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Com-1/P8 in oestrogen regulated growth of breast cancer cells, the ER-β connection

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Abstract

Background: Com-1 is a molecule that has recently discovered to have putative action on the metastatic nature of cancer cells. The current study investigated the impact of Com-1 on oestrogen regulated cell growth of breast cancer cells and explored the potential link between Com-1 and ER-β.

Method: Full length Com-1 cDNA was isolated from normal mammary tissues. Ribozyme transgenes that specifically targeted human Com-1 were constructed using the pEF6/V5-His vector. Expression of Com-1 was assessed at both mRNA and protein levels. Interaction of Com-1 with other candidate molecules was studied using immunoprecipitation and Western blotting.

Results: Elimination of Com-1 by way of ribozyme transgenes results in breast cancer cells with increased rate of growth and increased invasive potential. In contrast, over-expression of Com-1 in the cancer cells had an opposite effect. In ER-α-negative/ER-β positive MDA MB-231 cells, elimination of Com-1 caused more vigorous growth in response to 17-β-estradiol. However, the effect of Com-1 modification on MCF-7, which is positive for both ER-α and ER-β, was less clear. Protein interaction analysis has indicated that the Com-1 and ER-β were mutually co-precipitated with each other in breast cancer cells. Immunocytochemical staining revealed that Com-1 was primarily present in the nucleus, with some degree of cytoplasmic staining, and that the distribution of Com-1 was identical to that of ER-β. 17-β-Estradiol stimulation resulted in reduction of nucleic staining of Com-1. This reduction of nucleic Com-1 can be reverted when ubiquitin inhibitor, ubiquitin aldehyde or the lactacystin proteosome inhibitor was present, suggesting a pivotal role of the ubiquitin–proteosome pathway in the Com-1/ER-β complex.

Conclusion: Com-1 plays a tumour suppressor role in breast cancer cells and is involved in oestrogen-regulated cell growth. This action is potentially exerted by interacting with ER-β, in human breast cancer cells. The fate of Com-1 can be dually regulated by oestrogen and ubiquitin pathway.
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Keywords: Com-1; p8; Breast cancer; Oestrogen; ER-β; Invasion; Ubiquitination; Proteosome inhibitor; Hepatocyte growth factor

Com-1, candidate of metastasis-1, otherwise known as p8, is a molecule initially identified from metastatic breast cancer, by differential display method [1]. This was subsequently found to be identical to another nucleus protein known as p8, which was highly expressed in leukaemic cells and was thought to have mitogenic activities [2,3].

The molecule was initially thought of to act as a pro-metastasis candidate of breast cancer, as it was first demonstrated in metastatic tumours and appears to be expressed at a higher level in aggressive tumours using conventional RT-PCR. This was partly supported by studies on pancreatic cancer in which pancreatic cancer cells expressed higher levels of Com-1/p8 [4,5]. However, recent evidence began to suggest a different role of Com-1/p8 otherwise. First, expression of Com-1/p8 in pancreatic cancer cells resulted in growth inhibition, rather

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than promotion, and knock-out of Com-1 using antisense generated a faster growth rate [5]. Fibroblasts, when transfected with Com-1, had slower growth rate and higher rate of apoptosis [6]. Second, different tumours appear to have different expression patterns of the molecule. For example, thyroid cancer has a very different expression pattern from that of pancreas. Third, some tumour inhibitory agent, such as 1,25-dihydroxyvitamin D3 and TGF-β, can up-regulate the expression Com-1/p8 [7,8]. This latter evidence thus suggests that Com-1 may behave very differently from what was initially anticipated and earlier results may either reflect the complex nature of the molecule and/or the different methods used in these early studies.

We have recently observed, using both immunohistochemistry and quantitative analysis of transcript of Com-1, that low levels of Com-1 in breast tumours were associated with poor prognosis and significantly reduced survival, particularly in ER-α negative and ER-β positive tumours [9]. This has inspired us to postulate that Com-1 may be strongly linked to ER-β, both being nucleus proteins. The nucleus connection is further indicated by a recent study showing that Com-1 is associated with 1,25-dihydroxyvitamin D3 induced growth inhibition of breast cancer cells, in that 1,25-dihydroxyvitamin D3 induced a rapid and substantial rise of Com-1, together with the reduction of the growth rate [10].

Here, we report that knock-out Com-1 from breast cancer resulted in cells with a faster growth rate. Interestingly, in ER-α negative/ER-β-positive MDA MB-231 cells, Com-1 knockout resulted in a more vigorous response to oestrogen. We went on to demonstrate that Com-1 interacted and co-localised with ER-β, and that oestrogen stimulation resulted in the reduction of nucleus Com-1 staining, possibly by ubiquitin dependent pathways.

Materials and methods

Materials. RNA extraction kit and RT kit were obtained from AbGene, Surrey, England, UK. PCR primers were designed using Beacon Designer (Palo Alto, California, USA) and synthesised by Invitrogen (Paisley, UK). Molecular biology grade agarose and DNA ladder were from Invitrogen. Master mix for routine PCR and quantitative PCR was from AbGene. Anti-human Com-1 (p8), anti-ER-β, and anti-ER antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, California, USA). Peroxidase conjugated anti-rabbit and anti-goat antibodies were from Sigma and a biotin universal staining kit was from Vector Laboratories (Nottingham, England, UK). 17-β-Estradiol and the ubiquitin inhibitor, ubiquitin aldehyde (Ual), and lactacystin proteosome inhibitor (LPI) were purchased from Sigma–Aldrich and Santa Cruz Biotechnologies, respectively.

RNA extraction and cDNA synthesis. Total RNA was extracted from cells using an RNA extraction kit (AbGene). The concentration of RNA was determined using a UV spectrophotometer. Reverse transcription was carried using a RT kit with an anchored oligo(dt) primer supplied by AbGene, using 1 μg total RNA. The quality of cDNA was verified by using β-actin primers (Table 1). cDNA was synthesised using a first strand synthesis kit with an oligo(dt) primer (Sigma, Poole, England, UK). PCR products were then separated on a 2% agarose gel, visualised under UV light, photographed using a Unisave camera (Wolf Laboratories, York, England, UK), and documented with Photoshop software.

