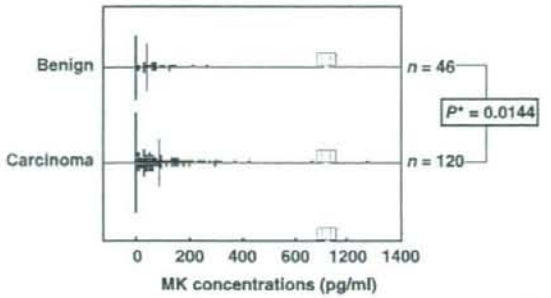

Fig. 3. Serum midkine (MK) protein concentrations from patients with benign gynecologic tumors or endometrial carcinoma. MK protein levels were measured by ELISA. The serum concentration for the carcinoma patients was significantly higher than that for the benign patients ($P = 0.014$, Mann–Whitney test).

esophageal squamous cell carcinoma, serum MK is a good marker of lymph node metastasis that correlates with serum levels of VEGF-C. Lymph node metastasis is a critical prognostic factor in endometrial carcinoma, and myometrial invasion and histological grade are correlated strongly with lymph node metastasis.[25,26] Thus, preoperative serum MK levels might prove to be useful for selecting high risk patients or predicting prognosis.

In conclusion, MK immunoreactivity in endometrial carcinoma is significantly higher than in normal endometrium. Additionally, preoperative serum MK levels are significantly correlated with prognosis and the presence of lymph node metastasis. Further, larger, prospective studies with longer follow-up periods are needed to fully understand the role of MK in endometrial carcinoma carcinogenesis.

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Estrogen signaling pathway and hormonal therapy

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Abstract Hormonal therapy, such as estrogen-targeting therapy, has undergone remarkable development in recent several years, using drugs such as LH–RH agonists, new SERMs and third-generation aromatase inhibitors. Several ongoing large-scale international clinical trials for hormonal therapy are establishing the standard protocol for treatments with these drugs. On the other hand, there have been attempts to predict the individual efficacy of hormonal therapy using classical molecular biomarkers such as ER and PgR. However, approximately one-third of ERα-positive patients do not respond to endocrine therapy, while some ERα-negative patients are responsive. These discrepancies may be due to the different estrogen-related intracellular signaling pathways in breast cancer cells. Furthermore, the ineffectiveness of hormonal therapy in some individuals (due to, for example, aromatase inhibitor resistance) may be caused by these mechanisms. In this paper, we discuss the molecular mechanisms of these different responses to hormonal therapies and their implications for the estrogen signaling pathway in breast cancer cells. Furthermore, we touch upon basic studies into predicting the efficacy of hormonal therapy and new strategies in this field.

Keywords Estrogen · Hormonal therapy · SERM · Aromatase · Phosphorylation

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Intracellular estrogen signaling pathways

Estrogen regulates various physiological responses in many target tissues, and is well known to play important roles in the development and progression of breast cancers [1]. As shown in Fig. 1, estrogen controls the expression of a wide variety of genes through distinct genomic and nongenomic pathways [2, 3]. In the classical genomic pathway, estrogen signals are mediated through the estrogen receptor (ER), which functions as a transcription factor for target genes. Estrogen also regulates the functions of factors in cells through various mechanisms, including protein phosphorylation, involving nongenomic and rapid actions [4]. ERα can be activated by the signal crosstalk between estrogen and growth factors such as epidermal growth factor (EGF) and insulin growth factor-1 (IGF-1) via receptor phosphorylation [5–7]. Recent findings have revealed that these extremely complicated signaling pathways are triggered by estrogen stimulation.

Estrogen signals via ligand-dependent genomic pathways

Estrogen exerts its biological effects by binding to ER, which mainly exists in the nucleus as a member of the nuclear receptor superfamily of transcription factors. ER acts through the formation of homo- or heterodimers of ERα and ERβ, and ERα has been widely used as a predictive marker for hormonal therapy. In the classical pathway, estrogen-bound ERs dimerize and function as a transcription

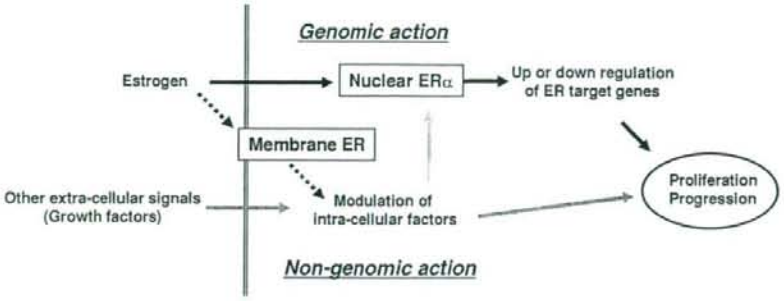

Fig. 1 Outline of estrogen signaling pathways. Estrogen evokes genomic and nongenomic actions via nuclear ER and membrane-associated ER. Moreover, these signals are also stimulated or modulated by crosstalk with the intracellular protein kinase-mediated phosphorylation signaling cascade

factor which binds to a specific DNA sequence named the estrogen response element (ERE) present in the promoter or enhancer regions of target genes. ER binds to ERE through its DNA-binding domain (DBD) and recruits coactivators such as SRC-1, AIB1 and p300/CBP to form a functional ER complex [8, 9]. It is now known that ER target genes which have full or half ERE sites include pS2, cathepsin D, PgR, Efp, EGR3, etc. [10–13].

In the genomic pathway, ER can also regulate transcription without binding directly to DNA. ER acts as a coactivator—ER interacts with other transcription factors such as AP-1, SP-1 and NF-κB via protein–protein interactions, and it could regulate the transcription of genes that lack ERE but has a binding element for its partner's protein. It has been suggested that genes activated in this way include ovalbumin, IGF-1, collagenase, VEGF, c-Myc, cyclin D1, c-fos, NF-κB, and LDL receptor [14–16].

