Knockdown of *ICB-1* gene enhanced estrogen responsiveness of ovarian and breast cancer cells

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**Abstract**

ICB-1 chromosome 1 open reading frame 38 (C1orf38) is a human gene initially described by our group to be involved in differentiation processes of cancer cells. Recently, we have reported ICB-1 as a novel estrogen target gene and identified an estrogen response element in its promoter. In this study, we examined the role of ICB-1 in regulation of proliferation of breast and ovarian cancer cells. We knocked down its expression in estrogen-dependent MCF-7 breast cancer cells and hormone-unresponsive SK-OV-3 ovarian cancer cells by stable transfection with a specific shRNA plasmid followed by G-418 selection. Knockdown of ICB-1 enabled a considerable estrogen response of SK-OV-3 cells in terms of proliferation. This transformation of SK-OV-3 cells into an estrogen-responsive phenotype was accompanied by upregulation of estrogen receptor α (ERα) expression and a significant decrease of ERβ expression on the mRNA level. Expression of ERα-dependent genes progesterone receptor, pS2, fibulin 1c, and c-fos was elevated in SK-OV-3 cells stably expressing ICB-1 shRNA. In MCF-7 cells, ICB-1 knockdown exerted similar effects on gene expression, supporting a general role of ICB-1 in estrogen responsiveness. Our data suggest that differentiation-associated gene ICB-1 might exert antagonistic actions on cellular estrogen response, which can result in inhibition of estradiol-triggered proliferation. The molecular mechanisms mediating this inhibitory effect of ICB-1 on estrogen signaling are suggested to be limitation of ERα transcript levels but sustaining high levels of ERβ, reducing both activation of ERα target genes and cellular proliferation. The identification of ICB-1 as a new player in endocrine-related cancer encourages further studies on the significance of this gene in cancer development and therapy.

*Endocrine-Related Cancer* (2010) 17 147–157

**Introduction**

Human gene *ICB-1*, also known as chromosome 1 open reading frame 38 (C1orf38), is a widely expressed gene, which has been shown to be involved in cellular differentiation processes. However, its exact function remains unknown and it does not share significant homologies to other genes. ICB-1 gene was initially cloned by our group in an attempt to identify differentially expressed genes during *in vitro* differentiation of endometrial tumor cells triggered by culture on an artificial basement membrane (Treeck *et al*. 1998). ICB-1 expression is involved both in monocytic and granulocytic *in vitro* differentiation of promyelocytic leukemia cells and also has been shown to be responsive to interferon-γ (Treeck *et al*. 2002, 2005).

Recently, our group has been able to identify an estrogen response element (ERE) in the promoter region of *ICB-1* gene. We demonstrated that 17-β estradiol (E2) activates *ICB-1* expression in breast and ovarian cancer cells in an estrogen receptor α (ERα) -dependent manner (Bollmann *et al*. 2008).

In the human reproductive system, estrogens are important regulators of growth and differentiation. Cancer cells remain estrogen-dependent at least for a distinct time during their process of dedifferentiation.
ERα and β often are expressed in cancer cells of the breast and ovary. The effects of 17-β E₂ on these tissues are either mediated through ER binding to EREs directly regulating gene expression, via ER interaction with other transcription factors or by nonnuclear actions that seem to be mediated by membrane-bound ERs (Rae & Johnson 2005, Kleuser et al. 2008, Peng & Jordan 2008). Estrogens are known to stimulate proliferation of hormone-dependent tissues for example by activation of cell cycle genes (Kashima et al. 2009).

In this study, we intended to find out to what extent the differentiation-associated, estrogen-responsive human gene ICB-1 plays a role in proliferation control of cancer cells derived from human breast and ovary. For this purpose, we used an RNAi-based approach to knockdown ICB-1 expression in estrogen-responsive MCF-7 breast cancer cells and hormone-unresponsive SK-OV-3 ovarian cancer cells. Employing these cell culture models, we studied the effect of an ICB-1 knockdown on cellular proliferation and gene expression.