Quantitative analysis of Com-1 transcript. The level of Com-1 transcripts from the above-prepared cDNA was determined using a real-time quantitative PCR, based on the Amplifluor technology as we recently reported [10,11], modified from a method previously reported [12]. Briefly, pairs of PCR primers were designed using the Beacon Designer software (version 2, California, USA) (sequence given in Table 1), but to one of the primer, an additional sequence, known as the Z sequence (5′actgaacctgaccgtaca′3, underlined in Table 1) which is complementary to the universal Z probe (Intergen, England, UK), was added. A Taqman detection kit for β-actin was purchased from Perkin-Elmer. The reaction was carried out using the following: Hot-start Q-master mix (Abgene), 10 pmol of specific forward primer, 1 pmol reverse primer which has the Z sequence, 10 pmol FAM-tagged probe (Intergen), and cDNA from approximate 50 ng RNA. The reaction was carried out using IcyclerIQ (Bio-Rad) which is equipped with an optic unit that allows real time detection of 96 reactions, using the following condition: 94 °C for 12 min, 50 cycles of 94 °C for 15 s, 55 °C for 40 s, and 72 °C for 20 s. The levels of the transcripts were generated from a standard that was simultaneously amplified with the samples.

Immunocytochemistry. Cells were seeded in a chamber slide and allowed to adhere overnight. They were then fixed with 4% buffered formalin and briefly permeabilised with 0.1% Triton X-100 in a TBS buffer for 5 min. The cells were incubated for 20 min in a blocking buffer containing 10% horse serum and probed with the primary antibodies. Following extensive washings, cells were incubated for 30 min in the secondary biotinylated antibody (Multilink Swine anti-goat/mouse/rabbit immunoglobulin, Vector Laboratories.). Following washings, Avidin–Biotin Complex (Vector Laboratories) was then applied to the cells followed by extensive washings. Diaminobenzidine chromogen (Vector Labs) was then added to the cells which were incubated in the dark for 5 min. The slides were then mounted and photographed for quantitation purpose (without counterstaining). The same slides were then de-mounted in xylene overnight to remove coverslip, then counterstained in Gill's Haematoxylin, and dehydrated in ascending grades of methanol before clearing in xylene and mounting under a coverslip for photography. Negative controls were wells without the primary antibody.

Table 1
PCR primer sequence

	Sense	Antisense
Com-1 quantitation and screening	cctggatgaatctgacctc	actgaacctgaccgtacacaagcagcttctctcttggtg
Com-1 expression	atggccaccttcccac	Actgcgccgtgcccctcg
Com-1 ribozyme-8	ctgcagcttctctcttggtgcctgatgagtccgtgagga	actagtggaggccggaaaggtttcgtcctcacggact
Com-1 ribozyme-9	ctgcagctggttgctggtggctgatgagtccgtgagga	actagtatggccacctttttcgtcctcacggact
Ribozyme screening	ctgatgagtccgtgaggacgaa	ttcgtcctcacggactcatcag
ER-α	cctactacctggagaacgag	ctcttcggtcttttcgtatg
ER-β	aaaagaatcattcaatgaca	attaacacctccatccaaca
β-Actin	gctgatttgatggagttgga	tcagctacttgttcttgagtgaa

Staining intensity was semi-quantified from non-counterstained images, using a method established in our laboratory [13,14]. Briefly, grey scale digitised images were imported into the Optimas software (Optimas 6.0). Staining intensity was analysed in both nucleus and cytoplasmic compartments. Control staining (without primary antibody) was used for extraction of the background staining. Intensity data were stored in or input into Excel for statistical analysis and are shown here as mean intensity of either the cytoplasmic region or nucleus staining.

Construction of hammerhead ribozyme transgenes targeting human Com-1/p8 and human Com-1 expression cassette. Hammerhead ribozymes that specifically target a GTC and a UUC site of human Com-1 (GenBank Accession AF135266, position 189 and 68, respectively), based on the secondary structure of Com-1, have been generated as previously described [15,16]. Touch-down PCR was used to generate the ribozymes with the following respective primers (Table 1). This was subsequently cloned into pEF6/V5-His vector (selection markers: ampicillin and blasticidin, for prokaryotic and mammalian cells, respectively), and amplified in *Escherichia coli*, purified, verified, and used for electroporation of breast cancer cells. Full-length human Com-1 cDNA was generated from normal mammary tissue using primers, Com1ExF and Com1ExR (Table 1). Sequence verified product was then TA cloned into pEF6/V5-His and similarly prepared as with ribozyme transgenes and used for transfection. The above procedure generated with the following stably transfected cells Com-1 knockout cells, MDA MB-231$^{\Delta com1}$ and MCF-7$^{\Delta com1}$, Com-1 over-expressing cells, MDA MB-231^{com1Exp} and MCF-7^{com1Exp}, and plasmid only control cells, MDA MB-231^{pEFa} and MCF-7^{pEFa}, used together with the respective wild types MDA MB-231-WT and MCF-7-WT.

Immunoprecipitation and Western blotting. Cells were treated with 17-β-estradiol with or without the presence of 10 μM lactacystin proteosome inhibitor (Santa Cruz Biotechnology) for overnight, before being extracted using a lysis buffer that contained 2.4 mg/ml Tris, 4.4 mg/ml NaCl, 5 mg/ml sodium deoxycholate, 20 μg/ml sodium azide, 1.5% Triton, 100 μg/ml PMSF, 1 μg/ml leupeptin, and 1 μg/ml aprotinin for 30 min [17]. Protein concentrations were measured using fluorescamine and quantified by using a multi-fluoroscanner (Denly, Sussex, UK). Equal amounts of protein from each cell sample (controls and treated) were prepared for electrophoresis. They were then boiled at 100 °C for 5 min before clarification at 13,000*g* for 10 min.

Immunoprecipitation was performed as we previously described [18]. To the total cell lysates was added either anti-Com-1 antibody or anti-ER-β antibody (20 μg) followed by mixing for 1 h. To the mixture was added 20 μl of protein A/G–agarose with additional mixing. The Ab/Ag/agarose complex was collected and washed twice, followed by solubilisation with a sample buffer that contained 5% 2-mercaptoethanol.

