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Estrogenic GPR30 signalling induces proliferation and migration of breast cancer cells through CTGF

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The steroid hormone oestrogen can signal through several receptors and pathways. Although the transcriptional responses mediated by the nuclear oestrogen receptors (ER) have been extensively characterized, the changes in gene expression elicited by signalling through the membrane-associated ER GPR30 have not been studied. We show here for ER-negative human breast cancer cells that the activation of GPR30 signalling by oestrogen or by hydroxytamoxifen (OHT), an ER antagonist but GPR30 agonist, induces a transcription factor network, which resembles that induced by serum in fibroblasts. The most strongly induced gene, *CTGF*, appears to be a target of these transcription factors. We found that the secreted factor connective tissue growth factor (CTGF) not only contributes to promote proliferation but also mediates the GPR30-induced stimulation of cell migration. These results provide a framework for understanding the physiological and pathological functions of GPR30. As the activation of GPR30 by OHT also induces *CTGF* in fibroblasts from breast tumour biopsies, these pathways may be involved in promoting aggressive behaviour of breast tumours in response to endogenous oestrogens or to OHT being used for endocrine therapy.

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Introduction

The steroid hormone oestrogen binds and activates the oestrogen receptors (ER) α and β, two members of the nuclear receptor superfamily. Activated ERs regulate the transcription of target genes by binding either directly to specific DNA sequences or by tethering to other DNA-bound transcription factors. ERs have been extensively studied at the molecular, cellular, physiological and pathological levels (reviewed by Dahlman-Wright *et al*, 2006; Deroo and Korach, 2006; Heldring *et al*, 2007). Tamoxifen and its hydroxylated active form hydroxytamoxifen (OHT) are synthetic ER ligands that compete with the physiological oestrogen 17β-estradiol (E2) for binding. Depending on promoter, cell and signalling context, OHT functions either as a partial agonist or as a partial antagonist. The latter mode has led to its use for endocrine therapy of ERα-positive breast tumours, the proliferation of which can be stimulated by E2 (reviewed by Jordan, 2004).

The early discovery of Filardo *et al* (2000) that the presence of the completely unrelated transmembrane receptor GPR30 can mediate oestrogen responsiveness of ER-negative breast cancer cells came as a big surprise. GPR30 was later shown to be a genuine ER (Revankar *et al*, 2005; Thomas *et al*, 2005). In addition to E2, OHT also functions as a GPR30 agonist (Revankar *et al*, 2005; Vivacqua *et al*, 2006a, b). The GPR30 signalling pathway has been studied in a variety of cell lines. GPR30 couples to a trimeric G protein, stimulating the cAMP pathway most likely through a $G_{\alpha s}$ (Thomas *et al*, 2005) and Src through Gβγ (Filardo, 2002). Subsequently, Src promotes the shedding of heparin-binding EGF-like growth factor and activation of the EGF receptor (Filardo *et al*, 2000). This in turn activates a whole series of intracellular signalling events, most notably the activation of mitogen-activated protein kinases (MAPK) Erk1/2, PI3 kinase and phospholipase C (reviewed by Prossnitz *et al*, 2008). Further cellular responses lie downstream of these signals, including the activation of the gene *FOS* (Maggiolini *et al*, 2004).

It is unlikely that the activation of *FOS* can account for all of the biological effects of GPR30 signalling that have been reported. For example, E2 is able to stimulate the proliferation of breast, thyroid and ovarian carcinomas through GPR30 (Vivacqua *et al*, 2006b; Albanito *et al*, 2007, 2008). This effect is clearly independent of ERs and can also be observed with a GPR30-specific ligand, but how GPR30 signalling stimulates proliferation remains unclear. Although the genomic effects of ERα have been extensively studied, and in particular in breast cancer cells (see Carroll and Brown, 2006; Dudek and Picard, 2008 and references therein), the global changes in gene expression triggered by GPR30 signalling are not known. Unlike a transcription factor such as ERα, GPR30 would have to effect these changes indirectly. Nevertheless, GPR30-mediated changes in gene expression patterns have to be considered a specific output of this signal-transduction pathway. Here, we report the transcriptional consequences of GPR30 signalling in human breast cancer cells. The most strongly induced gene suggested a new function of GPR30 signalling in cell migration and proved to be functionally relevant for our understanding of the biological effects of GPR30 signalling.

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Results

Gene expression profiling of GPR30 signalling

To determine the changes in gene expression that GPR30 signalling elicits, we chose human SKBr3 breast cancer cells as our model system. These cells lack both ERα and ERβ but express GPR30 and display GPR30 signalling (Filardo *et al*, 2000; Maggiolini *et al*, 2004). Despite the absence of other known ERs in SKBr3 cells, we knocked down *GPR30* expression with an antisense strategy (Revankar *et al*, 2005; Vivacqua *et al*, 2006b) (Supplementary Figure 1A) to ascertain that any observed ligand-induced changes in gene expression are mediated by GPR30. Serum-deprived cells were treated for only 1 h with E2 or OHT to capture the primary responses. It should be pointed out here, that the OHT concentration (10 μM) used for induction is comparable to the micromolar OHT concentrations that are reached in breast tissue of patients undergoing tamoxifen therapy (Kisanga *et al*, 2004).

The mRNA levels of a total of 175 genes were induced by at least 1.3-fold by one of the treatments by comparison with uninduced control cells (Supplementary Figure 1B). At this point, we decided that we would only consider those genes as potential GPR30 target genes that fulfilled the following stringent criteria: at least 1.3-fold induction by both E2 and OHT, and at least a 1.3-fold reduction of the OHT response by antisense-mediated *GPR30* knockdown. These criteria defined 36 genes as GPR30 target genes (Figure 1; Supplementary Table 1). In total, 19 of these 36 genes were induced by more than two-fold by OHT. Within the short time frame of the treatment, no gene was significantly repressed according to the same criteria (data not shown).