Materials and methods

Materials

Phenol red-free DMEM culture medium was obtained from Invitrogen; FCS was purchased from PAA (Pasching, Austria). 17-β E₂, 4-OH tamoxifen, ICI 182 780, staurosporine, and serum replacement 2 (SR2) were obtained from Sigma; MCF-7 breast cancer cells and SK-OV-3 ovarian cancer cells were obtained from American Type Culture Collection (Manassas, VA, USA). Moloney murine leukemia virus-P (M-MLV-P) reverse transcriptase (RT), Cell Titer Blue kit, Caspase-Glo 3/7 kit, and iMPrO-II RT were purchased from Promega. RNeasy Mini Kit, RNase-Free DNase Set, and Quantitect SYBR Green PCR Kit were obtained from Qiagen. PCR primers were synthesized at Metabion (Planegg–Martinsried, Germany). Transfectin reagent was obtained from BioRad. Platinum PfX Polymerase and OptiMEM medium were purchased at Invitrogen. SureSilencing shRNA plasmid for human ICB-1 (C1orf38) gene was purchased from SABiosciences, Frederick, MD, USA.

Cell culture and transfections

MCF-7 breast cancer cells were maintained in DMEM/F12 medium supplemented with 10% FCS, 1 mM sodium pyruvate, and 10 ng/ml insulin, and SK-OV-3 cells were cultured in DMEM/F12 medium with 10% FCS. For analysis of cell growth, cells were cultured in DMEM/F12 supplemented with defined SR2 and treated with E₂, 4-OH tamoxifen, or ICI 182 780. Cells were cultured with 5% CO₂ at 37 °C in a humidified incubator. For transfection, 10⁶ cells per well of a 6-well dish were seeded in DMEM/F12 containing 10% FCS. The next day, 1.2 ml fresh culture medium was added to the cells and transfection solution was prepared by mixing 8 μl Transfectin reagent (BioRad), and 1 μg of the shRNA plasmid mix or control plasmid DNA in OptiMEM reduced serum medium (Invitrogen) and added to the cultured cells. For transfection with ICB-1 shRNA, a mixture of four plasmids (SureSilencing, SABiosciences) was used containing the ICB-1-specific shRNA insert sequences 5’-gggagtagctttgtggaact-3’, 5’-caacctgagctctcataaatg-3’, 5’-gtagcacacgctctgataag-3’, and 5’-cattgattgaattgtggaact-3’, which bind to ICB-1 exon 6 present in all splice variants. As a negative control, the same plasmid with the nonspecific insert 5’-ggatctctgcctgatatc-3’ was used. For generation of stable clones, G418 selection (300 μg/ml) was started at 48 h after transfection and lasted for about 6 weeks before the first clones were isolated.

Reverse transcription and PCR

Total RNA was isolated by means of the SV Total RNA Isolation System (Promega) according to the manufacturer’s instructions. From 0.3 μg total RNA, cDNA was synthesized using 100 U M-MLV-P RT (Promega), 2.5 mM dNTP mixture, and 50 pM random primers (Invitrogen). For real-time PCR detection of ICB-1 mRNA or expression of estrogen target genes in an intron-spanning manner (primer sequences in Table 1), 2 μl cDNA was amplified using the Quantitect SYBR Green PCR Kit (Qiagen) and the LightCycler PCR device (Roche Diagnostics). The PCR program was 95 °C for 15 min, followed by 45 PCR cycles (95 °C for 10 s, 56 °C for 30 s, and 72 °C for 30 s) and a final extension for 5 min at 72 °C, followed by a standard melting curve analysis. In order to optimize results, we then used LightCycler FastStart DNA Master SYBR Green I (Roche) for another set of experiments. Here, the PCR program was 95 °C for 15 min (denaturation) followed by 37 cycles (95 °C for 10 s, 60 °C for 1 s, and 72 °C for 12 s, including measurement) completed by a standard melting curve analysis. In all RT-PCR experiments, a 190 bp β-actin fragment was amplified as reference gene using intron-spanning primers actin-2573 and actin-2876. After performing dilution experiments with sample cDNA over a 100-fold range confirming the PCR efficiencies of all primer pairs to be approximately...
equal (Ståhlberg et al., 2003), data were analyzed using the comparative DDCT method (Livak & Schmittgen 2001) calculating the difference between the threshold cycle (Ct) values of the target and reference gene of each sample and then comparing the resulting DCT values between different samples. In these experiments, mRNA not subjected to RT was used as a negative control to distinguish cDNA and vector or genomic DNA amplification.