For detection of anti-Com-1 immunoprecipitate, the agarose samples were separated over 10% SDS–PAGE gel and subsequently probed with anti-ER-β or other respective antibodies. For detection of Com-1, which is a small protein, from the cell lysate and from ER-β precipitate, a modified 12% Tricine PAGE gel was used. Separation was carried out using a Tricine-based cathode buffer and a Tris–HCl anode buffer.

In vitro invasion analysis and cell growth assay. This was performed as previously reported and modified in our laboratory [19]. Briefly, transwell inserts (upper chamber) with 8 μm pore size were coated with 50 μg/insert of Matrigel and air-dried, before being rehydrated. 20,000 cells were added to each well with or without hepatocyte growth factor (HGF). After 96 h, cells that had migrated through the matrix and adhered to other side of the insert were fixed and stained with 0.5%(w/v) crystal violet. Cells that invaded were stained with crystal and counted under a microscope.

For cell growth assay, control cells and stably transfected cells were plated in 96-well plate at 2500 cells/well, with or without treatment with 17-β-estradiol (10^{-12}–10^{-6} M final concentration). Cells were fixed in 10% formaldehyde on the day of plating, day 1, 2, 3, 4, 5, and 7 after plating, and then stained with 0.5%(w/v) crystal violet. Following washing, stained crystal was extracted with 10%(v/v) acetic acid and the absorbance was determined using a multiplate reader. The growth of cells is shown here as absorbance (mean ± SD).

Statistical analysis was carried out using a Mann–Whitney *U* test and the Kruskal–Wallis test.

Results

Com-1 modification linked to altered growth and invasiveness

Com-1 transcript was detected in both MCF-7 and MDA MB-231 cells. As previously reported [15], MCF-7 was positive for ER-α and negative for ER-β, MDA MB-231 was negative for ER but positive for ER-β (Fig. 1A).

Com-1 ribozyme transgenes and Com-1 expression vector were used to transfect both cells and have generated the following stably transfected cells (Figs. 1B and C): Com-1 knockout cells, MDA MB-231$^{\Delta com1}$ and MCF-7$^{\Delta com1}$, Com-1 over-expressing cells, MDA MB-231^{com1Exp} and MCF-7^{com1Exp}, and plasmid only control cells, MDA MB-231^{pEFa} and MCF-7^{pEFa}, used together with the respective wild types MDA MB-231-WT and MCF-7-WT (Fig. 1B). Quantitative analysis of the Com-1 transcript, using real time quantitative PCR, has shown that both ribozymes have significantly reduced the levels of the Com-1 transcript from both cells (Fig. 1C, top-MCF-7 cells and bottom-MDA MB-231 cell). Ribozyme [9] appeared to be more effective and was chosen in the subsequent analysis. Furthermore, expression has significantly increased the levels of Com-1 transcripts in both cells as shown in Fig. 1C.

The growth of these breast cancer cells was assessed over a 7 day period. As revealed in Fig. 2, MDA MB-231$^{\Delta com1}$ and MCF-7$^{\Delta com1}$ cells exhibited a faster rate of growth compared with the wild type and control cells, MDA MB-231^{pEFa} and MCF-7^{pEFa}, used together with the respective wild types MDA MB-231-WT and MCF-7-WT, respectively (Fig. 2). In contrast, over-expression of Com-1 in both cells has rendered them to a slower growth rate compared with controls and wild type.

In addition, we analysed the invasiveness of these Com-1 modified cells. Knock-out Com-1 from MDA MB-231 cells resulted in a significant increase in the invasiveness of MDA MB-231 cells (MDA MB-231$^{\Delta com1}$) (Fig. 3). Interestingly, changes with MCF-7 were not significant. In addition, knock-out of Com-1 has not changed the cells' response to HGF. Forced over-expression of Com-1 in both cells had resulted in lower invasiveness, however the difference is yet to reach statistical significance ($p = 0.0575$ for MDA MB-231 and $p = 0.093$ for MCF-7 cells). However, these Com-1 over-expression cells all had reduced their responses to HGF, for MDA MB 231 cells—32.8 ± 7.2 for the wild type and 18.3 ± 7.1 for the Com1Exp with HGF