We then undertook a Q-PCR experiment with the same RNA samples for a panel of genes to validate the microarray results and to obtain more quantitative data. Qualitatively, GPR30-mediated induction could be confirmed for all of them, although, not surprisingly, larger quantitative differences were obtained by Q-PCR (Figure 2). The gene encoding the connective tissue growth factor (CTGF, also known as CCN2) proved to be induced 15- to 16-fold by OHT and E2. It is a technical limitation of microarray analyses that some genes with a relatively modest induction fall through the cracks. This is the case, for example, for *JUN*. Our short list of 36 genes contains the genes *FOS* and *FOSB* (Supplementary Table 1). These encode components of the heterodimeric transcription factor AP1. Surprisingly, our gene list contains none of the genes, such as *JUN*, that encode heterodimeric partner proteins of Fos proteins. Although *JUN* did not pass the third stringent criterion (reduction by at least 1.3-fold in the *GPR30* knockdown sample) in the microarray analysis, it easily passed all criteria for a GPR30 target gene in the Q-PCR experiment, including a two-fold induction by both ligands (Figure 2). We therefore include *JUN* as a GPR30 target gene and consider it very likely that there are other false negatives in the microarray data.

CTGF is a GPR30 target gene

CTGF is by far the gene most strongly induced by E2 or OHT. We performed an immunoblot analysis to determine whether the dramatic induction seen at the mRNA level leads to increased CTGF protein expression in SKBr3 cells.

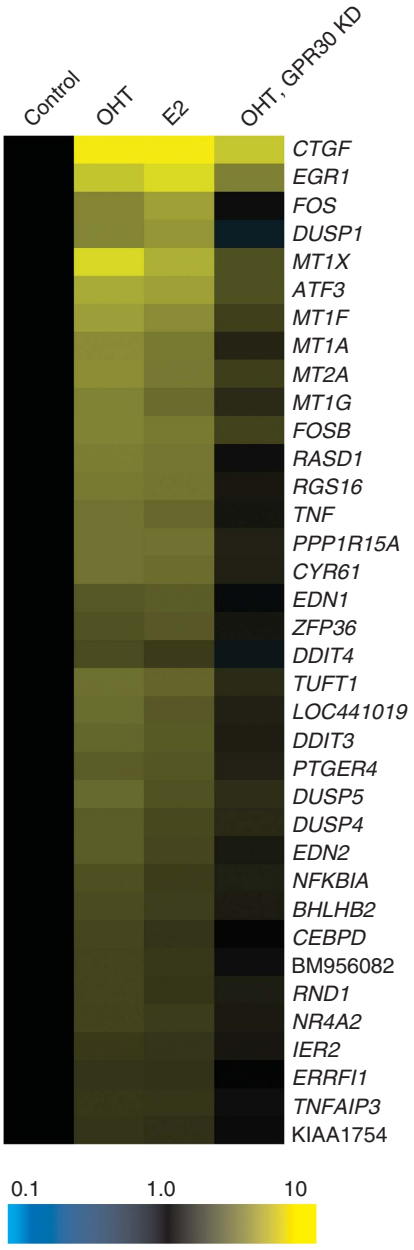

Figure 1 Colour-coded map of hierarchically clustered gene expression profiles. For each gene and condition, the colour indicates the ratio of the values obtained for the treated and untreated samples (as listed in Supplementary Table 1). GPR30 KD, *GPR30* knockdown.

Figure 3A shows that this is the case and that this increase can be blunted by an shRNA-mediated knock down of *GPR30*. The requirement for GPR30 and the specificity of the GPR30 knockdown are further emphasized by the fact that the co-transfection of an shRNA-resistant version of *GPR30* ('GPR30 rescue') restores the response. The increase at the protein level might seem modest, but note that only cell-associated proteins, and not proteins already released into the medium, were immunoblotted. We further explored the generality of this response with other cell lines and the GPR30-specific ligand G-1 (Bologa *et al*, 2006) (see Figure 3B and C). *CTGF* is induced by OHT in the human breast cancer cell lines MCF7 and BT-20, which are ERα positive and negative, respectively.

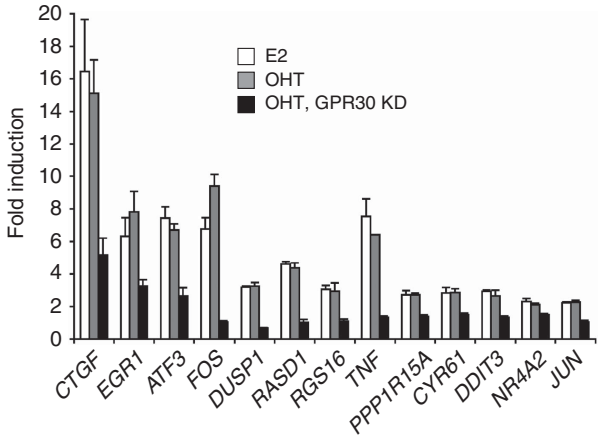

Figure 2 Q-PCR validation of a subset of GPR30-regulated genes. 'Fold induction' denotes the ratio of the values obtained for the treated and untreated samples. Error bars indicate standard deviations of measurements of triplicate samples. GPR30 KD, *GPR30* knockdown.