Cell viability assay

ICB-1 knockdown clones and negative control clones of the cell lines MCF-7 and SK-OV-3 were seeded in 96-well plates (1000 SK-OV-3 cells/well and 2500 MCF-7 cells/well). Cells were cultured in DMEM containing 1!SR2 and for stimulation they were treated with 3 nM 17-bE2 alone or in combination with 4-OH tamoxifen (0.1, 1, 5, and 10 mM) or pure anti-estrogen ICI 182 780 (0.1, 1, and 10 mM). Alternatively, cells were treated with the ERa-specific agonist 4,40,400-(4-propyl-[1H]-pyrazole-1,3,5-triyl) triphenol (PPT) (1–1000 nM) or the ERb-specific agonist diarylpropionitrile (DPN) (1–1000 nM). On days 0, 3, and 5 or on days 0 and 5 for experiments with PPT and DPN, relative numbers of viable cells were measured in comparison to negative control clones and solvent controls using the fluorimetical, resazurin-based Cell Titer Blue assay (Promega) according to the manufacturer’s instructions at 560Ex/590Em nm in a Victor3 multilabel counter (Perkin–Elmer, Waltham, MA, USA). Cell growth was expressed as percentage of day 0 or percentage of the vehicle control. Statistical analysis of the data was performed by one-way ANOVA using Prism 2.0 Software (Graph Pad, San Diego, CA, USA), with statistical significance accepted at P < 0.05.

Antibodies and western blot analysis

MCF-7 and SK-OV-3 clones were lysed in RIPA buffer (1% (v/v) Igepal CA-630, 0.5% (w/v) sodium deoxycholate, and 0.1% (w/v) SDS in PBS containing aprotonin and sodium orthovanadate). Aliquots containing 15 μg protein were resolved by 10% (w/v) SDS-PAGE, followed by electrotransfer to a PVDF hybond (Amersham) membrane. Immunodetection was carried out using ERa antibody (clone 6F11, Acris, Herford, Germany) or -actin antibody (8226, ABCAM, Cambridge, UK) diluted 1:5000 in PBS containing 5% skim milk (w/v) followed by HRP-conjugated secondary antibody, which was detected using chemiluminescence system (Amersham).

Results

Knockdown of ICB-1 gene in MCF-7 breast cancer and SK-OV-3 ovarian cancer cells

To study the function of ICB-1 in human breast and ovarian cancer cells with regard to proliferation, we performed a siRNA-mediated knockdown of this gene. For this purpose, we transfected MCF-7 and SK-OV-3 cells with a shRNA plasmid for human ICB-1 (SureSilencing, SABiosciences) or with a negative control plasmid coding for a nonspecific shRNA. After 6 weeks of G418 selection, we picked three clones of each cell line and determined the transcript levels of ICB-1 by real-time RT-PCR analysis. Stable expression of ICB-1 shRNA led to reduction of ICB-1 transcript levels by about 80% in both cell lines (Fig. 1). However, we did not observe significant differences in the ICB-1 expression levels between the three clones of each cell line. The generated cell lines were termed MCF-7/icbKD or SK-OV/icbKD respectively. We were not able to verify ICB-1 knockdown on the protein level, because today no functional antibody to ICB-1 protein exists. Thus, we had to limit this verification to the RNA level.