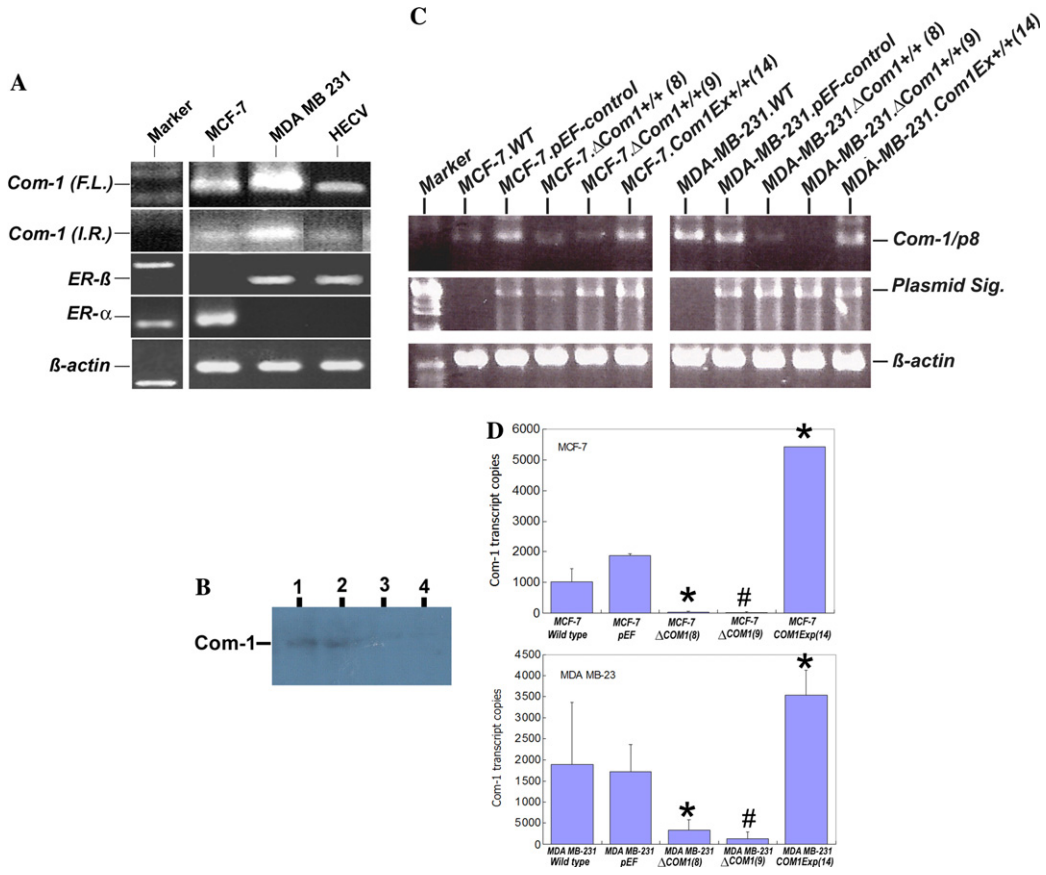

Fig. 1. (A) Expression of Com-1 and oestrogen receptors in breast cancer cells. FL—full coding region of Com-1, IR—an internal region of Com-1. (B) Genetically modified MDA MB-231 and MCF-1 cells. Both ribozyme transgenes worked equally well in MDA MB-231 cells (8 and 9). Transgene 9 worked more efficiently in MCF-7. (C) Ribozyme transgenes to Com-1 successfully reduced the levels of Com-1 protein (lanes 3 and 4 for transgene 8 (MDA MB-231$^{\Delta com1}$-8) and 9 (MDA MB-231$^{\Delta com1}$-9), respectively), compared with wild type (lane 1) and plasmid control (lane 2). Western blotting was conducted with 12% Tris–Tricine PAGE. (D) Quantitative analysis of Com-1 transcript in breast cancers using real time quantitative PCR. $^{*}p < 0.05$ vs wild type; $^{\#}p < 0.02$ vs wild type.

($p = 0.00915$), and for MCF-7 cells 11.2 ± 1.3 wild type with HGF and 3.6 ± 2.5 for Com1Exp with HGF ($p = 0.0016$).

Differential response to oestrogen in Com-1 modified breast cancer cells

The growth response of both cells, Com-1 modified and the respective control cells, to 17-β-estradiol, was assessed over a period of 7 days. Despite the moderate response of MDA MB-231-WT and MDA MB 231^{pEFa} to oestrogen, knock-out Com-1 (MDA MB-231$^{\Delta com1}$) has resulted in a dramatic increase in the growth rate, in response to 17-β-estradiol. Com-1 over-expressing MDA MB-231^{com1Exp} had a similar response to oestrogen, compared with the wild type and control cells (Fig. 4).

In wild type MCF-7 and MCF-7^{pEFa} control cells, oestrogen elicited an increase in the growth rate. Knock-out Com-1 and over-expressing Com-1 failed to impact on the response to oestrogen (Fig. 5).

Com-1 co-precipitates with ER-β, and not ER-α

To determine if Com-1 may interact with oestrogen receptors, we conducted co-precipitation between Com-1 and potential targets. Using a pool of 64 respective antibodies against potential nucleus proteins (data not shown), it was revealed that ER-β was one of the very few proteins that co-precipitated with Com-1/p8 (Fig. 6). It is worth noting that ER-α did not co-precipitate with Com-1. The interaction between ER-β and Com-1 was further confirmed using a reverse precipitation by anti-ER-β and probing with anti-Com-1 in a modified Tris–Tricine gel (Fig. 6).

Co-localisation of Com-1 with ER-β, the effect of oestrogen

We conducted immunocytochemical staining of Com-1 and ER-β in MDA MB 231 cells. Com-1 staining in MDA MB-231 cells was primarily in the nucleus, but was also seen in cytoplasm, although much weaker.

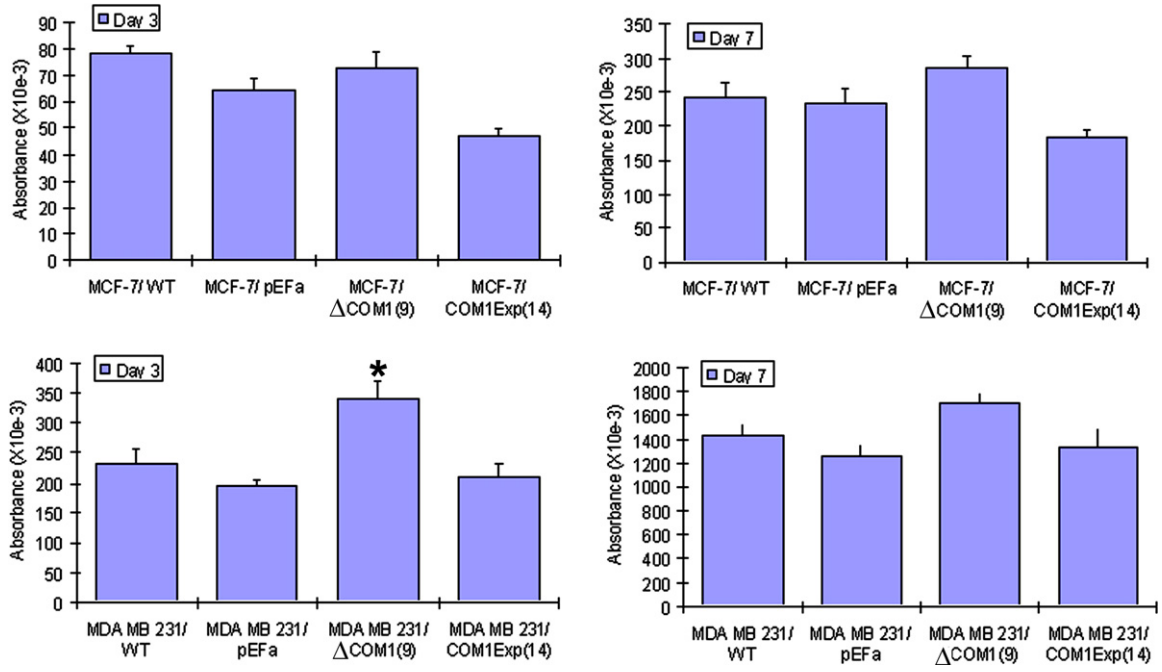

Fig. 2. Growth of Com-1 modified cells. Equal number of cells (as in Fig. 1B) were plated into 96-well plate and cultured for up to 7 days. Shown is absorbance of the stained cells. $*p < 0.05$ vs control.