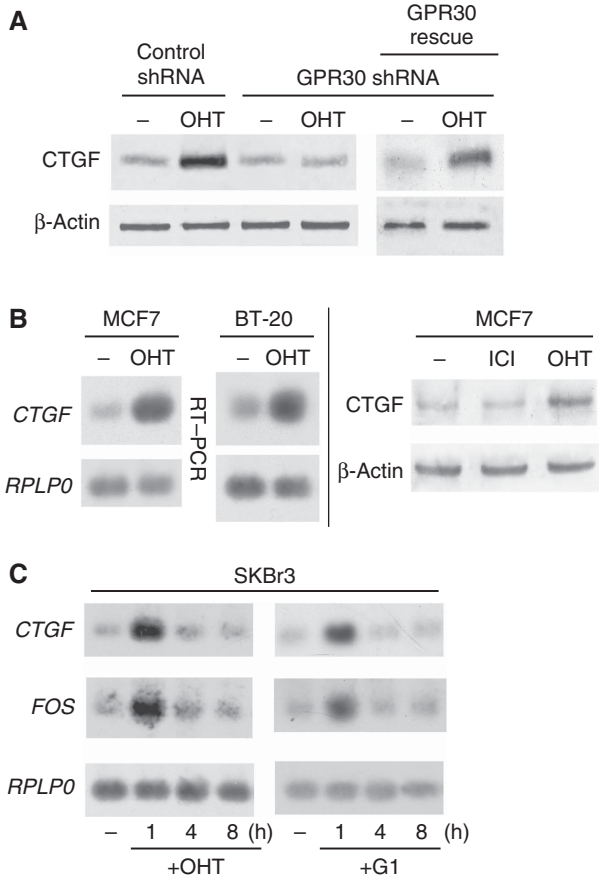

Figure 3 Induction of *CTGF* mRNA and protein in a variety of breast cancer cell lines. (**A**) Immunoblot analysis of CTGF expressed by SKBr3 cells. Cells were transfected with shRNA constructs and the GPR30 rescue vector, and treated with OHT as indicated. (**B**) Immunoblots of semiquantitative RT–PCR products ('RT–PCR') and CTGF protein (rightmost panel). (**C**) Time course of *CTGF* and *FOS* induction; semiquantitative RT–PCR analysis of SKBr3 cells treated with OHT or G1. β-Actin and the *RPLP0* mRNA served as internal standards for the immunoblot and RT–PCR experiments, respectively.

The induction is seen both at the mRNA and protein levels, and it is not elicited by the antioestrogen ICI 182′780 (ICI) (Figure 3B), at least not in MCF7 cells under our experimental conditions. Importantly, the OHT induction of *CTGF* in MCF7 cells is independent of ERα as it can still be observed when ERα is knocked down (Supplementary Figure 2). The time course experiment confirms the activation of the *CTGF* and *FOS* genes by OHT, and shows an identical activation by G-1. Induction at the mRNA level is transient in that it is clearly observed after 1 h but has subsided 3 h later. Note that the microarray analysis was performed with RNA from cells treated for 1 h.

Mediators of the transcriptional response to GPR30 signalling

The GPR30-mediated activation of target genes must be indirect. Previous analyses had indicated that GPR30 leads to the activation of Erk1/2 (Filardo *et al*, 2000; Maggiolini *et al*, 2004). MAPK can activate transcription factors such as the serum response factor (SRF) and members of the ETS family by direct phosphorylation (see for example, Posern and Treisman, 2006; Gutierrez-Hartmann *et al*, 2007). Moreover, it has been pointed out that the increase in cAMP elicited by GPR30 signalling could be expected to activate CREB (Prossnitz *et al*, 2008). These factors in turn activate the expression of the second tier of transcription factors such as c-Fos, FosB, c-Jun, EGR1, ATF3, C/EBPδ and NR4A2 (Nurr1). In addition to *FOS*, which we already knew to be activated by GPR30 signalling (Maggiolini *et al*, 2004), the genes for the aforementioned second tier transcription factors are all in our list of GPR30-induced genes (complemented with *JUN* from the Q-PCR experiment).

As a first step towards elucidating the signalling and transcription factor network that might underlie the transcriptional response to GPR30 signalling, we downloaded 5 kb of upstream sequences (relative to the start sites of transcription) for 34 of the 36 target genes of Supplementary Table 1. We scanned them for the presence of common sequence motifs and compared those with the known DNA-binding sequences of the TRANSFAC database to identify the corresponding transcription factors. The results of these analyses are displayed in Figure 4A for *CTGF* and for the complete set of target genes in Supplementary Table 2. SRF is by far the most over-represented transcription factor with EGR2, CREB and ATF among the runners up. Overall, the results of this bioinformatic analysis are entirely compatible with the aforementioned activation scheme.

Binding sites for AP1, of which c-Fos can be a component, are also highly represented, although not over-represented by more than two-fold, in promoters of GPR30 target genes (data not shown). For *CTGF* (Figure 4A), whose upstream sequences contain AP1 sites, we experimentally verified the role of c-Fos. The expression of a dominant-negative variant of c-Fos in SKBr3 cells abolishes the induction of *CTGF* by OHT or E2 (Figure 4B). To assess whether 5′ flanking sequences of the *CTGF* gene would be sufficient to mediate the GPR30 response, we used a reporter gene containing a 2 kb *CTGF* promoter fragment upstream of the luciferase-coding region (Chaqour *et al*, 2006; see Figure 4A). Upon transfection into SKBr3 cells, this reporter gene could be induced more than two-fold with E2 in a GPR30-dependent manner (Figure 4C). The response to OHT, which appears to