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Table 1 Oligonucleotide sequences of the intron-spanning PCR primers used in this study

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Primer sequence (5’–3’)</th>
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<tr>
<td>ICB-1</td>
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<tr>
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<td>PR</td>
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<tr>
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Knockdown of ICB-1 enables a proliferative E₂ response of SK-OV-3 ovarian cancer cells

Next, we compared the growth of MCF-7/icbKD or SK-OV/icbKD cells with the negative control clone of each cell line. As determined in proliferation assays using medium containing a defined SR, knockdown of ICB-1 expression significantly increased the growth of ovarian SK-OV-3 cells by 29% on day 5, whereas the growth of MCF-7 was only increased by 12% (Fig. 2). Additionally, we performed growth assays with or without steroid hormone E₂ (3 nM) to analyse the effect of ICB-1 knockdown on E₂-triggered proliferation. Addition of E₂ further increased proliferation of MCF-7/icbKD cells, but the relative estrogen response of this cell line was not changed (Fig. 2a). In contrast, ICB-1 knockdown enabled a strong proliferative effect of E₂ on initially estrogen-unresponsive SK-OV-3 ovarian cancer cells (Fig. 2b). Dose–response analysis revealed that the maximum proliferative effect of E₂ was triggered by a 3 × 10⁻¹⁰ M concentration of this steroid hormone (Fig. 3a).

This transformation of ovarian cancer cells from hormone independence to an estrogen-responsive phenotype by knockdown of a single gene was further validated by proliferation experiments combining E₂ with the pure anti-estrogen ICI 182 780 or the selective ER modulator (SERM) tamoxifen. Treatment with ICI 182 780 (0.1, 1, and 10 μM) did not affect the growth of SK-OV-3 cells at all. In contrast, knockdown of ICB-1 in this cell line enabled a strong inhibitory effect
of ICI 182 780 on E2-triggered growth by about 80% after 5 days of treatment (Fig. 2c). The same was true for treatment with 4-OH tamoxifen (1 and 5 \( \mu \)M). Whereas this drug did not significantly affect the growth of negative control SK-OV-3 cells, after ICB-1 knockdown, it inhibited E2-stimulated cell growth by 22% (1 \( \mu \)M) or 42% (5 \( \mu \)M) after 7 days of treatment.

Estrogen-triggered proliferation of MCF-7 breast cancer cells was not significantly increased after ICB-1 knockdown (Fig. 3b). When we added the pure anti-estrogen ICI 182 780 (1 \( \mu \)M), MCF-7 growth was reduced by 60% after 5 days of treatment irrespective of ICB-1 expression. Treatment with 4-OH tamoxifen resulted in the expected anti-proliferative effect on this cell line, which also was not dependent on ICB-1 mRNA level (data not shown).

To distinguish between ER\( \alpha \)- and ER\( \beta \)-mediated mechanisms in cellular estrogen response, we also studied the effect of ICB-1 knockdown on the action of ER\( \alpha \) agonist PPT and ER\( \beta \) agonist DPN in both cell lines. Whereas both drugs did not significantly affect the growth of SK-OV-3 control cells, in SK-OV-3/icbKD cells, treatment with high doses of PPT or DPN (10–1000 nM) significantly accelerated cell growth, with PPT showing a more pronounced effect, an about threefold increase of proliferation when compared to negative control cells (Fig. 3c). Small doses of DPN (1 nM) did not significantly increase growth of this cell line (Fig. 3d). In MCF-7 cells, ER\( \alpha \) agonist PPT increased cell growth by about 50% irrespective of ICB-1 expression. In contrast, after knockdown of ICB-1, the proliferative effect of ER\( \beta \) ligand DPN (10–100 nM) on MCF-7 cells was diminished by about 15% (data not shown).