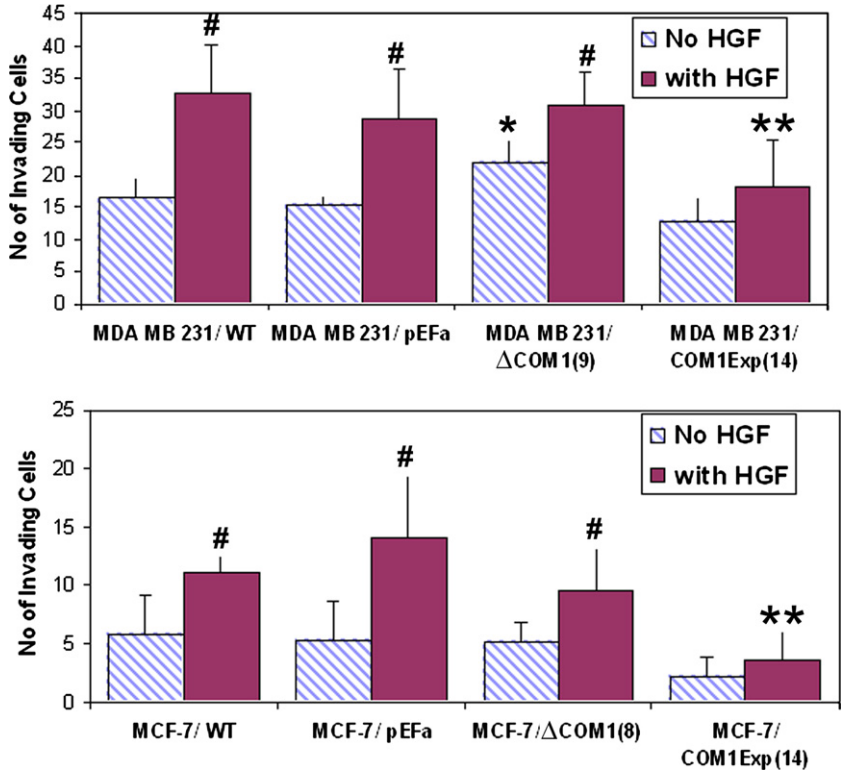

Fig. 3. Invasiveness of Com-1 modified cells. Equal number of cells (as in Fig. 1B) were used in the Matrigel invasion assay. Shown are the numbers of invading cells after 96 h. $*p < 0.05$ vs control. $^{\#}p < 0.05$ vs without HGF (hepatocyte growth factor). $**p < 0.05$ vs wild type with HGF.

Treatment with 17-β-estradiol resulted in loss of the nucleus Com-1, compared with control ($*p = 0.034$ vs without 17-β-estradiol), however changes in cytoplasmic Com-1 were minimal (Figs. 7A and C). ER-β was primarily nucleic and treatment with 17-β-estradiol caused a similar change (Figs. 7B and D).