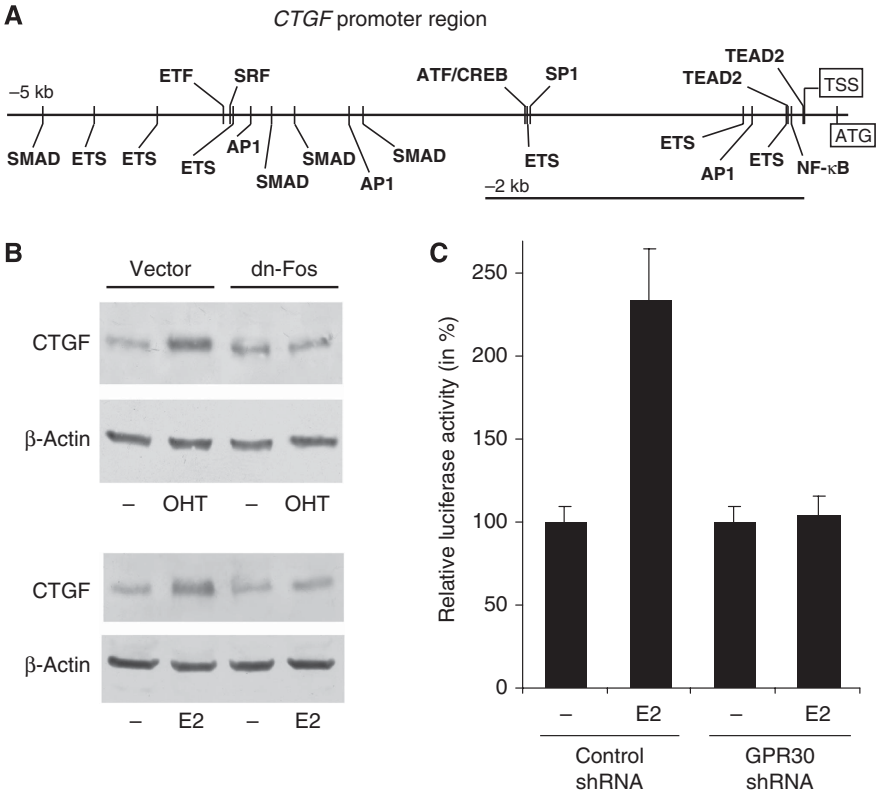

Figure 4 Transcriptional control of *CTGF* induction. (**A**) Transcription factor map of *CTGF* promoter region. Sites with factors indicated above the promoter line are over-represented at least two-fold in all GPR30 target genes (see Supplementary Table 2). AP1 and some additional factors reported in the literature are indicated below the line (see Leask and Abraham, 2006). The shorter line indicates the 2-kb fragment present in the *CTGF* luciferase reporter construct. The start sites of transcription (TSS) and translation (ATG) are indicated. (**B**) Immunoblot analysis of CTGF from SKBr3 cells expressing a dominant-negative version of c-Fos (dn-Fos). (**C**) E2 activation of a *CTGF* luciferase reporter gene co-transfected with shRNA constructs into SKBr3 cells as indicated. For each pair of samples, the values of the uninduced one were set to 100%. Error bars indicate standard deviations of normalized luciferase activities of triplicate samples.

be a stronger inducer of GPR30 signalling, could not be determined. The prolonged exposure of the cells to OHT, which this transactivation assay requires, turned out to be too toxic for SKBr3 cells. As observed for the induction of endogenous CTGF protein, AP1 turned out to be important for induction of the *CTGF* reporter gene, and this observation could be extended to ETS and SRF (Supplementary Figure 3A). A preliminary survey of signalling mediators that are required for the induction of *CTGF* highlights the role of the EGF receptor (see also Filardo *et al*, 2000) and the MAPK signalling cascade (Supplementary Figure 3B), mirroring our previous findings related to the induction of *FOS* (Albanito *et al*, 2008). In contrast to *FOS*, the induction of *CTGF* further depends on actin dynamics, a well-known regulator of SRF activity (Sotiropoulos *et al*, 1999). These results are compatible with the notion that these signalling pathways mediate the GPR30-induced activation of these transcription factors leading to the activation of *CTGF* and possibly other target genes.

GPR30 signalling promotes migration through CTGF induction

We next wondered what the biological significance of the potent induction of CTGF by GPR30 signalling might be. As CTGF had already been reported to be both sufficient and necessary for the stimulation of migration of other breast cancer cell lines (Chen *et al*, 2007), we considered the possibility that GPR30 signalling might promote migration through the induction of CTGF. The migration of SKBr3 cells was analysed with a Boyden chamber migration assay. With this assay, the number of SKBr3 cells that are able to migrate through a polycarbonate filter during a 3-h treatment period is counted. Figure 5A demonstrates that both OHT and CTGF stimulate the migration of SKBr3 cells more than two-fold. In the following experiment, we determined whether the stimulation of migration by OHT is indeed mediated by GPR30 signalling and the induction of CTGF. The transient knockdown of *GPR30* or *CTGF* expression using transfected shRNA constructs completely abolishes the stimulation of migration by OHT (Figure 5B). The addition of CTGF to the medium of cells, in which *GPR30* or *CTGF* are knocked down, rescues their migration defect. This result along with an immunoblot confirming the knockdown of *CTGF* at the protein level (Figure 5C) attests to the specificity of the RNA interference experiment. Thus, OHT stimulates the migration of SKBr3 cells through GPR30, and the GPR30-dependent induction of *CTGF* expression is necessary for this stimulatory effect. The fact that added CTGF stimulates migration to a similar extent as OHT, both in control and *GPR30* knockdown cells, indicates that the GPR30-mediated induction of *CTGF* is also sufficient for this stimulation.