**Knockdown of ICB-1 increases expression of ER\( \alpha \) and progesterone receptor, but reduces expression of ER\( \beta \) in MCF-7 and SK-OV-3 cells**

Given that ICB-1 knockdown transformed a hormone-unresponsive ovarian cancer cell line in an estrogen-responsive one, we were eager to know how this would be reflected on the level of gene expression. For this purpose, we studied expression of ER\( \alpha \) and three ER\( \beta \) transcript isoforms as well as expression of estrogen-responsive genes like progesterone receptor (PR) and \( p52 \) gene on the mRNA level by means of real-time RT-PCR. To examine the molecular mechanisms underlying the observed changes in
cellular proliferation, we also compared the expression of cell cycle regulators like cyclin D1 (Bates & Peters 1995, Hodges et al. 2003), cyclin A2 (Hall et al. 1993, Vendrell et al. 2004), and p21/WAF-1 (Arooz et al. 2000) in both cell lines with and without ICB-1 shRNA. Last, we analyzed expression of two genes that were described to be associated with differentiation of breast or ovarian cancer cells, epithelial cell marker cytokeratin 8 (KRT8) (Fuchs et al. 2002), and fibulin 1 (FIB1/FBLN1) encoding an extracellular matrix protein (Clinton et al. 1996, Moll et al. 2002).

Knockdown of ICB-1 expression led to a significantly increased level of ERα mRNA both in MCF-7 and SK-OV-3 cells (Fig. 4a). In SK-OV/icbKD cells, ERα transcript levels were more than doubled, whereas in MCF-7/icbKD cells, an increase by about 50% was observed. In contrast, expression of the ERβ isoform 2 was strongly decreased in both cell lines, and ERβ1 mRNA levels were significantly lowered in SK-OV/icbKD cells. Transcript levels of estrogen target genes PR and pS2 both were elevated about threefold in SK-OV/icbKD cells when compared to negative control cells, and were increased by about 80% (40%) in MCF-7/icbKD cells (Fig. 4b). We then intended to confirm upregulation of ERα expression on the protein level by means of western blot analysis. MCF-7 cells exhibited strong ERα expression as indicated by intensive ERα bands of about 65 kDa, but did not show any difference between MCF-7/icbKD and control cells. ERα bands in SK-OV-3 cells were more than fivefold weaker, and expression of ERα was slightly higher in the icbKD clone of this cell line. However, after densitometrical analysis of three experiments, this difference did not reach statistical significance (Fig. 5).

In SK-OV/icbKD cells, expression of epithelial cell marker KRT8 was significantly smaller than in the negative control, and the level of fibulin 1c transcript coding for an extracellular matrix protein was increased more than twofold. In contrast, in MCF-7/icbKD cells, transcript levels of negative cell cycle regulator p21/WAF-1 were significantly diminished by about 50% (Fig. 4b).

Given that SK-OV-3 ovarian cancer cells are characterized by HER2 overexpression (Hsieh et al. 2000) determining their fast, hormone-independent growth, we also examined HER2 transcript levels subject to ICB-1 knockdown. After analysis of HER2 expression by real-time RT-PCR, we observed a slight, but significant decrease of HER2 mRNA levels in SK-OV/icbKD cells by about 25% (Fig. 4c).

**Figure 4** Gene expression changes in SK-OV-3 and MCF-7 cells triggered by ICB-1 knockdown as determined by real-time RT-PCR analysis. The transcript levels of estrogen receptors (a), other genes indicated (b), or HER2 (c) are shown in percent of the respective expression in negative control cells, which was defined as 100%. (n=4), *P<0.05 versus control.
Knockdown of ICB-1 increases estrogen responsiveness of gene expression in MCF-7 and SK-OV-3 cancer cells