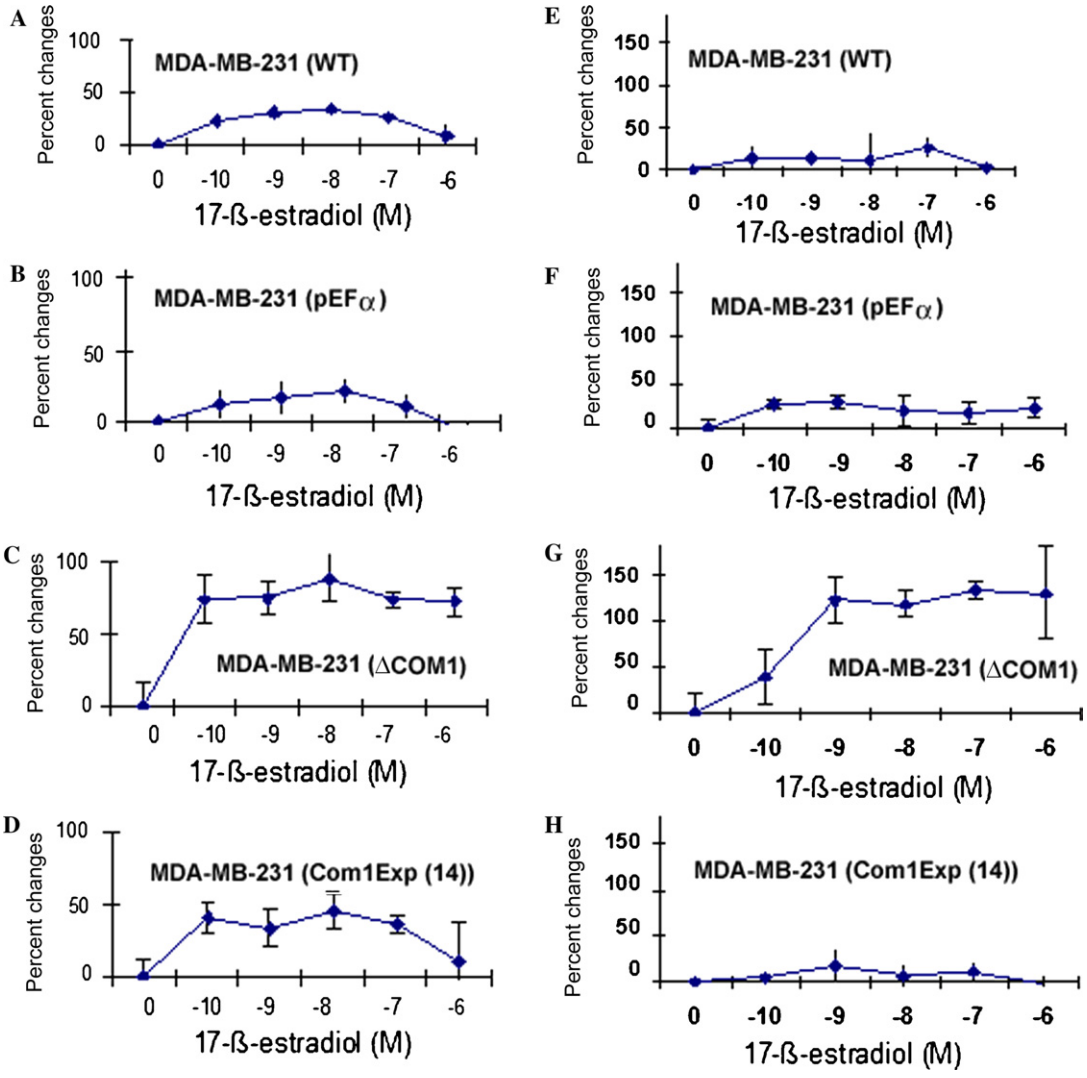

Fig. 4. The growth response to 17-β-estradiol of Com-1 modified MDA MB-231 cell over a 5 day (left panel, A,B,C, and D) and 7 day (right panel, E,F,G, and H) period. Shown are of MDA MB-231-WT and MDA MB-231^{pEFa} (top 2 panels), Com-1 knock-out MDA MB-231$^{\Delta com1}$ and Com-1 over-expressing MDA MB-231^{com1Exp} (bottom 2 panels). Elimination of Com-1 (MB-231$^{\Delta com1}$) resulted in a marked increase in the growth rate, in response to 17-β-estradiol.

The ubiquitin pathway and the fate of Com-1 in cancer cells

To elucidate if the ubiquitin–proteosome pathway plays a role in the regulation of Com-1, cells were treated with oestrogen, with or without the presence of a specific ubiquitin inhibitor, ubiquitin aldehyde. At 1 μm, the ubiquitin inhibitor appeared to weakly increase the staining of nucleic Com-1 ($p = 0.19$), however, it reversed the loss of Com-1 following treatment with 17-β-estradiol (**$p < 0.01$ vs without 17-β-estradiol) (Figs. 7A and C). There was no visible change in the staining in cytoplasmic staining of Com-1 under these conditions. As previously reported, 17-β-estradiol resulted in significant reduction of ER-β (Figs. 7B and D). This was reversed by the ubiquitin inhibitor (Fig. 7D, **$p < 0.001$). The test with lactacystin proteosome inhibitor yielded similar results, in that the inhibitor between 1 and 15 μM resulted in a similar increase in Com-1, in both 17-β-estradiol treated and untreated cells. However, the Inhibitor resulted in substantial cytotoxicity at concentration over 20 μM. The changes of the staining pattern were partially reflected in Western blotting, in which the lactacystin proteosome inhibitor increased the overall levels of Com-1 in total cell lysate (Fig. 7E).

Discussion

The current study has shown that Com-1, in concert with ER-β, plays a role in the invasiveness and oestrogen regulated cell growth of breast cancer. By interacting with ER-β, Com-1 acts as a potential tumour suppressor.

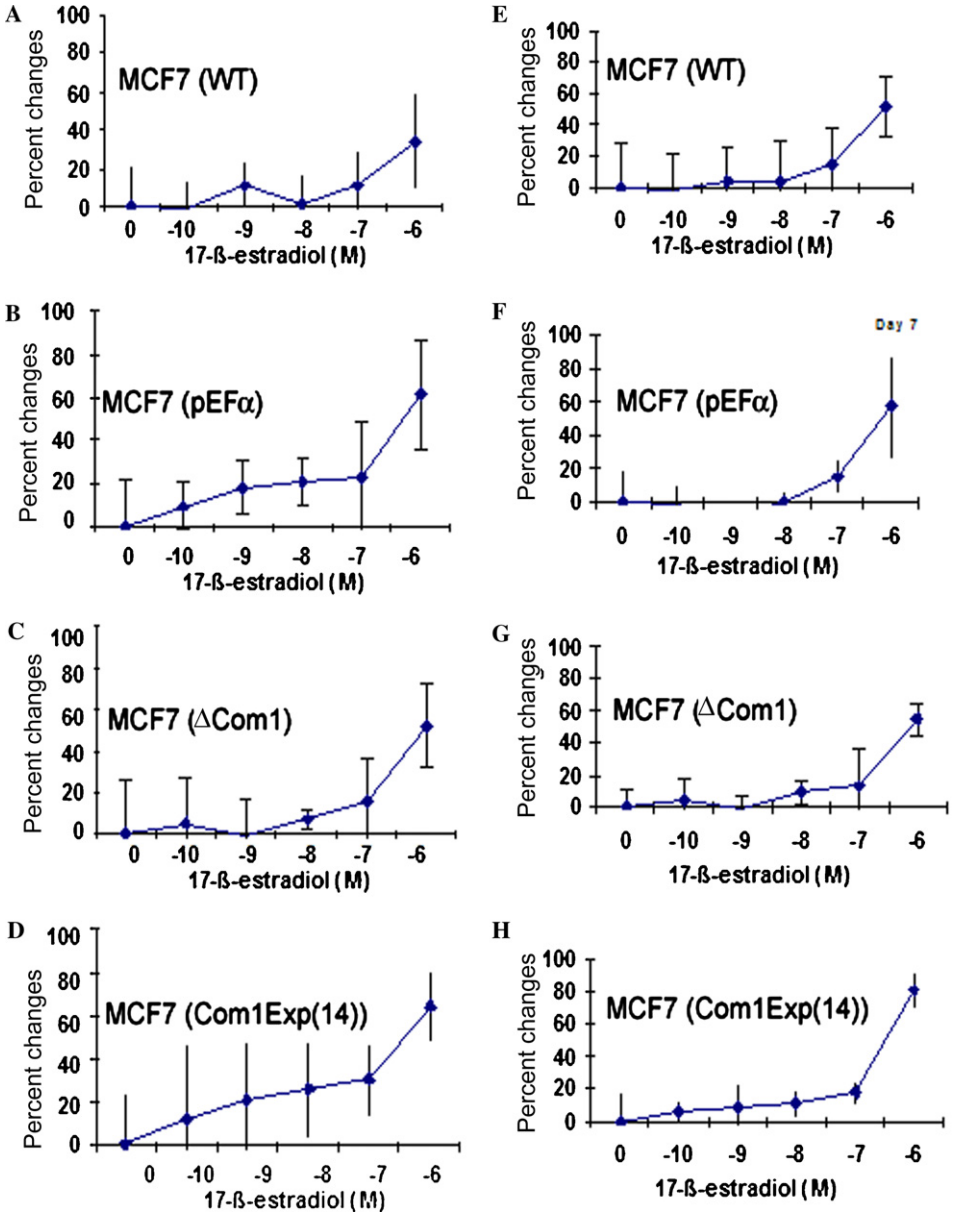

Fig. 5. The growth response to 17-β-estradiol of Com-1 modified MCF-7 cells over a 5 day (left panel, A,B,C, and D) and 7 day (right panel, E,F,G, and H) period. The layout is similar to that of Fig. 4. Manipulation of Com-1 did not alter the response of MCF-7 cells to 17-β-estradiol.