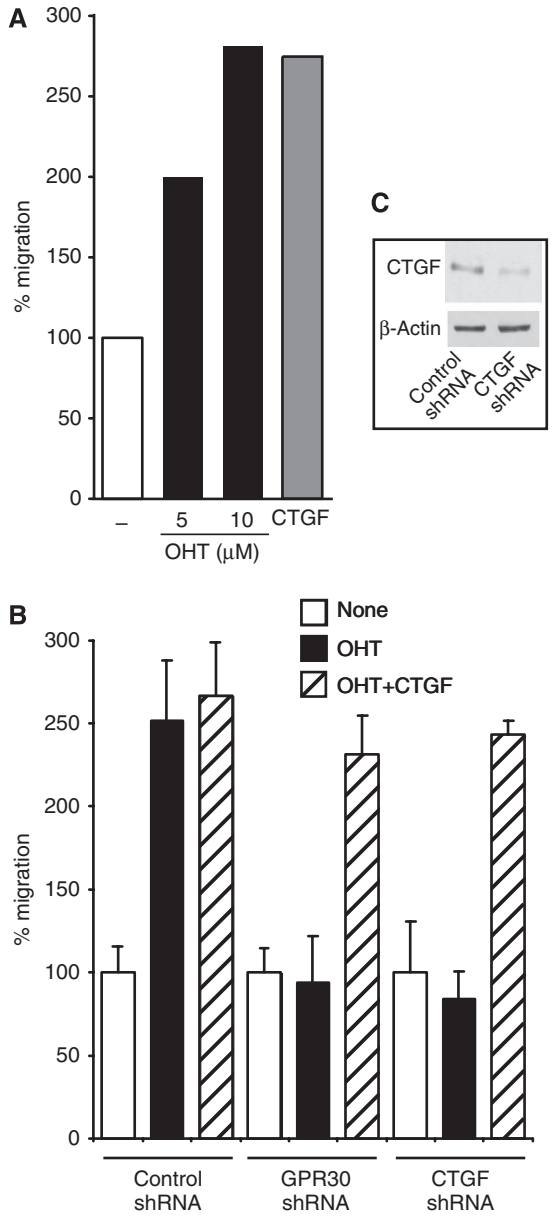

Figure 5 CTGF-dependent stimulation of cell migration by GPR30 signalling. (**A**) OHT and CTGF added to the medium induce the migration of SKBr3 cells. Bar graph shows a representative experiment with means of duplicate samples, standardized to the untreated control set to 100%. (**B**) Transient knockdown of *GPR30* or *CTGF* in SKBr3 cells blocks OHT stimulation of migration, and CTGF added to the medium restores it. Bar graph shows a representative experiment with means of triplicate samples, standardized to the respective untreated controls set to 100%. Error bars show standard deviations. (**C**) Immunoblot illustrating the extent of CTGF knockdown of a typical experiment.

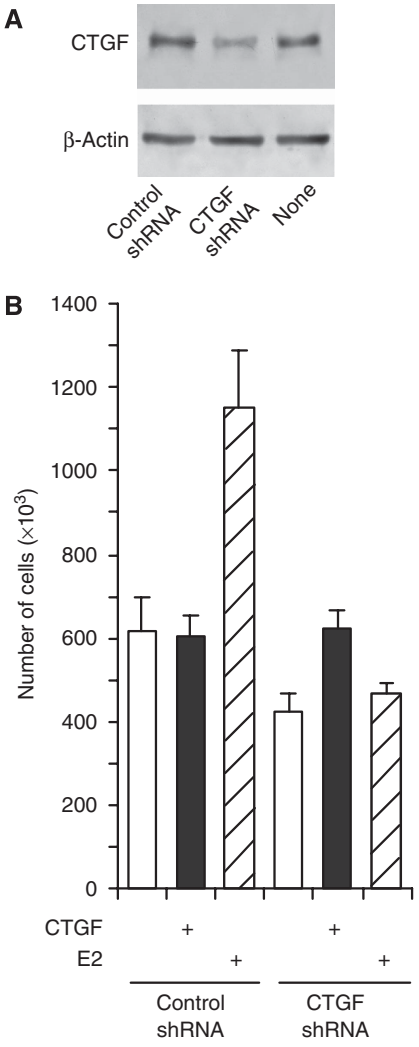

Figure 6 CTGF is required for the proliferative stimulation of SKBr3 by E2. (**A**) CTGF immunoblot of stable *CTGF* knockdown SKBr3 cells. They are compared with SKBr3 cells stably transduced with an unrelated control shRNA and untransfected wild-type SKBr3 cells. (**B**) Proliferation assay with SKBr3 cells stably transduced with shRNA constructs and treated with CTGF or E2 as indicated. Bar graph shows means of triplicate samples with standard deviations.

A possible role for CTGF in promoting proliferation

We have previously reported that GPR30 signalling stimulates the proliferation of cell lines, including SKBr3 cells, representing a variety of different carcinomas (Vivacqua *et al*, 2006b; Albanito *et al*, 2007, 2008) and mouse spermatogonia (Sirianni *et al*, 2008). It is very likely that the activation of growth-related transcription factors, such as the ones mentioned above, constitutes a cell-autonomous proliferative stimulus. In addition, primary or secondary GPR30 target genes that encode secreted factors such as CTGF might stimulate proliferation in an autocrine or paracrine manner. Indeed, there is evidence in the literature for a proliferative effect of CTGF on a variety of cell types (Rachfal and Brigstock, 2005; Leask and Abraham, 2006; De Winter *et al*, 2008), but its effects on normal or cancerous breast epithelial cells are less clear and possibly more complex (see below and Discussion). Pilot experiments indicated that CTGF added to the medium does not stimulate the proliferation of SKBr3 cells (data not shown; see also Figure 6). Although this finding is compatible with the observation that CTGF does not stimulate the proliferation of MCF7 cells (Chen *et al*, 2007), we noticed that SKBr3 cells grow poorly when *CTGF* is stably knocked-down below the basal level by a virally transduced shRNA construct (Figure 6). Remarkably, this defect can be corrected by adding CTGF to the medium. In contrast, unlike wild-type SKBr3 cells (Albanito *et al*, 2008), *CTGF* knockdown cells are resistant to the proliferative stimulus of E2. These results indicate that CTGF can have a

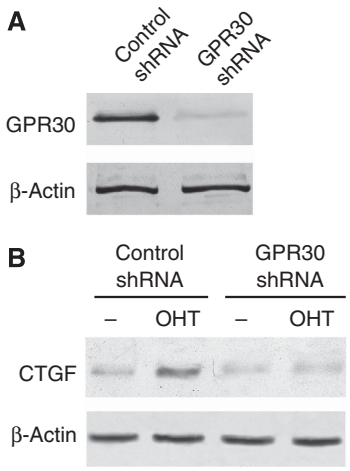

Figure 7 GPR30 expression and signalling in fibroblasts from breast tumours. The data shown are from a representative experiment of three experiments performed with samples from different patients. (**A**) Immunoblot analysis showing expression and knock down of GPR30 in transfected fibroblasts. (**B**) Immunoblot analysis of CTGF expressed by fibroblasts. Cells were transfected with indicated shRNA constructs and treated with OHT as indicated.

proliferative effect on such breast carcinoma cells under certain conditions, and that CTGF is required for the proliferative response to E2.