Activation of ER by protein phosphorylation

Nuclear ERα has several different phosphorylation sites, as shown in Fig. 2, and is targeted by several kinases, including MAPK and PI3K/Akt. In particular, serines 104/106, 118 and 167 are located within the activation function 1 (AF-1) region of ERα, in the A/B domain. Thus, these phosphorylation sites could play an important role in the regulation of AF-1 transcription activity. For example, it has been shown that Ser118 of ERα is a major site of phosphorylation by MAPK cascade, and this phosphorylation exerts or upregulates the estrogen-responsive transcription activity of ERα [17, 18]. Some other reports have shown that PI3k-Akt pathway-stimulating Ser167 phosphorylation is a major estrogen-induced phosphorylation event [19]. It has been reported that this phosphorylation is also provided by the MAPK pathway [20]. Furthermore, phosphorylation signaling pathways are also suggested to stimulate ERα transcription activity through the phosphorylation of coactivators for ERα, such as AIB1, a member of the p160 family [21, 22].

These alternative ERα activation pathways arising from the intracellular phosphorylation signaling cascade could be related to the clinical response of breast cancer to hormonal therapy. It has recently been reported that the phosphorylation of Ser167 is related to the response to hormonal therapy, based on analysis by an immunohistochemical technique [23]. Although inter-individual differences in the estrogen signaling pathway in breast cancer have not yet been investigated, the analysis of this alternative pathway in patients is important for the personalized treatment of breast cancer in the future. For some ERα-positive cases in which this alternative pathway is dominant, growth factor inhibitors or protein-kinase inhibitors may be useful for combination therapies with hormone (Fig. 3).

Estrogen signals via nongenomic pathways

Estrogen also exerts rapid effects that are not accounted for by transcriptional mechanisms. As some signals from several ligands for the steroid receptor superfamily have been shown to be mediated through plasma membrane, there is accumulating evidence to support the idea that estrogen receptors are also located at the plasma membrane and are responsible for extranuclear, rapid and nongenomic actions. The existence of membrane ER has been demonstrated using cell-membrane impermeable BSA-conjugated estradiol [24, 25], although some concerns have been pointed out regarding these agents. Membrane ER has been reported to associate with many growth factor receptors, such as IGF-1R, EGFR, HER2. In the activation of IGF-1R, E2 induces the formation of a ternary complex among ERα, IGF1-R and Shc, the adaptor protein, in the plasma membrane, which induces phosphorylation of IGF-1R [26]. The estrogen-bounded membrane ER rapidly activates several signals in a cell type-specific manner, including calcium currents, cAMP, inositol phosphate, G proteins, Src, and Shc, which leads to the activation of downstream kinases, such as mitogen-activated protein kianse (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt [4].

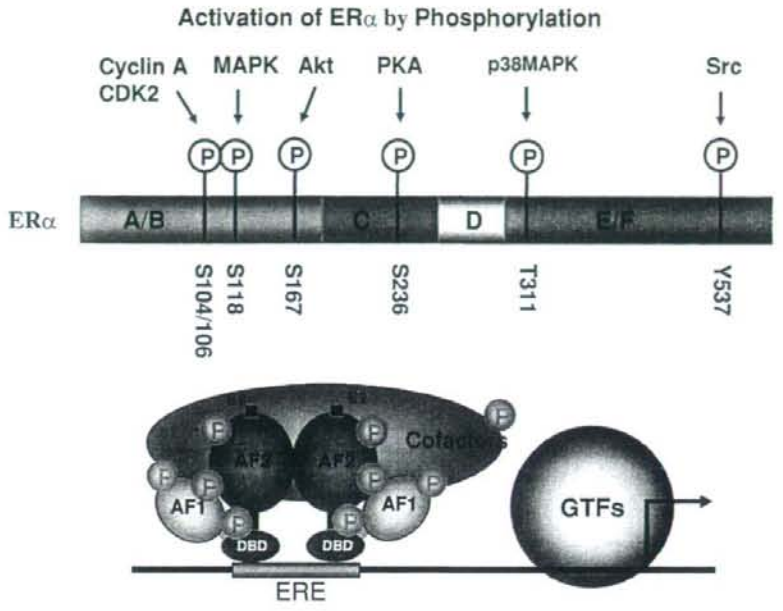

Fig. 2 Phosphorylation sites in ERα. Six amino acid residues have been reported to be phosphorylated in the ERα protein, and various specific protein kinase cascades are involved with their phosphorylation. The phosphorylated protein forms a more stable transcription complex with the coactivators and upregulates the transcription activity of ERα

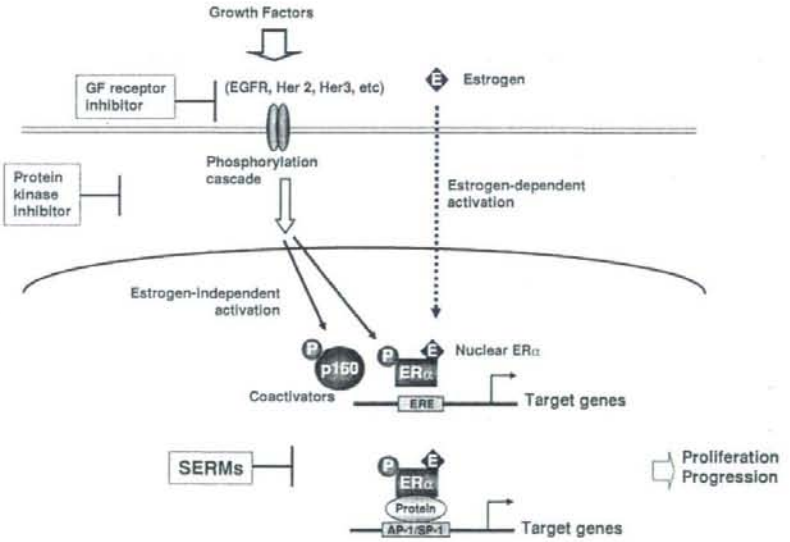

Fig. 3 Intracellular cross-talk of the estrogen signaling pathway with the phosphorylation cascade. In addition to ligand-dependent activation, ERα activity is modulated by post-translational modifications through the direct phosphorylation of ERα or coactivators in a ligand-independent manner. ERα activation by growth factors such as EGF, IGF, heregulin and TGF-α has been reported. The activated ER binds to not only ERE but also AP-1 or SP-1 binding elements, together with each specific protein

Although the origin of membrane ER is not completely resolved, most of the evidence supports the idea that the membrane ER is the same protein as the nuclear ER, based on evidence such as the detection of membrane ER by antibodies to nuclear ER and the codetection of membrane and nuclear ERs after nuclear ER cDNA transfection in ER

null cells. In either case, the membrane ER was only a small fraction of the total ER. Recently, several reports have suggested that GPR30, a G protein-coupled receptor, is another candidate for the rapid estrogen signals [27, 28]. However, one report indicated that estrogen signals could not be induced in human breast cancer SKBR-3 (ER negative, GPR30 positive) cell signals [29], and further studies are needed to clarify the role of GPR30 in rapid signaling by estrogen at the plasma membrane in breast cancer cells.