Location of Com-1

Although Com-1 staining was more intense in thyroid cancer than normal thyroid, there is little bearing to the differentiation [20]. Interestingly, cytoplasmic staining of Com-1 is frequently seen in poorly differentiated tumour cells, compared with well- and moderately differentiated tumours and normal cells which frequently display a nucleus staining pattern. The current study has shown that Com-1 is primarily localised in the nucleus of breast cancer cells, although limited amount was seen in the cytoplasmic compartment. This was also reflected in our studies in clinical breast cancer, which showed lower levels of nuclear Com-1 and relatively higher levels of cytoplasmic Com-1 [9]. It also indicates that Com-1 primarily acts as nuclear protein and may regulate events such as transcription.

Com-1 acts as a regulator of oestrogen-induced cell growth and cellular invasion

To clarify the potential function of Com-1 in breast cancer, we have constructed Com-1 knockout cells by way of using ribozyme transgene. MDA MB-231, on losing its expression of Com-1, becomes sensitive to oestrogen by increasing the growth rate. In contrast, loss of Com-1 from MCF-7 cells has not resulted in significant changes in its response to oestrogen, although

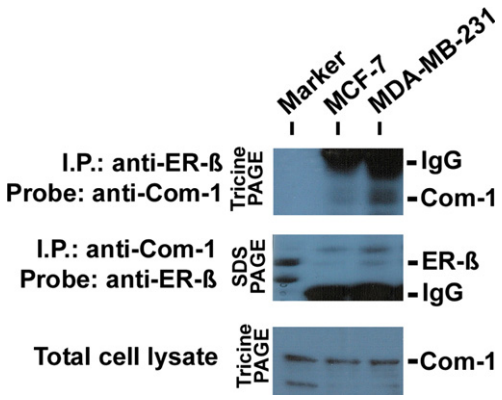

Fig. 6. Interaction between Com-1 and ER-β. Lysate from wild type MDA MB-231 or MCF-7 was either precipitated with anti-ER-β antibody and probed with an anti-Com-1 using a 12% Tris–Tricine PAGE (top panel), or precipitated with an anti-Com-1 antibody and probed with an anti-ER-β using a 10% SDS–PAGE (middle panel). ER-β was seen to co-precipitate with Com-1 in both cases. Bottom: Com-1 in total cell lysate in respective cells using a 12% Tris–Tricine gel.

over-expression of Com-1 in the cell has resulted in a slower but not statistically significant growth. Given the ER expression profiling of these two cells, i.e., ER-α(−)/ER-β(+) for MDA MB-231 and ER-α(+)/ER-β(+) for MCF-7, it points to a potential interplay between Com-1 and ER-β. It is noteworthy that both MCF-7 and MDA MB-231 cells responded to estrogen in the current study. Although MCF-7 cell has been traditionally regarded as an oestrogen dependent cell line, and MDA MB-231 has not been, changes in cell characteristics over a period of continued culture may well have change certain aspects of cellular behaviours as widely reported. The other interesting observation was that manipulation of Com-1 expression affected cellular invasion. Loss of Com-1 from MDA MB-231 has increased its spontaneous invasion into the matrix. In contrast, over-expression of Com-1 (Com1Exp) resulted in the loss of cell's response to an invasion inducing cytokine, hepatocyte growth factor [21]. This has provided further evidence that Com-1 is a molecule widely affecting the aggressive behaviour of breast cancer cells. In addition, it indicates that Com-1 may also influence the downstream event in HGF induced cell invasion.

Formation of Com-1/ER-β complex and proposed mechanism of Com-1

To search for the potential partner for Com-1 in cancer cells, we conducted immunoprecipitation first using anti-Com-1 antibody and probed the precipitate with over 60 specific antibodies to other proteins. Unexpectedly, ER-β was one of the very few proteins that co-precipitated with Com-1. The interaction between Com-1 and ER-β is further supported by the reverse precipitation.

Given the potential role of ER-β in breast cancer, we propose the following potential mechanisms for the molecular action of Com-1 in breast cancer cells. Com-1 forms a critical part for the ER-β mediated action. From both clinical and the current studies, it is suggested that formation of a Com-1/ER-β complex is critical to ER-ß. When ER-β forms as complex with Com-1, cells' response to oestrogen may be either blocked, reduced or diverted. However, as revealed in the current study, when Com-1 is absent in cancer cells, cells may use the ER-β pathway to generate growth signal thus rendering themselves to a faster rapid growth rate. The latter is an important clinical question, in ER-negative, but ER-β positive, tumours, lack of Com-1 may indicate an aggressive nature of the tumour, as indeed shown in our recent studies. The current study has deliberately avoided using ER-α positive tumours, as the interplay between the two ER types is currently unclear. It is therefore impossible to deduce the role of Com-1 in ER-α signalling and its biological actions. ER-β positive tumours are likely to be associated with endocrine-insensitive breast tumours and inversely correlated with ER-α [22]. ER-β is able to reduce 17-β-estradiol stimulated proliferation of breast cancer cells, T47D [23,24], thus further projecting its counteractive role from that of ER-α.

The roles of ER-α [25] and ER-β [26–28] have been recently documented. Despite the fact that the mechanism of the action of ER-β on cancer cells is not clear, evidence suggests that ER-β may be inhibitory to the growth of breast cancer cells [29]. ER-β has been shown to repress the expression of c-myc- cyclin-D1, cyclin-E, cyclin-A, and CDKs, while increasing the expression of $p21^{cip1}$ and $p27^{kip1}$ [23,24]. Lack of Com-1 also results in high CDKs and low $p27^{kip1}$ [3]. Collectively, it suggests that the inhibitory action of ER-β is dependent on the presence of Com-1/p8.