Contribution to GPR30 signalling from the stroma

Given that secreted factors regulating proliferation and migration need not only be made by the breast carcinoma cells themselves, we examined the expression and signalling of GPR30 in fibroblasts obtained from biopsies of primary breast tumours. GPR30 is clearly expressed in these fibroblasts, and its activation by OHT stimulates the expression of CTGF (Figure 7).

Discussion

In the process of characterizing the transcriptional response to GPR30 signalling, we have found a set of target genes that contribute to mediating the proliferative stimulus of GPR30 activators for carcinoma cell lines. Moreover, the prominent induction of *CTGF* by both E2 and OHT led us to the discovery that GPR30 signalling stimulates cell migration through CTGF. As these responses may occur in breast cancer cells independently of their ERα status as well as in the surrounding stromal cells, these findings may have important implications for our understanding of endocrine resistance in breast cancer.

GPR30 signalling activates a transcription factor network

Our microarray analysis indicated that GPR30 signalling triggers the activation of a network of transcription factors. A first tier, including SRF, members of the ETS family and CREB, is directly activated through post-translational modifications by kinases that are activated by GPR30 signalling. These transcription factors are the ones that transcriptionally activate the expression of a second tier of transcription factors (for example, Fos and Jun proteins, EGR1, ATF3, C/EBPδ and NR4A2). The response is most likely further amplified by GPR30-stimulated post-translational modifications of these proteins and positive feedback loops between many of these transcription factors. GPR30 itself may be part of a positive reinforcement loop as we have found that its expression is induced through MAPK and c-Fos in response to the growth factor EGF (Albanito *et al*, 2008). It remains to be seen whether these mechanisms, possibly involving other transcription factors mentioned above, also operate in response to GRP30 signalling, which incidentally depends on the EGF receptor (Filardo *et al*, 2000).

Considering the GPR30 signalling pathway, it is not surprising that the GPR30 response resembles the well-characterized transcriptional 'immediate early response' of fibroblasts to serum (Iyer *et al*, 1999). The strong induction of *CTGF* is consistent with the following: (i) *CTGF* is known to be activated by a panel of different extracellular signals and its promoter contains binding sites for transcription factors of the 'immediate early response', notably SRF, ETS, ATF, AP1, and TEAD2/ETF (Leask and Abraham, 2006; Cooper *et al*, 2007; Figure 4A); (ii) the inhibitory effect of a dominant-negative Fos points to a role for AP1, and additional preliminary functional results suggest that ETS and SRF are also required; and (iii) the activation of MAPK and actin dynamics are essential for the activation of both the full complement of these transcription factors and *CTGF*.

GPR30 expression and signalling in carcinomas

Activation of GPR30 by oestrogens elicits proliferative responses of breast and other carcinomas (Vivacqua *et al*, 2006b; Albanito *et al*, 2007, 2008). Although GPR30 is also expressed in normal breast epithelium, a survey of 321 primary breast tumours showed that 60% maintain GPR30 expression, including half of all ER-negative tumours (Filardo *et al*, 2006, 2008). Unlike ERα, the expression of which correlates with good prognosis, GPR30 expression was found to correlate very strongly with tumour size, HER-2 expression and distant metastasis. Remarkably, another study on breast cancer failed to find a similar correlation (Kuo *et al*, 2007), but methodological differences and a smaller cohort compared with the aforementioned studies call for more investigations. In the meantime, it is noteworthy that GPR30 expression was also associated with poor prognosis in a survey of endometrial carcinomas (Smith *et al*, 2007). Even in GPR30-positive cells, GPR30 is not expressed abundantly at the mRNA and protein levels (data not shown). Rather than monitoring only the expression of GPR30 itself, it might be more informative to combine it with measurements of the expression of a set of GPR30 target genes. Hence, our results highlight a set of GPR30 target genes that might both provide a signature for GPR30 signalling and a mechanistic underpinning of the aforementioned phenomena.

GPR30 target genes and cancer

Our experiments with SKBr3 cells demonstrate that CTGF is necessary for the stimulation of proliferation and migration by GPR30 signalling. Although CTGF is sufficient to stimulate migration, steady-state levels of CTGF might normally be sufficient to sustain a basal level of proliferation. The stimulation of proliferation by GPR30 signalling might arise from the combined induction of *CTGF* with multiple other GPR30 target genes. How the highly transient activation of GPR30 target genes that we have observed at the mRNA level is

converted into this long-term response remains to be further analysed. Perhaps not a single, but repeated pulses of GPR30 signalling may be sufficient to produce relatively persistent higher levels of key proteins such as CTGF.