ER does not have a transmembrane domain, and is localized at the inner face of the plasma membrane through binding to other proteins such as caveolin-1 [30], the major structural protein of caveolae. Shc and growth factor receptors also facilitate the membrane localization of ER as described above, and the cytoplasmic proteins MTA1-S or MNAR (modulator of the nongenomic activity of ER)/PELP1 can sequester ER outside of the nucleus [31–33]. Overexpression of HER2 in ER-positive breast cancer cells, which is associated with the development of hormone-resistant breast cancer, promotes the cytoplasmic sequestration of ER, and the interaction of ER with HER2 activates mitogen-activated protein kinase extracellular signal-regulated kinase 1/2 (ERK1/2) [34].

As described above, the molecular mechanism of nongenomic estrogen action has not yet been established. Moreover, the clinical significance of this action has not been assessed in breast cancer, because of methodological issues.

Extracellular factors for estrogen signal stimulation

Recent data have demonstrated that the intercellular communication between tumor and stromal cells profoundly affects the proliferation of breast cancer cells, which is mediated by the production of estrogen, growth factors, chemokines, and cytokines [35–37]. Among the stromal cells, fibroblasts are the most abundant cell type, and tumor-associated fibroblasts are known to express aromatase, a key enzyme of estrogen synthesis, resulting in the intratumoral estrogen production frequently observed in postmenopausal breast cancers [38, 39]. Aromatase expression levels in breast cancer tissues are significantly higher than those in benign breast lesions. Several reports suggest that the tumor–stromal interaction regulates aromatase gene expression via the production of various factors such as prostaglandin E_2, COX2, tumor necrosis factor-α, interleukin-6 and interleukin-11 [40]. Therefore, aromatase is a target of endocrine therapy for breast cancers, and several aromatase inhibitors have been developed to attenuate estrogen biosynthesis, such as anastrozole, letrozole, and exemestane. The recent reports on the efficacies of these drugs again strongly indicate that the estrogen is a crucial factor in the survival of breast cancer [41].

ERE-GFP reporter cells (E10) and estrogen-depletion resistance

To investigate estrogen signaling in breast cancer and in order to improve predictions of the efficacy of aromatase inhibitors for individual breast cancers, we developed a comprehensive system to visualize the ER-activating ability (not only of estrogen, but also of other crosstalk signals, as described above) stimulated by the adjacent microenvironment. We first established a stable transformant, named E10, of human breast cancer MCF-7 cells which had an integrated ERE-GFP reporter gene. These E10 cells express GFP when endogenous ERα is activated. We characterized the stromal fibroblasts of individual breast cancers by establishing a co-culture system with E10 cells, and found that the induced GFP expression varied among the cases, indicating that the stromal fibroblasts in each case had their own properties with respect to the activation of estrogen signals [42].

Furthermore, we recently isolated and established estrogen-depletion resistant (EDR) E10 cells by long-term culture and passage under estrogen-depletion medium. Figure 4 shows the effect of antiestrogen on two established EDR-E10 cells. The 277-1 cells grew well under estrogen-depletion medium, and antiestrogen did not

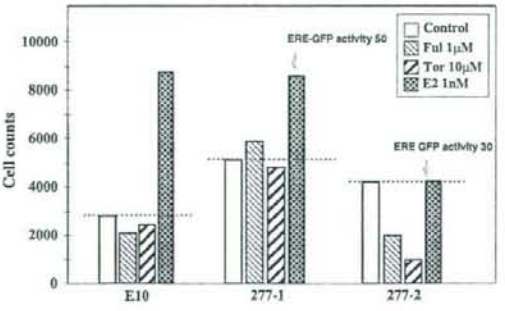

Fig. 4 Effect of antiestrogen on the growth of estrogen-depletion resistant (EDR) cells. The EDR cells, which had high ERE transactivation activities, were established from MCF-7-ERE-GFP-E10 cells by passing them through estrogen-depleting medium and cloning them, and then their ERE-GFP activities were monitored. Antiestrogens (toremifene and fulvestrant) were added to the estrogen-depleted culture medium, and the cell growth was assayed after two days. The ERE-GFP activities of cells to which 1 nM estradiol was added were also assayed, and the percentages of positive cells are shown in the plot

inhibit the cell growth, while the addition of estrogen even upregulated cell growth. On the other hand, the 277-2 cells showed significant growth inhibition with fulvestrant or a high dose of toremifen, and the growth of this cell line was not upregulated by the addition of estrogen. Moreover, they showed substantial ERE-GFP expression, indicating the presence of activated endogenous ERα. These observations evoked interesting speculation that the ERα in the 277-2 cells may be fully activated by an alternative signaling pathway such as a phosphorylation cascade. While the growth of the 277-1 cells is not critically dependent on the action of the estrogen receptor, the estrogen signaling pathway is active. These cell lines could provide a useful model for the study of multiple estrogen signaling pathways and the eligibility of hormonal therapy. The value of selective estrogen receptor modulators (SERMs) for the treatment of breast cancer must be revaluated to include aromatase inhibitor-resistant cases.

Prediction of hormonal therapy

Recent advances in molecular diagnostic tools such as Oncotype DX (Genomic Health Inc.) and MammaPrint (Agendia Inc.) have opened up a new vista for personalized medicine in the future. On the other hand, a more accurate predictive method or a novel predictive biomarker from among ER and PgR is also desired for predicting the individual efficacy of hormonal therapy. To address this issue, we studied the gene expression profiles of estrogen-responsive genes in breast cancer using a DNA microarray technique [43], and further study revealed several candidate genes for assessing response to hormonal therapy [44]. Recently, a molecular signature of neoadjuvant hormonal therapy for ER-positive patients has been reported [45]. The identified 50-gene subset could be useful for monitoring the short-term effects of hormonal therapy on ER-positive cancers. A new predictive biomarker for hormonal therapy may be found in these genes. Further studies using comprehensive techniques such as microarray analysis to search for new biomarkers will be common in the future.