Down-regulation of Com-1 by oestrogen is likely via the ubiquitination pathway

The p8 polypeptide is a phosphoprotein subjected to constitutive degradation by the ubiquitin/proteosome system and is mediated by phosphatidylinositol 3-kinase and protein kinase B/Akt. In contrast, stabilization of the p8 protein requires glycogen synthase kinase-3 [30]. The current study has shown that oestrogen induced loss of Com-1 may indeed be via the ubiquitin pathway, as both inhibitors, ubiquitin aldehyde and lactacystin proteosome inhibitor, have reverted the reduction of Com-1. This observation is also interesting when interpreting the loss of Com-1 in cancer cells. Given the observations that aggressive thyroid cancer cells have more cytoplasmic staining and less nucleus staining, and that non-aggressive tumour cells and normal cells have more nucleus staining, one would argue if the loss of Com-1 from the nucleus following oestrogen stimulation may be the result of translocation from nucleus to cytoplasm.

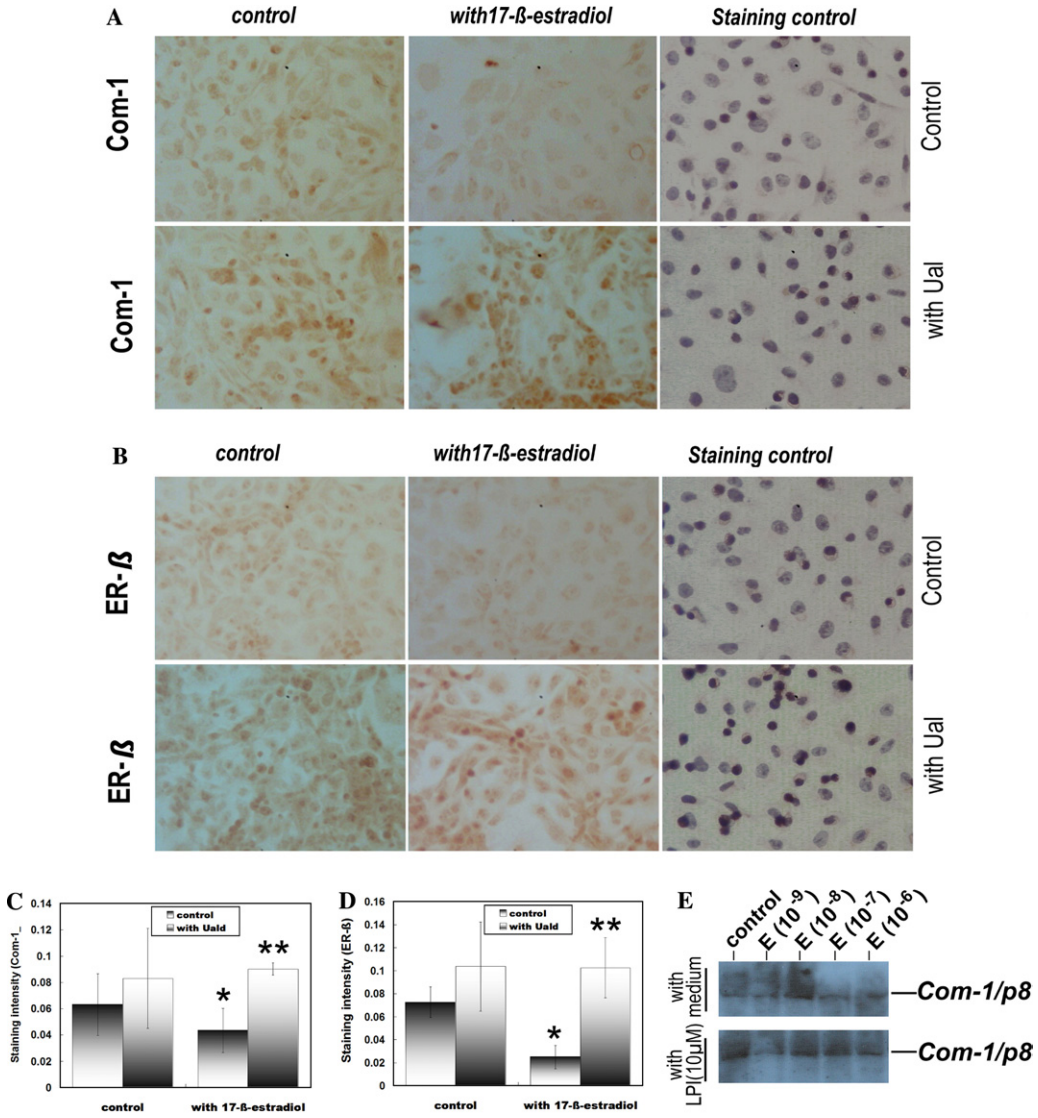

Fig. 7. (A) Immunohistochemical staining of Com-1 in wild type MDA MB 231 cells. Ual: ubiquitin aldehyde. Shown are cells treated with 10^{-7} M 17-β-estradiol and 1 μM Ual. Staining control: negative control (omitting the primary antibody) and nucleus counter-stained with haematoxylin. (B) Immunohistochemical staining of ER-β in wild type MDA MB-231 cells. Staining control: negative control (omitting the primary antibody) and nucleus counter-stained with haematoxylin. (C) Analysis of staining intensity of Com-1. $*p < 0.05$ vs no oestrogen, $**p < 0.05$ vs without Ual. (D) Analysis of staining intensity of ER-β. $*p < 0.05$ vs no oestrogen, $**p < 0.05$ vs without Ual. Ual significantly reverted the loss of the oestrogen receptor caused by 17-β-estradiol. (E) Effects of lactacystin proteosome inhibitor on Com-1 using Tris–Tricine-based Western blotting.

However, the current study failed to notice such a translocation. It is further suggested therefore that oestrogen induced loss of Com-1 is through the ubiquitin pathway.

In summary, the current study has demonstrated that Com-1 is an important tumour suppressor in breast cancer. Lack of Com-1 in breast cancer cells results in cells with a faster growth rate in response to oestrogen and exhibiting a more aggressive phenotype. This is at least in part orchestrated by the interaction between Com-1 and ER-β. It is proposed that formation of the Com-1/ER-β complex blocks ER-β mediated response to oestrogen and loss of Com-1 from the complex renders cells vigorously responding to oestrogen stimulation.

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