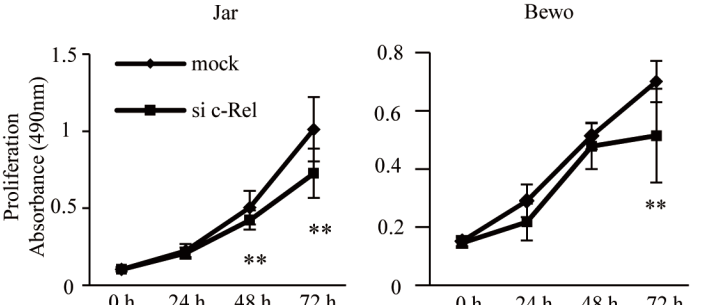
a


Jar
Bewo
REL mRNA Relative expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
**
**
mock
si c-Rel
mock
si c-Rel

b


Jar
Bewo
mock
si c-Rel
mock
si c-Rel
24 h
48 h

c


Jar
Bewo
mock
si c-Rel
Proliferation
Absorbance (490nm)
1.5
1
0.5
0
0.8
0.6
0.4
0.2
0
**
**
**
0 h
24 h
48 h
72 h
0 h
24 h
48 h
72 h

d

Fig. 4

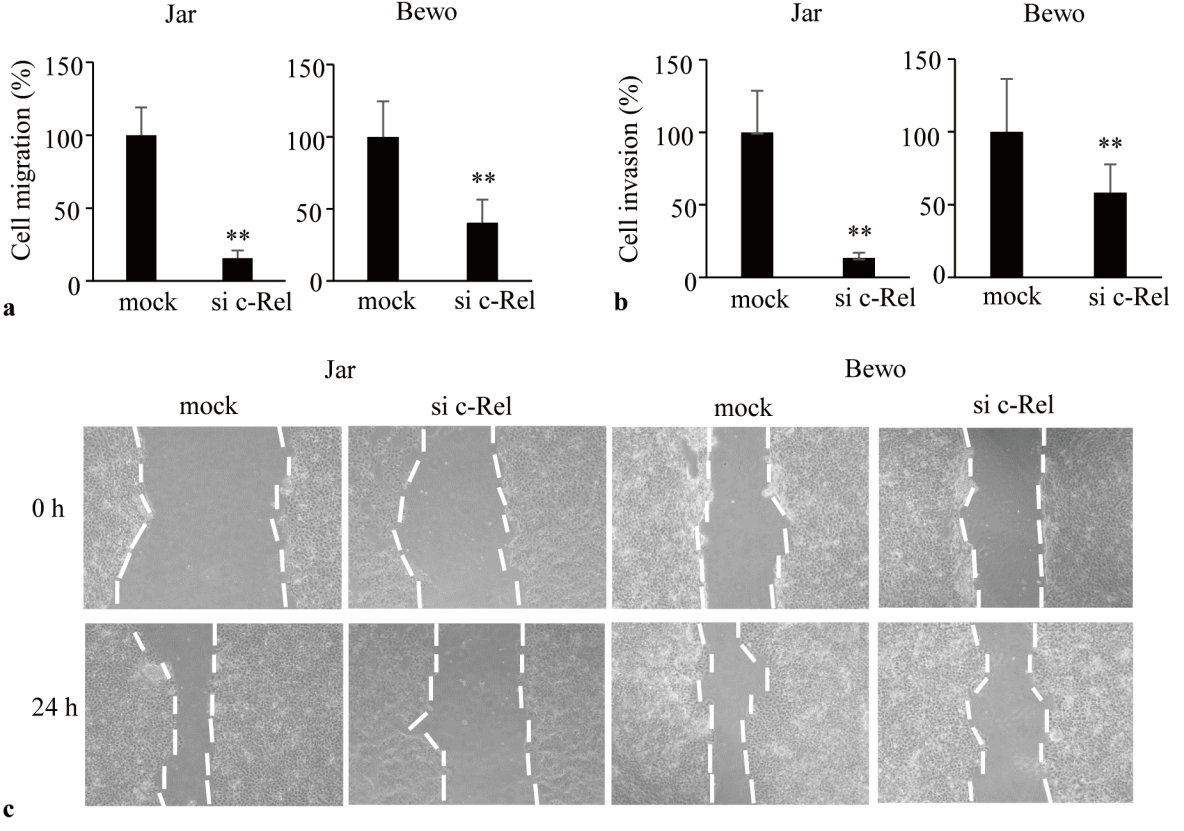
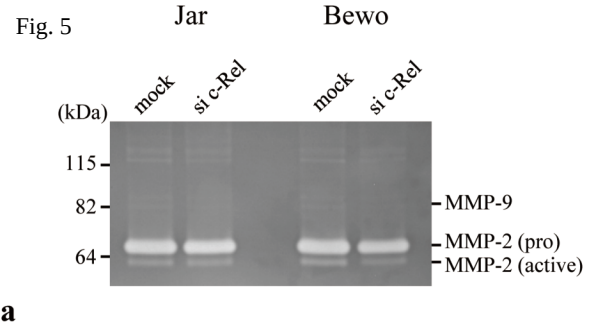
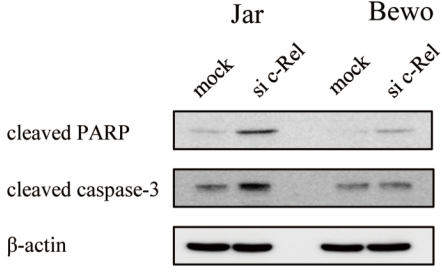