However, these expression analyses are not able to reveal the specific pathway of estrogen signaling in the cells. Other novel methods, such as phosphorylation-specific proteomics or the in vitro bioassay system using GFP that we reported previously [42], will be needed to analyze the signaling pathway at work in individual cancer cells. In order to be able to predict the individual response to aromatase inhibitor or SERMs, the ability to distinguish between a so-called ligand (estrogen)-independent/ERα-dependent signaling pathway and a ligand-dependent/ERα-dependent signaling pathway is crucial.

Conclusion

The estrogen signaling pathway must be closely associated to the efficacy of hormonal therapy. The discrepancy between ERα expression status and response to hormonal therapy probably derives from the different estrogen signaling pathway conditions in the cells. The resistance to aromatase inhibitor or antiestrogen may be also caused by changes in this pathway. The last two decades have revealed the structure and function of the estrogen receptor to be a nuclear transcription factor which controls its target genes and plays an important role in various physiologic functions. However, as described in this paper, there are still many questions about and ambiguities regarding the estrogen signaling pathway. In particular, the elucidation of the mechanism of ligand-independent ER-activation and nongenomic estrogen action is extremely important for translational research in breast cancer. Further studies of the estrogen signaling pathway may provide new clues about the estrogen-dependent mechanisms of breast cancer development, and may dramatically advance the accuracy with which the efficacy of molecular therapeutics can be predicted, as well as new strategies in this field.

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REVIEW

Estrogen-Related Cancer Microenvironment of Breast Carcinoma

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Abstract. Stromal cells establish the microenvironment based on interaction with tumor cells, which is essential for cancer growth, invasion and metastasis. Fibroblasts are the primary component of stroma, and carcinoma-associated fibroblasts (CAFs) demonstrate tumor-promoting activities compared with normal counterparts. In breast cancers, stromal fibroblasts adjacent to the tumor express aromatase, a key enzyme in the synthesis of estrogen, resulting in in situ estrogen production, and aromatase is a primary target of endocrine therapy. Thus, stromal fibroblasts demonstrate multiple functions in the genesis and progression of breast cancers. In this paper, we discuss the importance of tumor-stromal crosstalk in breast cancers, and describe our system to predict the efficacy of endocrine therapy and to analyze estrogen signals in individual breast cancers.

Key words: Stromal fibroblast, Estrogen signal, Aromatase, Breast cancer

IN the microenvironment of carcinomas, tumor cells recruit various types of stromal cells, such as fibroblasts, inflammatory cells and endothelial cells, and their cellular interactions play a crucial role in the establishment of a supportive microenvironment to progress tumor growth. Expansion of tumor stroma is often observed in invasive carcinoma, which is identified as desmoplasia where tumor and stroma actively interact [1]. Among stromal cells, fibroblasts are the major cellular component, and have a profound influence on the phenotype of various carcinomas, inducing the production of various growth factors and chemokines in the microenvironment [2-4]. Fibroblasts isolated from carcinoma, named carcinoma-associated fibroblasts (CAF), show biological functions distinct from those of normal fibroblasts in tumor progression and angiogenesis [5, 6]. A recent study also reported that stromal fibroblasts lead the process of collective invasion of cancer cells via remodeling of the extracellular matrix [7].

In addition to growth factors, stromal fibroblasts in breast carcinomas actively induce local estrogen production, which contributes to the genesis and progression of breast carcinomas [8, 9], and signal crosstalk between estrogen and growth factors affects the progression of breast carcinoma in many aspects [10-12]. This review describes the roles of stromal fibroblasts in the progression of breast carcinoma based on interaction with tumor cells, and shows our system to analyze the function of these fibroblasts in individual breast cancers.

Role of tumor-stromal interaction in estrogen signaling in breast cancers

Estrogen regulates the expression of a wide variety of genes by binding to ER, a member of the nuclear receptor superfamily of transcription factors, through genomic and

non-genomic pathways, resulting in the progression of breast cancers [4, 13-16]. The incidence of breast cancer is high even in postmenopausal women when the ovaries have ceased to produce estrogen. This is due to estrogen production by adipose stromal fibroblasts adjacent to tumors which express aromatase, a key enzyme in estrogen biosynthesis [8, 9]. Aromatase expression in stromal fibroblasts is regulated by the production of various factors, such as prostaglandin E_2, COX2, tumor necrosis factor-alpha, interleukin-6 and interleukin-11 under tumor-stromal crosstalks [4, 17, 18] (Fig. 1). Expression levels of aromatse are significantly higher in the stroma in malignant breast carcinoma than in benign breast lesions [8, 9]. Endocrine therapies designed to block estrogen signals are the most effective treatments for breast cancers expressing the estrogen receptor (ER); therefore, aromatase is a target of endocrine therapy, and aromatase inhibitors, such as anastrozole, letrozole and exemestane, have been developed to attenuate the biosynthesis of estrogen. Several large-scale clinical trials have reported that aromatase inhibitors are effective for ER-positive advanced breast cancer in post-menopausal women [18-21].

In addition, tumor-stromal interactions provide various growth factors such as EGF and IGF-1, in the microenvironment (Fig. 1), and their signaling pathways could activate ERα via phosphorylation at several sites, including Ser118 and Ser167 [22]. Phosphorylated ERs dimerize and act as a transcription factor. Growth factors induce phosphorylation through activation of their downstream kinases, such as mitogen-activated protein kinas (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt [23-26]. Estrogen itself also stimulates breast cancer cells to produce growth factors [27, 28].

System to detect ER activating ability of stromal fibroblasts

Several tumor-stromal interactions affect ERα function in both estrogen-dependent and -independent pathways, as described above, but an appropriate system to detect overall ERα activity has not been available. To predict the efficacy of aromatase inhibitors and to analyze estrogen signals in individual breast cancers, we developed a comprehensive system to visualize the ER-activating ability of stromal fibroblasts. We first established a reporter cell line, named E10, derived from human breast cancer MCF-7 cells by transfection with the ERE-GFP gene. E10 cells express GFP when cocultured with stromal fibroblasts isolated from breast cancer tissues in the presence of testosterone, a substrate for aromatase [29] (Fig. 2A). Using this system, we characterized the stromal fibroblasts of individual human breast cancers, and found that the levels of GFP expression induced by coculture varied among the cases, indicating that stromal fibroblasts in each case have their own properties with respect to the activation of estrogen signals (Fig. 2B).