Given that reduced levels of ICB-1 mRNA were accompanied by altered ERα and ERβ expression, we now examined the effect of E2 on gene expression subject to ICB-1 mRNA levels. For this purpose, cells cultured in defined, serum-, and phenol red-free SR2 medium were treated with 3 nM E2 for 24 h prior to RNA isolation. Gene expression was analyzed by means of real-time RT-PCR as described above. In hormone-unresponsive SK-OV-3 cells, treatment with E2 did not affect expression of the genes tested in a significant manner. In contrast, E2 treatment induced expression of ERα target gene PR by about 40% in SK-OV-3/icbKD cells (Fig. 6a). In MCF-7 breast cancer cells, PR transcript levels were upregulated about fourfold after E2 treatment irrespective of ICB-1 expression level (Fig. 6b). In SK-OV-3, but not MCF-7, cell clones exhibiting reduced ICB-1 transcript levels, E2 also triggered a significant upregulation of c-fos mRNA expression when compared to the negative control. In MCF-7 cells, ICB-1 knockdown enabled a significant E2-triggered increase of cyclin D1 transcript levels. The estrogen effect on expression of the other genes tested did not differ between cells with or without ICB-1 shRNA.

Figure 5 Expression of ERα after knockdown of ICB-1 gene. Western blot analysis of total protein isolated from the indicated cell lines. Ten microgram protein was resolved by SDS-PAGE and blotting was performed as described in the materials and methods section. Shown are a representative blot and below a densitometric analysis of three experiments (n=3).

Figure 6 Effect of 17β estradiol on gene expression subject to ICB-1 knockdown. SK-OV-3 (a) or MCF-7 (b) clones cultured in serum- and phenol red-free-defined SR2 medium were treated with 3 nM E2 for 18 h prior to isolation of total RNA and subsequent real-time RT-PCR analysis. The shown relative mRNA levels are expressed in percent of the solvent control (n=3), *P<0.05 versus EtOH control, *P<0.05 versus expression in negative control cells.

Discussion

We report differentiation-associated gene ICB-1 to considerably affect steroid hormone receptor transcript levels both in MCF-7 breast cancer and in SK-OV-3 ovarian cancer cells. Knockdown of ICB-1 enhanced the estrogen responsiveness of both cell lines, increasing the effect of E2 on gene expression or proliferation. Most strikingly, knockdown of ICB-1 gene was sufficient to transform the hormone-unresponsive cell line SK-OV-3 into an estrogen-dependent one, which exhibits a strong proliferative estrogen response.
ICB-1 is a widely expressed gene, which is involved in differentiation processes such as in vitro differentiation by growth on an artificial basement membrane (Treeck et al. 1998) or by differentiation-inducing substances like 1, 25-dihydroxyvitamin D3 or all-trans retinoic acid (Treeck et al. 2002). Given that our group has recently demonstrated ICB-1 expression being estrogen-responsive in an ERα-dependent manner (Bollmann et al. 2008) and that estrogens are known to stimulate growth of responsive cells, in this study, we further examined the role of ICB-1 in proliferation control of breast and ovarian cancer cells. Differentiation on the one hand and proliferation on the other hand are two basic cellular functions resulting from complex gene expression patterns being activated by specific signals. These functions are at least in part opposing, as differentiation processes often are associated with cell cycle arrest (Borellini & Oka 1989). Given that stimulation of cellular differentiation significantly enhances expression of ICB-1 (Treeck et al. 1998, 2002), which is an estrogen target gene, we hypothesized that knockdown of ICB-1 might also affect cellular proliferation.

For this study, we employed the hormone-responsive, ERα-positive breast cancer cell line MCF-7 (Brooks et al. 1973, Dickson et al. 1986) and ovarian carcinoma cell line SK-OV-3, which is unresponsive to estrogens and anti-estrogens, overexpresses HER2, and is known as a rather aggressive and fast-growing cell type (Hua et al. 1995, Lau et al. 1999). Both cell lines express similar levels of ICB-1 mRNA (Treeck et al. 2005).