Jar
Bewo
Cell migration (%)
150
100
50
0
**
mock
si c-Rel
a
Cell invasion (%)
b
0 h
24 h
c

Fig. 5


Jar
Bewo
mock
si c-Rel
mock
si c-Rel
(kDa)
115
82
64
MMP-9
MMP-2 (pro)
MMP-2 (active)
a


Jar
Bewo
mock
si c-Rel
mock
si c-Rel
cleaved PARP
cleaved caspase-3
β-actin
c

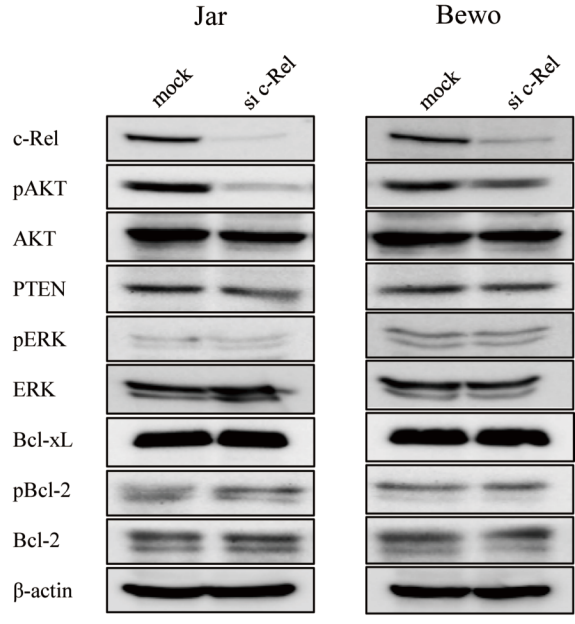
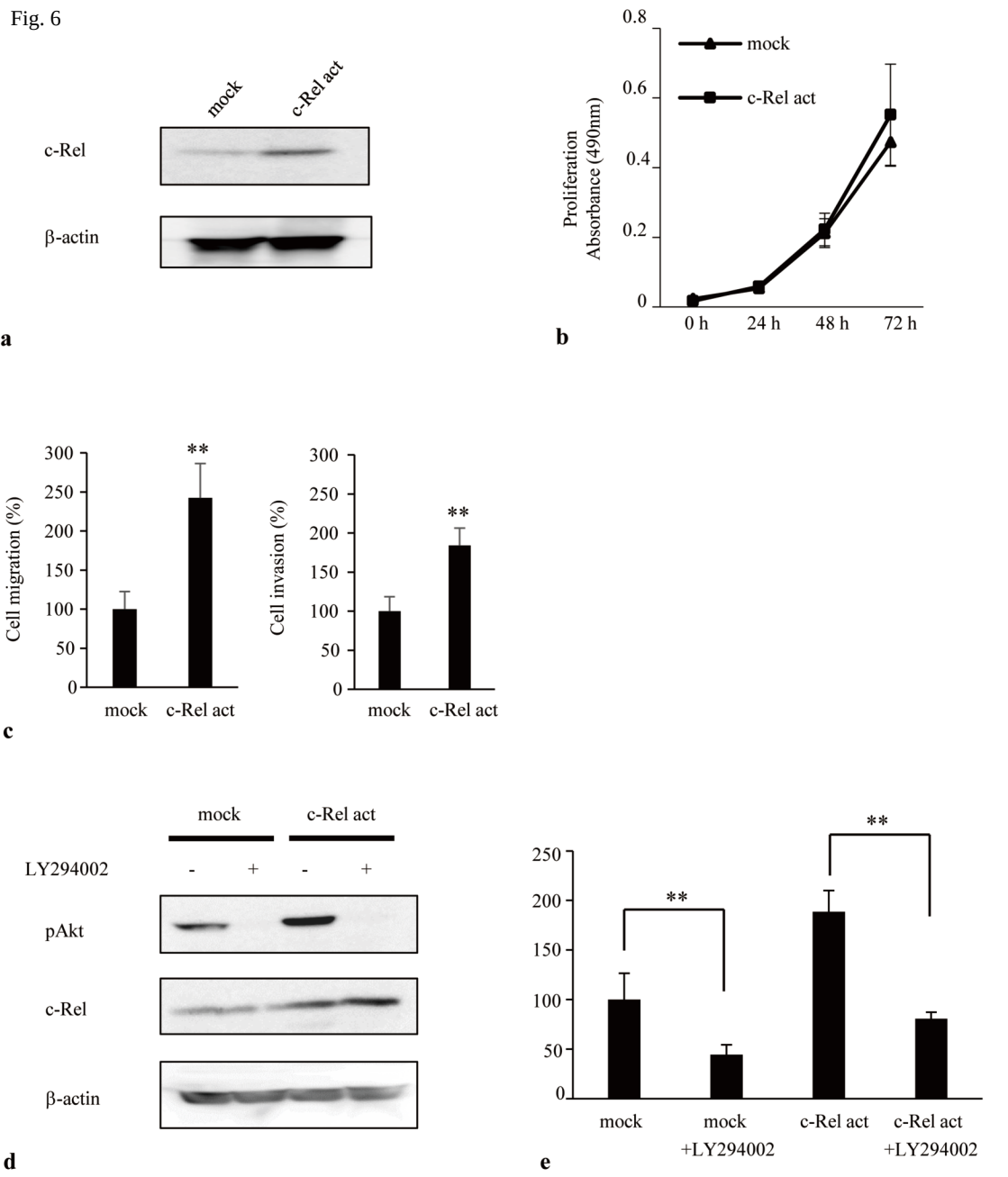
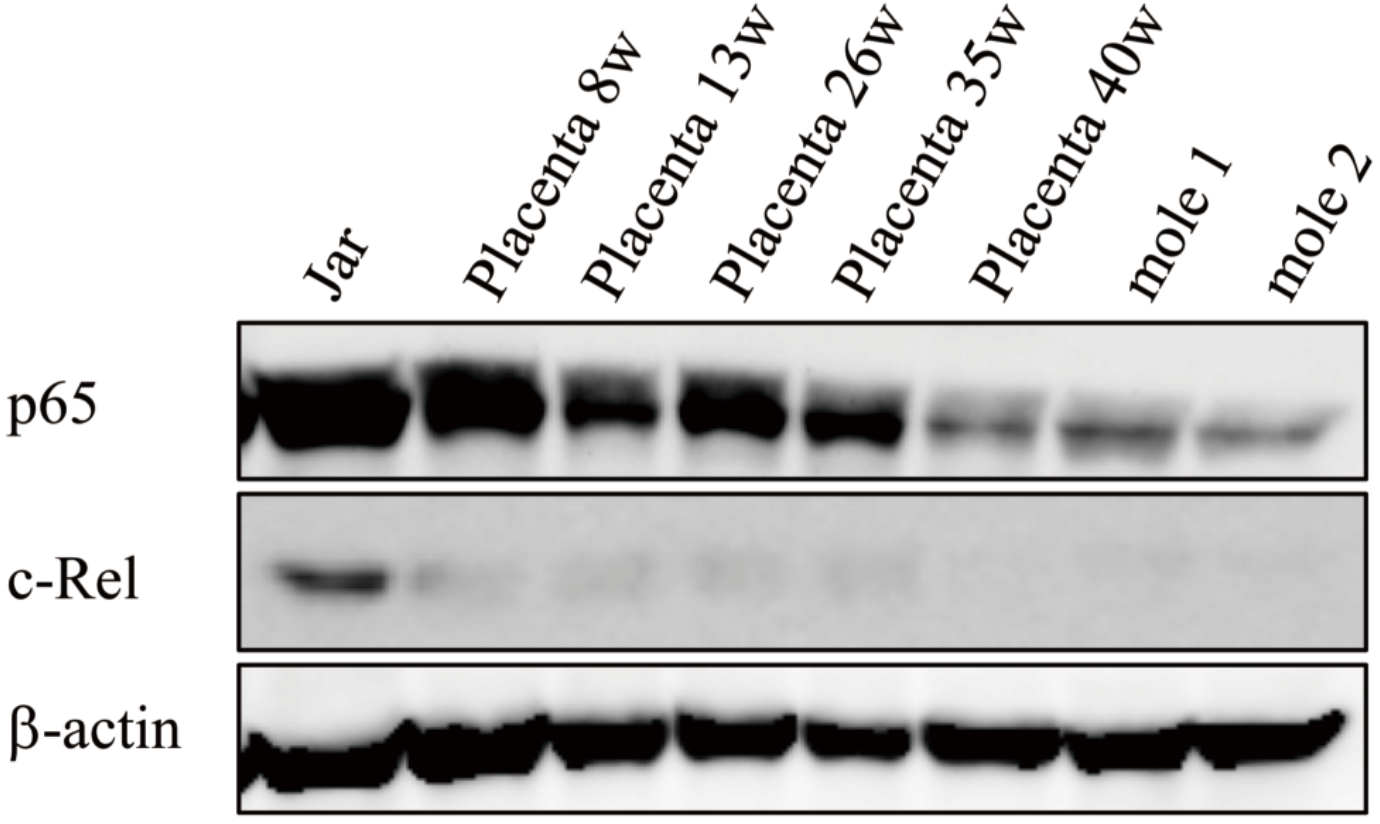