We analyzed the relationship between the ER-activating ability of stromal fibroblasts and clinicopathological variables, and found that ER-activating ability is higher in postmenopausal patients than in premenopausal patients, which is consistent with a report that intratumoral estrogen production causes the progression of postmenopausal breast cancers (Table 1). Furthermore, grade 3 cases showed lower ER activation than those of grade 1(Table 1), suggesting that estrogen signals are not active within grade 3 cases, and that their microenvironment may supply other growth factors to stimulate the proliferation of breast cancer cells. Aromatase inhibitors dose-dependently inhibit GFP expression, suggesting that this system might be useful for predicting individual responses to them (Fig. 2C).

Automated system to detect ER-activating ability of stromal fibroblasts

ER activation levels in our system are determined manually by counting the number of cells expressing GFP, but this process is time consuming and rather complicated. To more easily evaluate GFP expression, we next developed an automated image analysis system of GFP expression in collaboration with Olympus Life Science Company (Tokyo, Japan) (Fig. 3A). Because stromal fibroblasts induce GFP expression in E10 cells without direct cell-to-cell contact, E10 cells in the outer wells of a 24-well culture plate are cocultured with stromal fibroblasts plated in insert wells with a 0.4 micron membrane at the bottom. The insert wells are removed after coculture, and the intensity of GFP in E10 cells is automatically quantified using fluorescence microscopy. Each cell is identified by pre-incubation with a cell -permeable DNA probe, DRAQ5 (Fig. 3 A).

In this system, estrogen-induced GFP expression and the growth of E10 cells were similarly observed (Fig. 3B), and the inhibitory effect of exesmestane, an aromatase inhibitor, could be detected (Fig. 3C). Recently, we observed the ER-stimulating ability of stromal fibroblasts isolated from breast cancer tissue obtained by core-needle biopsy. Although further detailed study is required, this ERE-GFP assay system might be a useful tool for analyzing the estrogen-related microenvironment in individual breast cancers and the mechanisms of tumor-stromal interactions in tumor progression.

Another role of carcinoma-associated fibroblast in breast cancers

CAFs exhibit biological characteristics distinct from normal fibroblasts, and are mainly identified as myofibroblasts expressing α-smooth-muscle actin [5, 6]. In a tumor xenograft model in which breast cancer cells were coimplanted with CAFs or normal fibroblasts, xenografts containing CAFs grew larger than those containing normal fibroblasts [5].

Recently, accumulated evidence indicated that the chemokine CXCL12/SDF-1 and its signal receptor CXCR4, a G-protein-coupled receptor, have prominent roles in the crosstalk between tumor cells and stromal cells in the progression of breast cancers, and stromal fibroblasts in human breast cancers (CAFs) express high levels of CXCL12/SDF-1 while breast cancer cells highly express CXCR4 [3, 5, 6, 30] (Fig. 4). CXCL12/SDF-1 has also been reported to be a target gene of estrogen action, which stimulates the growth of breast cancer cells directly through binding to its receptor, CXCR4, via an autocrine mechanism [31]. Furthermore, CXCL12/SDF-1 can recruit endothelial progenitor cells to breast carcinomas that are required for angiogenesis [5, 6] (Fig. 4). Another study based on gene-expression profiles reported that the expression of CXCL1/SDF-12 is upregulated in stromal fibroblasts found in invasive breast cancers [3]. CXCR4 activates several signal transduction pathways including PI3K and MAP kinase [30]. CXCL12/SDF-1 transactivates HER2, which is overexpressed in 20－25% of invasive breast cancers and is an important therapeutic target [32], while HER2 enhances the expression of CXCR4 [33]. Hepatocyte growth factor (HGF), known to stimulate the growth of mouse breast cancer cells, induces the expression of CXCR4 [34]. Thus, CXCL12/SDF-1 and CXCR4 signaling pathways play an important role in the proliferation, immigration and invasion of breast cancer cells, and are now recognized as having a key role in breast cancer progression in vivo. These pathways might be involved in phosphorylation-induced ER activation and the tumor-supporting ability of the microenvironment of grade 3 breast cancers in which stromal fibroblasts showed low ER-activating ability, as shown in our coculture system.

Conclusion

The expression status of ERα is a primary determinant in endocrine therapy for breast cancers, but approximately one third of ERα-positive patients do not respond to

endocrine therapy. This is due to the fact that the progression of breast carcinoma is associated with the effects of various cellular components of the microenvironment. Fibroblasts, the major component of stroma, play an important role in the process of tumor growth, and recent studies have suggested that CAFs are potential therapeutic targets for a variety of carcinomas. In the microenviroment, fibroblasts might be highly heterogeneous, and a study on the relationship between stromal fibroblasts expressing aromatase and CAFs stimulating tumor growth will be useful to improve hormone therapy for breast cancers. The ERE-GFP assay system described in this review might be useful to explore the complex bi-directional signaling between breast cancer cells and stromal fibroblasts involved in modulating the tumor phenotype. Furthermore, to overcome the problem of breast carcinoma refractory to endocrine therapy, combination therapy of endocrine therapy and molecular-targeting therapy to attenuate tumor-stromal interactions using growth factor receptor kinase inhibitors, COX-2 inhibitors, or chemotherapy drugs might be useful for breast cancer treatment.

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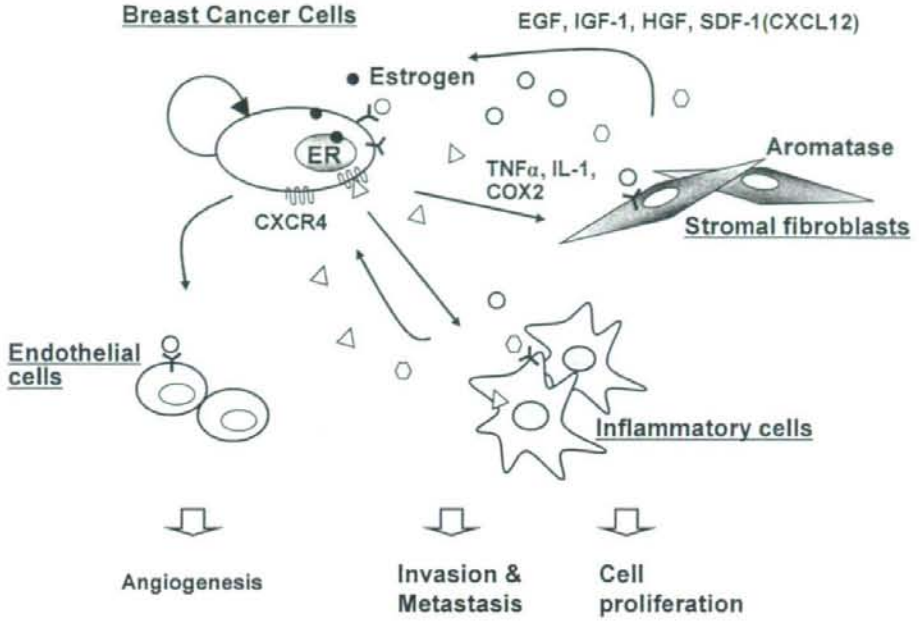