In this study, we first generated data confirming the relevance of ICB-1 gene for tumor cell differentiation. In SK-OV-3 cells, knockdown of ICB-1 led to downregulation of KRT8, a cytokeratin that is highly expressed in the ovarian epithelium and generally is considered as an epithelial cell marker (Gava et al. 2008). Reduction of KRT8 transcript levels clearly suggests a differentiation loss triggered by ICB-1 knockdown and supports the suggested role of this gene in the context of cellular differentiation. Loss of epithelial cell marker KRT8 has been detected in different cancer types and has been correlated with short survival (Fuchs et al. 2002, Walker et al. 2007, Abdel-Fatah et al. 2008). However, there are studies indicating contrary effects, describing overexpression of KRT8 in epithelial tumors (Casanova et al. 2004). Furthermore, knockdown of ICB-1 resulted in elevated transcript levels of fibulin 1c in SK-OV-3 cells, whereas this was not the case in MCF-7 cells. Fibulin1c gene codes for an extracellular matrix glycoprotein secreted by ovarian cancer cells (Clinton et al. 1996), which can be induced by estrogens in an ERα-dependent manner (Moll et al. 2002). Thus, the observed elevated levels of fibulin1c mRNA are suggested to be the consequence of the increased estrogen responsiveness of SK-OV-3 cells. Since fibulin 1c overexpression often occurs during ovarian carcinogenesis and is associated with differentiation loss, the observed upregulation of fibulin 1c after ICB-1 knockdown also supports the relevance of ICB-1 expression in cellular differentiation.

ICB-1 knockdown accelerated proliferation of SK-OV-3 ovarian cancer cells and, more strikingly, switched on a strong proliferative estrogen response in this estrogen-unresponsive cell line (Hua et al. 1995, Lau et al. 1999). Our gene expression data clearly provide an explanation for the changed growth properties of this cell line after ICB-1 knockdown. SK-OV/icbKD cells exhibited a significantly elevated ERα transcript level, which was demonstrated to be of biological relevance due to considerable upregulation of E2-responsive genes PR, pS2, and fibulin 1c, although our attempt to verify ERα upregulation on the protein level did not reach statistical significance. ERα is known to stimulate proliferation in response to estrogens – on the one hand by increased expression of genes connected with cell cycle progression like cyclin D1 or growth factors and on the other hand by downregulation of anti-proliferative and pro-apoptotic genes (Sabbah et al. 1999, Frasor et al. 2003). Thus, upregulation of this receptor is suggested to be one reason for estrogen-triggered proliferation of the KD clones. In contrast, expression of ERβ isoforms 1 and 2 was downregulated in SK-OV-3 cells with ICB-1 knockdown. ERβ has been described to act as an antagonist of ERα in certain settings and to act as a tumor suppressor with anti-proliferative properties (Lazennec et al. 2001, Bardin et al. 2004, Cheng et al. 2004, Treeck et al. 2007). Furthermore, loss of ERβ in ovarian epithelial cells has been connected to tumorigenesis and has been shown to increase proliferation of ovarian cancer cells (Pujol et al. 1998, Treeck et al. 2007). Thus, downregulation of this receptor caused by suppression of ICB-1 is suggested to be the second mechanism underlying the accelerated growth of these cells. Conferring estrogen responsiveness in terms of proliferation to SK-OV-3 cells by differential regulation of ERα and ERβ resulted from an enabled estrogenic gene regulation. The E2-triggered increase of PR expression observed after ICB-1 knockdown clearly demonstrates an enhanced activity of ERα. Thus, ERα is suggested to be the main trigger of the proliferative response, which was at least partially mediated by estrogenic upregulation of c-fos.
expression in SK-OV/ibKD cells. The role of ERα as main trigger was further confirmed in experiments with specific ERα agonist PPT. This drug strongly accelerated the growth of SK-OV/ibKD cells even to a larger extent than E2, clearly suggesting an ERα-dependent mechanism. Higher concentrations of ERβ agonist DPN (10–1000 nM) also increased proliferation of this cell line, although ERβ is considered to mediate anti-proliferative effects in breast and ovarian cancer cells and its expression decreased in SK-OV-3 cells stably expressing ICB-1 shRNA. Small concentrations of DPN (1 nM) did not significantly increase the growth of this clone. These observations can be explained by the fact that the selectivity of DPN for ERβ is only about 70-fold higher than for ERα, suggesting that the observed proliferative action of higher DPN doses is mediated by activation of ERα. To further examine the molecular mechanism underlying the observed E2 effect on proliferation, we co-treated these cells with the active metabolite of SERM tamoxifen, 4-OH tamoxifen (Clemons et al. 2002), and with pure anti-estrogen ICI 182 780 (Dauvois et al. 1993). Both 4-OH tamoxifen and to an even greater extent ICI 182 780 significantly inhibited E2-triggered growth of SK-OV/ibKD cells, thereby confirming their proliferation to be ERα-dependent. These findings are interesting, because ovarian cancer usually does not strongly respond to treatment with anti-estrogens (Hatch et al. 1991). The ESR1 gene in SK-OV-3 ovarian cancer cells has been described to contain a deletion in exon 1, but is reported to code for an at least in part functional ERα protein that binds both to E2 and an ERE, thereby mediating estrogen effects on early response gene like c-fos, but not on proliferation or PR expression (Hua et al. 1995). Our data suggest that the observed upregulation of ERα and downregulation of ERβ are at least two mechanisms underlying the observed gain of function of ERα in this cell line in terms of proliferation control and gene regulation. Due to our results from the analysis of ERα splice variant mRNA expression, we can rule out that a differential alteration of ERα-variant profile is responsible for the increased estrogen responsiveness of this cell line.

