

# Assessment of CXC ligand 12-mediated calcium signalling and its regulators in basal-like breast cancer cells

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**Abstract.** CXC ligand (L)12 is a chemokine implicated in the migration, invasion and metastasis of cancer cells via interaction with its receptors CXC chemokine receptor (CXCR)4 and CXCR7. In the present study, CXCL12-mediated Ca<sup>2+</sup> signalling was compared with two basal-like breast cancer cell lines, MDA-MB-231 and MDA-MB-468, which demonstrate distinct metastatic potential. CXCL12 treatment induced Ca<sup>2+</sup> responses in the more metastatic MDA-MB-231 cells but not in the less metastatic MDA-MB-468 cells. Assessment of mRNA levels of CXCL12 receptors and their potential modulators in both cell lines revealed that CXCR4 and CXCR7 levels were increased in MDA-MB-231 cells compared with MDA-MB-468 cells. Cluster of differentiation (CD)24, the negative regulator of CXCL12 responses, demonstrated increased expression in MDA-MB-468 cells compared with MDA-MB-231 cells, and the two cell lines expressed comparable levels of hypoxia-inducible

factor (HIF)2 $\alpha$ , a CXCR4 regulator. Induction of epithelial-mesenchymal transition (EMT) by epidermal growth factor exhibited opposite effects on CXCR4 mRNA levels compared with hypoxia-induced EMT. Neither EMT inducer exhibited an effect on CXCR7 expression, however hypoxia increased HIF2 $\alpha$  expression levels in MDA-MB-468 cells. Analysis of the gene expression profiles of breast tumours revealed that the highest expression levels of CXCR4 and CXCR7 were in the Claudin-Low molecular subtype, which is markedly associated with EMT features.

## Introduction

Chemokines are a superfamily of small (~8-14 kDa) molecules that mediate numerous cellular functions by activating G protein-coupled receptors (1). Chemokines and their respective receptors are also associated with metastasis in different types of cancer, including osteosarcoma (2) and neuroblastoma (3), as well as prostate (4) and breast (5) cancer. The chemokine CXC ligand 12 (CXCL12; also known as stromal cell-derived factor-1) is implicated in numerous cellular processes that are important in aspects of tumour progression. It interacts with its cognate receptors CXC chemokine receptor (R) type 4 (1) and CXCR7 (6) to regulate cell trafficking and adhesion, tumour vascularisation, cell proliferation and survival (7,8). CXCL12 enhances the invasiveness and migratory properties of breast cancer cells, particularly when these cells also express CXCR4 (9). Indeed, CXCR4 expression is upregulated in primary breast tumours compared with normal mammary epithelial cells (5) indicating that it serves an important function in the progression and metastasis of breast cancer (10,11).

CXCL12 responses are regulated by other factors beyond its receptors CXCR4 and CXCR7. Among these factors is cluster of differentiation (CD)24, a glycosylated cell surface protein that acts as a signal transducer in modulating responses to B cell activation (12). Schabath *et al* (13) demonstrated that MDA-MB-231 breast cancer cells with low CD24 expression exhibit augmented CXCL12/CXCR4-mediated cell migration

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**Abbreviations:** C-Low, Claudin-Low; EGF, epidermal growth factor; EMT, epithelial-mesenchymal transition; HER2, human epidermal growth factor receptor 2; HIF2 $\alpha$ , hypoxia-inducible factor-2 $\alpha$ ; FBS, foetal bovine serum; FLIPR, fluorescence imaging plate reader; LumA, luminal A; LumB, luminal B; N-Like, normal-like

**Key words:** breast cancer, calcium signalling, cluster of differentiation 24, CXC chemokine receptor type 4, CXC chemokine receptor type 7, hypoxia-inducible factor 2 $\alpha$

and enhanced tumour growth compared with MDA-MB-231 cells that express high exogenous levels of CD24, suggesting that higher CD24 expression decreases CXCL12 responses in breast cancer cells. Hypoxia-inducible factor-2 $\alpha$  (HIF2 $\alpha$ ) also regulates CXCR4 expression (14) and may therefore influence CXCL12 responsiveness.