Fig. 1. In the microenvironment of breast carcinoma, cancer cells interact with various types of stromal cells through the secretion of growth factors and cytokines. Among stromal cells, fibroblasts adjacent to the tumor produce estrogen via the expression of aromatase, a key enzyme of estrogen synthesis, which is induced by factors such as TNFα, IL-1 and COX2. These interactions finally lead to tumor cell proliferation, angiogenesis and metastasis.

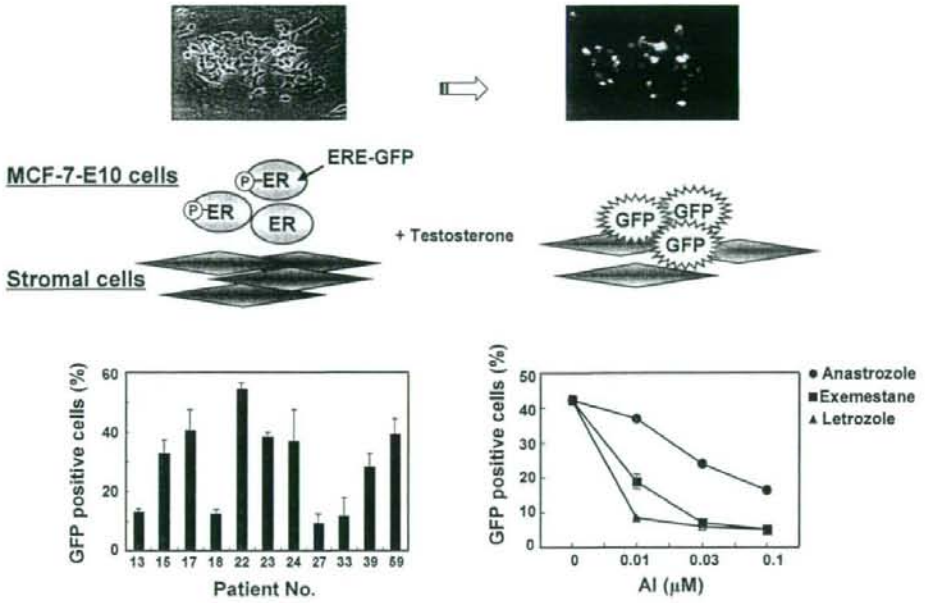

Fig. 2. System to detect the ability of stromal fibroblasts isolated from breast cancer tissues to activate ER. (A) In the presence of testosterone, a substrate of aromatase, MCF-7-ERE-GFP-E10 cells express GFP when cocultured with stromal fibroblasts with the ability to activate ER via both estrogen-dependent and -independent pathways. (B) GFP expression levels varied among cases. (C) Aromatase inhibitors effectively inhibited ER activation in the coculture system.

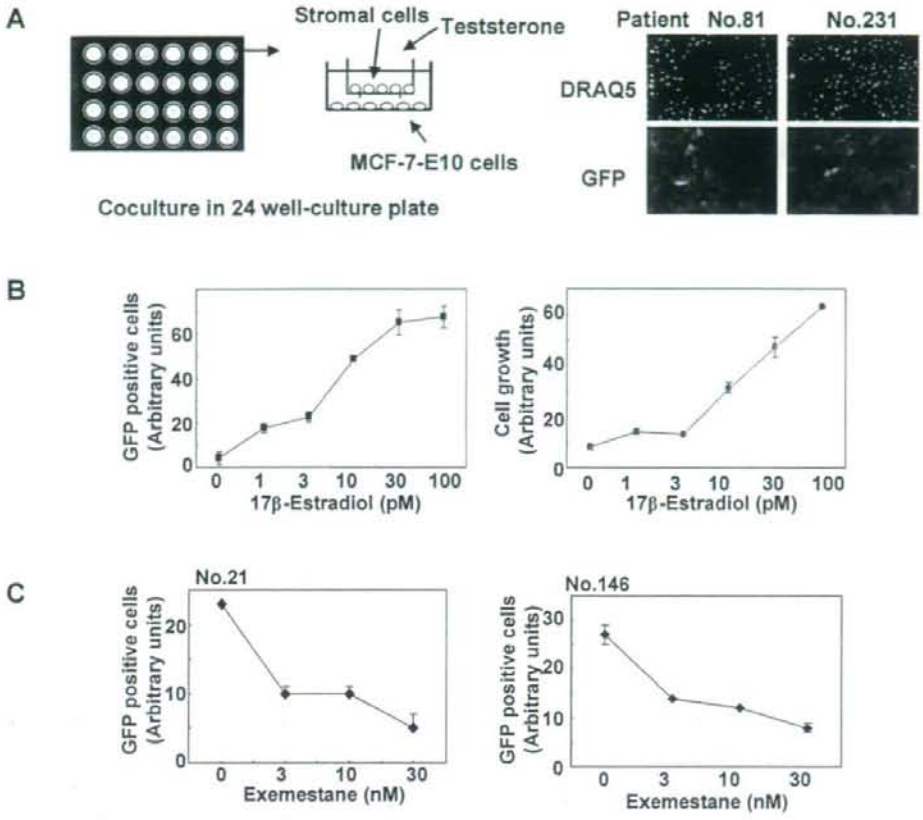

Fig. 3. Automated system to detect ER-activating ability of stromal fibroblasts. (A) MCF-7-ERE-GFP-E10 cells are separately cocultured with stromal fibroblasts in inner wells in the presence of testosterone. After coculture, the inner wells are removed, and the intensities of GFP in E10 cells plated in outer wells are automatically quantified using fluorescence microscopy. Each cell is identified by pre-incubation with a cell-permeable DNA probe, DRAQ5. (B) The effects of estrogen on GFP expression and growth stimulation in E10 cells were observed in a similar dose-dependent manner in this system. (C) The inhibitory effects of exemestane on GFP expression were detected.