Jar
Bewo
mock
si c-Rel
mock
si c-Rel
c-Rel
pAKT
AKT
PTEN
pERK
ERK
Bcl-xL
pBcl-2
Bcl-2
β-actin
b

Fig. 6


mock
c-Rel act
c-Rel
β-actin
a
0.8
0.6
0.4
0.2
0
Proliferation
Absorbance (490nm)
mock
c-Rel act
0 h
24 h
48 h
72 h
b
300
250
200
150
100
50
0
Cell migration (%)
**
mock
c-Rel act
Cell invasion (%)
**
c
mock
c-Rel act
LY294002
-
+
-
+
pAkt
c-Rel
β-actin
d
250
200
150
100
50
0
**
**
mock
mock
+LY294002
c-Rel act
c-Rel act
+LY294002
e

Supplementary Fig. 1. The expression of p65 and c-Rel proteins in normal placenta and hydatidiform mole. Tissues of normal placentas in various gestational weeks (n = 5) and hydatidiform mole (n = 2) were used for western blot analysis. p65 was expressed in all tissues of normal placenta and hydatidiform mole. The expression of c-Rel was very weak or none in placenta and hydatidiform mole.



Supplementary Fig. 2. The siRNA-mediated knockdown of c-Rel in Jar and Bewo cells decreased their migration and invasion abilities. a The expression of c-Rel protein in c-Rel siRNAs transfectants (si-Rel1 and si-Rel6) was effectively down-regulated compared with that in control siRNA transfectants (mock) in Jar and Bewo cells at 48 h after transfection. b The mRNA levels of c-Rel in si-Rel1 and si-Rel6 cells were determined using quantitative real-time PCR. The mRNA expression of c-Rel was decreased in si-Rel1 and si-Rel6 cells compared with that in mock transfectants. Graphical depictions of data obtained from (c) migration assay (n = 3) and (d) matrigel invasion assay (n = 3) after c-Rel knockdown, exhibiting the decreases in the relative mean distance migrated by si-Rel1 and si-Rel6 cells compared with that of mock transfectants. Each bar represents the mean distance migrated as a percentage of the distance migrated by the mock transfectants ± standard deviation **p < 0.01.