CTGF defines the cystein knot family of proteins along with Cyr61 (also known as CCN1) and Nov. Note that *CYR61* is also induced by GPR30 signalling in SKBr3 cells, albeit much less than *CTGF* (Figures 1 and 2; Supplementary Table 1). Cyr61 and CTGF bind integrins and heparan sulphate-containing proteoglycans. CTGF also binds receptors or co-receptors for other signalling molecules such as Wnt, and CCN proteins even bind growth factors and cytokines themselves. These findings have led to the notion that CCN proteins exert their biological functions by modifying the action of other signals and the interactions with the extracellular matrix (reviewed by Rachfal and Brigstock, 2005; Leask and Abraham, 2006; De Winter *et al*, 2008; Holbourn *et al*, 2008). This may explain why the effects of CCN proteins are exquisitely dependent on cell type and experimental conditions, and why they may at times be seemingly contradictory. For example, low concentrations of CTGF can promote angiogenesis, but high concentrations inhibit the angiogenic effects of VEGF (Inoki *et al*, 2002). The evidence for an implication of CTGF in breast cancer is equally confusing. Although we have observed stimulatory effects of CTGF with SKBr3 cells, CTGF was shown to mediate TGFβ-induced apoptosis of MCF7 cells (Hishikawa *et al*, 1999). Nevertheless, *CTGF* was found to be part of a gene expression signature of osteolytic metastatic variants of the ER-negative breast cancer cell line MDA-MB-231, and to contribute to metastasis upon overexpression (Kang *et al*, 2003). A recent study confirmed this association and notably demonstrated that the overexpression of the genome organizer protein SATB1, which is overexpressed by aggressive breast cancer cells, induces CTGF expression (Han *et al*, 2008). In contrast, in a survey of 122 human breast tumours, Cyr61 and CTGF were found to be oppositely correlated with poor outcome, being high and low, respectively (Jiang *et al*, 2004). Elevated Cyr61 and CTGF levels have been reported to be characteristic of a number of other carcinomas (Bleau *et al*, 2005; Rachfal and Brigstock, 2005; Deng *et al*, 2007; Liu *et al*, 2008; Mullis *et al*, 2008). Whether the ERα status of breast tumours and cell lines may influence the effects of CCN proteins and account for some of the apparent discrepancies remains to be investigated.

There is yet another set of GPR30 target genes, the expression of which may contribute to breast cancer progression. Of the 11 most strongly induced genes, 5 are metallothionein genes. These include four members of the *MT1* gene cluster and one, *MT2A*, from another chromosome. Interestingly, high expression of metallothioneins in breast tumours correlates with poor outcome, perhaps owing to their protective and proliferative functions (Bay *et al*, 2006).

Relevance to tamoxifen resistance of breast cancer and to physiology

Tamoxifen is used clinically for the endocrine treatment of ERα-positive breast cancer. How and why many of these tumours ultimately become resistant to this therapy remain poorly understood (reviewed by Herynk and Fuqua, 2007; Riggins *et al*, 2007). On the basis of our findings, we would suggest that GPR30 continues to mediate stimulatory oestrogen signals for proliferation and migration even in tumours that lose ERα expression. Indeed, GPR30 may become a risk factor in patients that are treated with tamoxifen as OHT is a potent GPR30 agonist. Thus, for a subset of breast tumours as well as other carcinomas, perhaps independently of ERα status, activation of GPR30 might actually promote poor outcome by stimulating tissue invasion and remodelling through CTGF. GPR30 antagonists, which are not yet available, could be of great therapeutic value. As our results with primary fibroblasts indicate, the stroma could contribute to relaying stimulatory signals of oestrogens or OHT to breast carcinoma cells, again independently of ERα status. It has been increasingly recognized that there is a decisive interplay between cancer cells and stroma (Bhowmick and Moses, 2005; Finak *et al*, 2008). Future studies should therefore include the evaluation of GPR30 levels in the cancer-associated stroma.

Although our transcriptome analysis was carried out with a breast cancer cell line, its results, which we have partially confirmed with primary fibroblasts, may be of heuristic value for understanding other biological GPR30 functions. The induction of a network of transcription factors and target genes such as *CTGF*, *CYR61* and metallothioneins may be relevant to the wide range of GPR30 functions currently being unravelled in thymic atrophy (Wang *et al*, 2008a), mechanical hyperalgesia (Kuhn *et al*, 2008), liver injury (Hsieh *et al*, 2007), in the hypothalamus (Qiu *et al*, 2006; Brailoiu *et al*, 2007; Canonaco *et al*, 2008), for primordial follicle formation (Wang *et al*, 2008b), and for the proliferation of urothelial cells (Teng *et al*, 2008) and osteoblasts (Teplyuk *et al*, 2008).

Materials and methods

Cell culture

SKBr3 breast cancer cells were maintained in RPMI-1640 without phenol red supplemented with 10% fetal bovine serum (FBS). MCF7 and BT-20 breast cancer cells were cultured in Dulbecco's modified Eagle's medium and MEM, respectively, supplemented with 10% FBS. At 24 h prior to induction experiments, MCF7 and BT-20 cells were switched to a medium without serum and without phenol red. Lentiviruses were produced by transient co-transfection of lentiviral shRNA constructs with the VSV-G envelope plasmid pMD2G and the packaging plasmid psPAX2 (gifts from Dr D Trono; see http://tronolab.epfl.ch) into 293T cells. SKBr3 cells with a stable knockdown of *CTGF* were generated by lentiviral transduction of an shRNA construct (see below). In parallel, we obtained cells stably transduced with an unrelated control shRNA. To this end, cells were consecutively infected twice overnight, washed twice and subjected to a selection with 3 μg/ml puromycin for at least 24 h to eliminate the small fraction of non-infected cells.

Generation of primary fibroblast cells from breast cancer tissues

Breast cancer specimens were collected from primary tumours of patients who signed informed consent and underwent surgery before any pharmacological treatment. Following tumour excision, small pieces (1–2 mm diameter) were placed in a digestion solution (400 IU collagenase, 100 IU hyalurodinase and 20% FBS in Hank's balanced salt solution; all components from Sigma) containing antibiotics and antimycotics (Sigma) and incubated at 37°C with 5% CO_2 for 5–6 h. After centrifugation at 90 *g* for 2 min, the supernatant containing fibroblast cells was centrifuged at 500 *g* for 8 min, then resuspended and cultured in RPMI-1640 medium supplemented with 20% FBS, antibiotics and antimycotics.