In MCF-7 breast cancer cells, ICB-1 knockdown did not significantly affect the growth. Our observation of increased ERα and PR mRNA levels and down-regulation of ERβ in MCF-7/ibKD cells demonstrated that ICB-1 gene also is able to affect steroid hormone receptor expression of breast cancer cells. Moreover, ICB-1 knockdown enhanced estrogen responsiveness of MCF-7 cells, as demonstrated by enhanced estrogenic activation of cyclin D1 expression (Sabbah et al. 1999, Lewis et al. 2005). However, upregulation of ERα and downregulation of ERβ were not sufficient to enhance the E2 effect on PR expression; regulation of cyclin D1 and anti-proliferative p21/WAF-1 was not sufficient to increase cellular proliferation. This might be due to the fact that MCF-7 cells already express high levels of functional ERα, resulting in a strong estrogen responsiveness of this cell line with regard to growth and PR expression. Thus, we propose that the elevated estrogen response, which we observed on the mRNA level, simply was not strong enough to further increase these E2 response markers in MCF-7 cells. Thus, it is plausible that treatment with 4-OH tamoxifen or ICI 182 780 as expected inhibited growth of ERα-positive MCF-7 cells in a dose-dependent manner, but did not differ with regard to ICB-1 expression.

In conclusion, we identified ICB-1 gene as a new player in hormone-dependent cancer. We demonstrated that ICB-1 gene function differentially affects regulation of steroid hormone receptor genes. We propose that ICB-1 is involved in limitation of ERα transcript levels and sustaining of ERβ expression, thereby reducing activation of ERα target genes and cellular proliferation. Furthermore, the results of this study clearly suggest that ICB-1 expression is at least one reason for the hormone unresponsiveness of SK-OV-3 ovarian cancer cells. Our data strongly encourage further studies on the role of ICB-1 gene in endocrine-related cancer.

Declaration of interest
We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This research did not receive any specific grant from any funding agency in the public, commercial, or not-for-profit sector.

Acknowledgements
We thank Gerhard Piendl and Helena Houlihan for expert technical assistance.

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