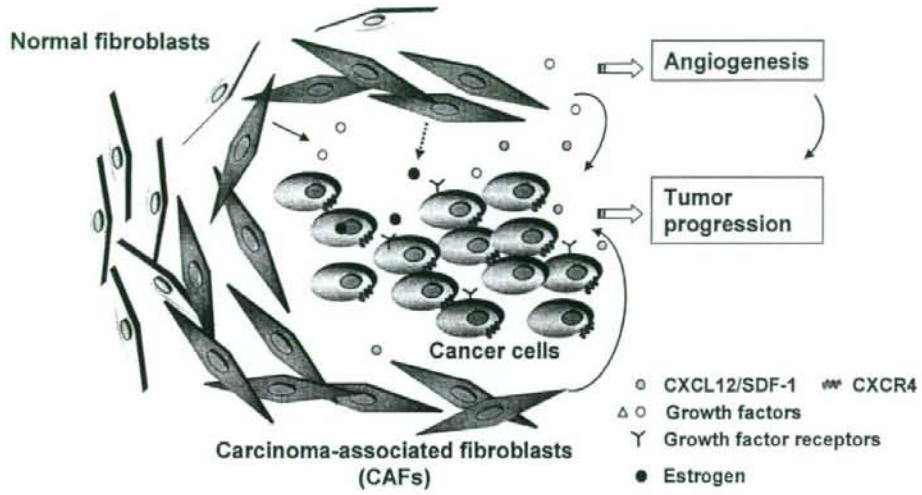

Fig. 4. Carcinoma-associated fibroblasts (CAFs) distinct from normal fibroblasts secrete CXCL12/SDF-1 and growth factors, leading to tumor progression and angiogenesis.

Table 1. Relationship between menopausal status or grade and high ER-stimulating ability of stromal cells

Parameters	Active cases/Total cases (%)	
Menopausal status		
Pre	2/25 (8.0)	P=0.0452
Post	12/42 (28.6)	
Grade		
1	6/13 (46.0)	P=0.0025
2	4/6 (66.7)	
3	3/36 (8.3)	P=0.0004

Active cases are those in which stromal fibroblasts induced GFP expression in >30% of E10 cells. Percentages of active cases are shown in parentheses.

nature
cell biology

The ubiquitin ligase CHIP acts as an upstream regulator of oncogenic pathways

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CHIP is a U-box-type ubiquitin ligase that induces ubiquitylation and degradation of its substrates, which include several oncogenic proteins[1–12]. The relationship between CHIP and tumour progression, however, has not been elucidated. Here, we show that CHIP suppresses tumour progression in human breast cancer by inhibiting oncogenic pathways. CHIP levels were negatively correlated with the malignancy of human breast tumour tissues. In a nude mouse xenograft model, tumour growth and metastasis were significantly inhibited by CHIP expression. In contrast, knockdown of *CHIP* (sh*CHIP*) in breast cancer cells resulted in rapid tumour growth and metastastic phenotypes in mice. In cell-based experiments, anchorage-independent growth and invasiveness of sh*CHIP* cells was significantly elevated due to increased expression of *Bcl2*, *Akt1*, *Smad* and *Twist*. Proteomic analysis identified the transcriptional co-activator SRC-3 (refs13–19) as a direct target for ubiquitylation and degradation by CHIP. Knocking down *SRC-3* in sh*CHIP* cells reduced the expression of *Smad* and *Twist*, and suppressed tumour metastasis *in vivo*. Conversely, SRC-3 co-expression prevented CHIP-induced suppression of metastasis formation. These observations demonstrate that CHIP inhibits anchorage-independent cell growth and metastatic potential by degrading oncogenic proteins including SRC-3.

We examined the mRNA and protein levels of CHIP (carboxyl terminus of Hsc70-interacting protein) in human breast cancer tissues, as it controls protein levels of several oncogenic proteins[6–12], including oestrogen receptor α (ERα). Samples of breast cancer tumours and normal tissue were obtained from 27 patients with breast cancer (Supplementary Information, Table S1), and the expression levels of *CHIP* mRNA were examined. In cancerous tissues from patients clinically determined to be stage I (Fig. 1a), or from node-negative patients (Fig. 1b), we found that the level of *CHIP* mRNA expression was comparable to that observed in the corresponding normal breast tissue ($P = 0.83$, Fig. 1a and $P = 0.16$, Fig. 1b). Tumours from stage II patients (Fig. 1a) or those from node-positive (N1a) patients (Fig. 1b) showed decreased levels of *CHIP* mRNA, compared with the non-cancerous tissues ($P < 0.001$, Fig. 1a, b). These results demonstrate a significant negative correlation between *CHIP* mRNA levels and tumour malignancy in human breast cancer tissues. We also compared the correlation between *CHIP* mRNA levels and tumour grade using the Oncomine database, which provides publicly available cancer gene expression datasets. Nine of 21 datasets that contain gene chip profiles classified by breast tumour grade, showed an inverse correlation between *CHIP* mRNA expression and tumour grade, consistent with our results. Two datasets characterized by large population sizes showed a significant inverse correlation between *CHIP* expression levels and tumour grade (Desmedt_Breast and vantVeer_Breast, $P = 0.00035$ and $P = 0.00093$, respectively). Immunohistochemical staining indicates that expression levels of CHIP proteins also decreased in malignant tumours (Fig. 1c). Thus, these results demonstrate that CHIP protein and mRNA levels were reduced in the invasive tumour region. As CHIP is a ubiquitin ligase for ERα, we expected the inverse correlation between *CHIP* mRNA and ERα protein levels. We did not observe a significant correlation between *CHIP* expression and ERα positivity ($P = 0.18$; Fig. 1d). Moreover, none of the 32 datasets from the Oncomine database (each dataset contains gene chip profiles classified by ERα positivity) demonstrated an inverse correlation between *CHIP* expression levels and ERα positivity.