Plasmids

The *GPR30* knockdown for the microarray analysis was carried out with the plasmid GPR30/AS (Revankar *et al*, 2005) using the related empty expression vector pRK5 (Schall *et al*, 1990) as a negative control. The *CTGF* luciferase reporter plasmid p(−1999/+36)-luc

(Chaqour *et al*, 2006), which is based on the backbone of vector pGL3-basic (Promega), was a gift from Dr B Chaqour. The plasmid A-FOS, which encodes a c-Fos mutant that heterodimerizes with c-Fos dimerization partners but does not allow DNA binding (Gerdes *et al*, 2006), was obtained from Dr C Vinson. We have previously reported the characteristics and the evaluation of an shRNA construct to knock down the expression of *GPR30* and of an unrelated shRNA control construct (Albanito *et al*, 2008). The shRNA construct for *CTGF* was obtained from the same supplier (Open Biosystems; www.Biocat.de). It has clone ID TRCN0000061950 and is based on the same lentiviral expression vector pLKO.1 as the other shRNA constructs. The targeting strand generated from the *CTGF* shRNA construct is TAGTACAGCGATT CAAAGATG. The expression vector for Flag-tagged human GPR30 has been described (Albanito *et al*, 2008). It was used to generate the GPR30 rescue vector containing silent mutations in the shRNA-targeted sequence: codons 293–297 were changed to CCG TGT AAA CAA AGT (changes underlined).

Microarray analysis

SKBr3 cells were transiently transfected either with the empty expression vector pRK5 or with the plasmid GPR30/AS using the FuGENE 6 transfection reagent (Roche) at 3 μl/μg DNA (5 μg DNA per 10 cm dish) in a medium with only 1% FBS. At 36 h after transfection, cells were switched to serum-free medium for 12 h before induction with 100 nM E2 or 10 μM OHT for 1 h. Total RNA from triplicate samples was extracted with Trizol (Invitrogen) according to the manufacturer's instructions. RNA quality was assessed with a BioAnalyzer (Bio-Rad). All RNA samples were stored at −80°C until required for further processing. Expression profiles were determined with HumanWG-6 v2.0 BeadChip microarrays (Illumina) at the genomics platform of the University of Geneva. The data have been submitted to GEO (access code GSE11567).

Promoter sequence analysis

Sequence retrieval: 5000 nucleotide sequences from upstream of the transcription start sites of the 34 genes in Supplementary Table 1 with a RefSeq accession number were retrieved from the UCSC genome browser (http://hgdownload.cse.ucsc.edu/goldenPath/hg18/bigZips/upstream5000.fa.gz). Similarly, a large set of promoter sequences, to be used as a background/control sequences, was retrieved from http://ani.embl.de/trawler. Transcription factor identification: TRANSFAC Professional 11.4 was downloaded from Biobase (http://www.biobase-international.com) and installed locally on a computer running Ubuntu. The set of all vertebrate transcription factor-binding sites was retrieved from matrix.dat in TRANSFAC. For these factors, a number of profiles were generated with matrix similarity ranging from 0.8 to 1.00 and core similarity ranging from 0.8 to 1.00. The profile minFP to minimize false positives was generated from MATCH on the website. MATCH was used locally to identify transcription factor-binding sites in the set of promoter sequences with the profile minFP. Thereafter, F-MATCH was used with a cutoff *P*-value of 0.001 to identify over-represented transcription factor-binding sites. These transcription factor-binding sites were sorted by their relative over-representation in the target sequence set. To retrieve the predicted sites for 5000 bp of *CTGF* upstream sequences, the profile with 0.95/0.95 matrix/core similarity was used.

PCR analyses

Quantitative real-time PCR analysis was performed for the triplicate RNA samples used for the microarray experiment. To identify appropriate control genes for standardization, the analysis was performed with three candidates that had proved useful in many other gene expression analyses (*TFRC*, *ALAS1* and *TBP*). As *TFRC* and *ALAS1* varied the least across all sample, all measurements were normalized to the geometric mean of these two control genes. Semiquantitative RT–PCR was carried out as described (Maggiolini *et al*, 1999) with total RNA from cells induced for 1 h. *FOS*, *CTGF* and the internal control *RPLP0* (also known as 36B4) cDNAs yielded bands of 420, 392 and 408 bp with 20, 20 and 10 PCR cycles, respectively. The primers are listed in Supplementary Table 3.

Immunoblotting

Total protein extracts were prepared from cells after a 2-h induction. For inhibition experiments with shRNAs or the dominant-negative c-Fos variant, cells were transiently transfected in a medium without serum and induced 24 h later. CTGF, β-actin and GPR30 were revealed by immunoblotting with the goat polyclonal antiserum sc-14939, the mouse monoclonal antibody sc-8432 (Santa Cruz Biotechnology) and the rabbit polyclonal antiserum LS-A1183 (MBL-Eppendorf), respectively.

Reporter gene assay

The *CTGF* luciferase construct was co-transfected with a *Renilla* luciferase expression plasmid as an internal transfection control into SKBr3 cells as described above for the microarray analysis. Induction with 100 nM E2 was carried out overnight. Luciferase activities of triplicate samples were measured with the Dual-Luciferase Reporter Assay system (Promega).

Migration and proliferation assays

Migration assays were performed with SKBr3 cells in triplicate using Boyden chambers (Costar Transwell, 8 μm polycarbonate membrane). For knockdown experiments, cells were transfected with shRNA constructs directed against GPR30 or *CTGF* or with an unrelated shRNA construct (3 μg DNA with FuGENE 6 at a 3 μl to 1 μg DNA ratio in 60 mm plates) in standard growth medium. After 24 h, they were seeded in the upper chambers. OHT or CTGF was added to the medium without serum in the bottom wells. After 3 h, cells on the bottom side of the membrane were fixed and counted. Human CTGF was purchased from MBL and added at 100 ng/ml. For proliferation assays, SKBr3 cells with a stable *CTGF* (or control) knockdown were cultured in a medium supplemented with 5% charcoal-treated FBS. Where indicated, CTGF was added each day. Cells were counted at day 6.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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