We then examined whether tumour progression was enhanced when the expression level of CHIP was downregulated. Protein levels of CHIP were much higher in MCF-7 cells, a non-aggressive cell line derived from human breast cancer cells, than in MDA-MB-231 cells, a highly aggressive cell line (Fig. 2a). We generated three MCF-7 clones in which endogenous *CHIP* expression was knocked down by a short hairpin RNA (shRNA; Fig. 2b). To investigate the effects of *CHIP* knockdown *in vivo*, ten nude

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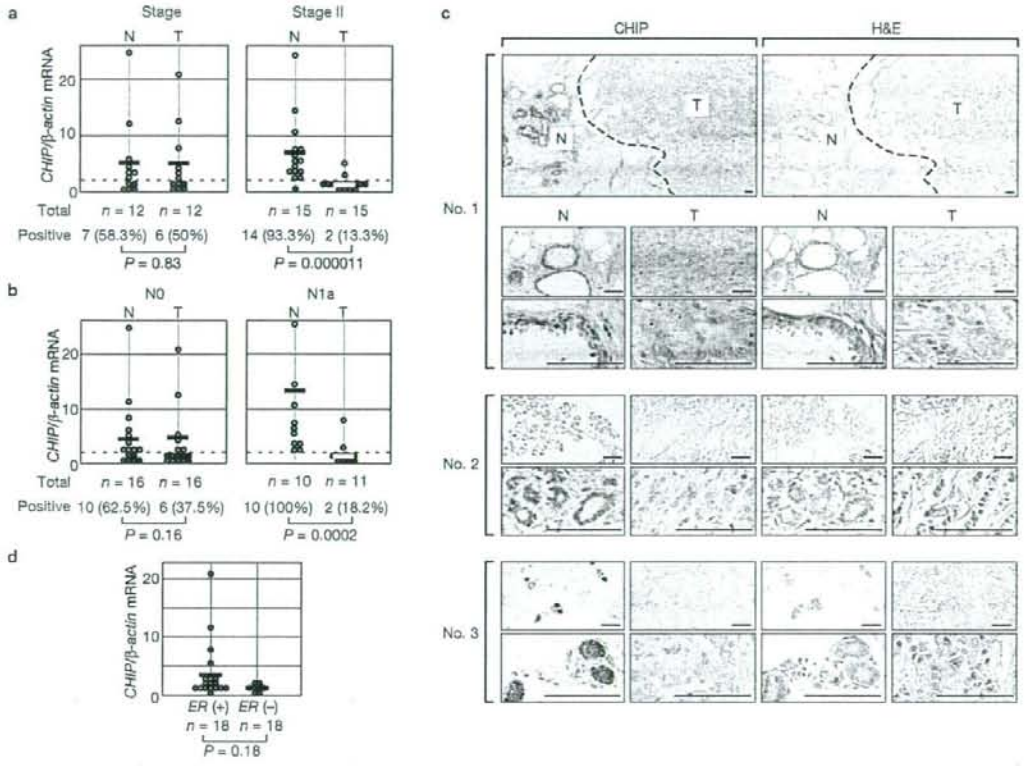

Figure 1 Decreased CHIP expression in malignant breast tumour tissues. (**a**, **b**) *CHIP* mRNA expression in breast tumours. Total RNA was prepared from normal (N) or tumour (T) tissues and the *CHIP* mRNA level was quantified using real-time RT–PCRs. Total RNA was prepared from tissues of stage I and stage II patients (**a**) or from node-negative (N0) and node-positive (N1a) patients (**b**). The cut-off points were determined using ROC (receiver operating characteristics) curve-based statistical analysis. Statistical significance of the differences between the groups was determined by *t*-test. Open circles, CHIP-positive; filled circles, CHIP-negative. (**c**) Representative images of immunohistochemical labelling of CHIP and haematoxylin and eosin (H&E) staining. The tumour tissues were derived from stage II or N1a patients (tissue samples nos 1–3). Normal (N) or tumour (T) tissues are marked with dotted lines. Scale bars, 100 μm. (**d**) Relationship between *ERα* and *CHIP* mRNA expression in breast tumours. Total RNA was prepared from *ERα*-positive or *ERα*-negative tumour tissues and the *CHIP* mRNA level was quantified using real-time RT–PCRs.

mice received bilateral subcutaneous flank injections of sh*CHIP* MCF-7 cells or control cells. The tumours in three groups that were injected with sh*CHIP* cells increased in size, whereas little tumour growth was observed in mice injected with control cells (Fig. 2c, d). Histological examination revealed that, compared with control cells, the sh*CHIP* tumour cells had a higher degree of nuclear atypia and a higher mitotic index (Fig. 2e). In addition, immunostaining of α-smooth muscle actin (SMA) demonstrated that the density of vessels in the tumours was higher in the sh*CHIP* tumours than in control tumours (Fig. 2f).

To further confirm the suppressive function of CHIP in tumour progression, we generated MDA-MB-231 cells that overexpressed CHIP ($CHIP^{OE}$; Fig. 2g). These cells were used in a nude mouse xenograft model. Tumours were significantly larger in mice injected with control MDA-MB-231 cells; however, little tumour growth was observed in the mice injected with $CHIP^{OE}$ cells (Fig. 2h, i). These results suggest that CHIP suppresses tumour progression.

We next used MCF-7 cells to investigate the roles of CHIP in anchorage-independent cell growth and invasiveness of cancer cells, both hallmarks of tumour progression. Control cells and sh*CHIP* cells proliferated at similar rates under normal culture conditions (Fig. 3a). sh*CHIP* increased the number of colonies in soft agar (colony-formation assay; Fig. 3b), suggesting that *CHIP* knockdown enhances anchorage-independent cell growth. Next, to examine the invasive and metastatic potential of these cells, we performed migration and invasion assays. In a migration assay, we found that sh*CHIP* increased the ability of cells to migrate (Fig. 3c). We also investigated the invasiveness of these cells (invasion assay), and found that sh*CHIP* significantly increased the number of cells that penetrated the Matrigel-coated membrane (Fig. 3d). The results of these assays indicate that the metastatic potential of the cancer cells was significantly enhanced by *CHIP* knockdown.

We performed a similar set of experiments using sh*CHIP* and $CHIP^{OE}$ MDA-MB-231 cells (Fig. 3e–3h). Changing the CHIP level did not markedly affect cell growth under normal culture conditions (Fig. 3f). However, knockdown increased, whereas overexpression decreased anchorage-independent cell growth (Fig. 3g) and metastatic potential (Fig.3h) of MDA-MB-231 cells. Thus, these results indicate that