Certain cells respond to CXCL12 activation by releasing  $\text{Ca}^{2+}$  from the endoplasmic reticulum internal  $\text{Ca}^{2+}$  store via G-protein coupled receptor, triggering phospholipase C activation and the generation of inositol trisphosphate and diacylglycerol (7).  $\text{Ca}^{2+}$  signalling is associated with processes that occur during metastasis, including cell migration and invasion (15,16), as well as the induction of an increasingly invasive phenotype by stimulating the epithelial-mesenchymal transition (EMT) (17). EMT is a process whereby epithelial cells undergo conversion to an increasingly mesenchymal (invasive) phenotype (18). However, the nexus between CXCL12,  $\text{Ca}^{2+}$  signalling, CXCL12 modulators and receptors and EMT has not yet been fully evaluated.

The nature of  $\text{Ca}^{2+}$  store release as a result of CXCL12/CXCR4 interaction may be tissue-dependent and vary between cell types (7). Changes in  $\text{Ca}^{2+}$  signalling and/or the expression of specific modulators of  $\text{Ca}^{2+}$  signalling are a feature of some subtypes of breast cancer and these changes often differ between different breast cancer subtypes. For example, the ratio of the calcium release-activated calcium channel protein (Orai)1 calcium influx pathway activators stromal interaction molecule 1/2 is higher in the basal molecular breast cancer subtype than in other subtypes (19). It has been demonstrated that Orai3 regulates store-operated  $\text{Ca}^{2+}$  entry in oestrogen receptor-positive breast cancer cell lines such as MCF-7 but does not in oestrogen receptor-negative breast cancer cell lines such as MDA-MB-231 (20). Elevated transient receptor potential cation channel V6 levels are more common in types of breast cancer that are oestrogen receptor-negative (21). Oestrogen receptor-negative breast cancer, particularly those of the triple-negative subtype, exhibit a significant overlap with molecularly defined basal breast cancer (22).

Basal breast cancer cell lines possess gene signatures that allow them to be divided into basal A and basal B subtypes (23). In the present study,  $\text{Ca}^{2+}$  signalling induced by CXCL12 was compared between two triple-negative basal breast cancer cell lines, MDA-MB-468 (basal A) and MDA-MB-231 (basal B). mRNA levels of CXCL12 receptors and their response modulators in EMT and in breast cancer cell lines of different molecular subtypes were also characterised. The present study therefore aimed to assess the potential heterogeneity of responses to CXCL12 in the context of induced  $\text{Ca}^{2+}$  increases in basal breast cancer.

## Materials and methods

**Cell culture.** The human basal-like triple-negative breast cancer cell lines MDA-MB-231 and MDA-MB-468 were obtained from the American Type Culture Collection (Manassas, VA, USA) and The Brisbane Breast Bank, University of Queensland Centre for Clinical Research, (Brisbane, Australia) respectively, and maintained in Dulbecco's modified Eagle's medium (DMEM; D6546;

Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% foetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA), L-glutamine (4 mM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), penicillin 100 U/ml and streptomycin 100  $\mu\text{g}/\text{ml}$  (Invitrogen; Thermo Fisher Scientific, Inc.) in a humidified incubator at 37°C in an atmosphere containing 5%  $\text{CO}_2$ . Cells were routinely screened for mycoplasma contamination using the MycoAlert Mycoplasma Detection kit (LT07-218; Lonza Group, Ltd., Basel, Switzerland) and validated by short tandem repeat profiling using the StemElite ID Profiling kit (Promega Corporation, Madison, WI, USA).

**Intracellular  $\text{Ca}^{2+}$  measurement.** For  $\text{Ca}^{2+}$  measurements, MDA-MB-231 ( $7.5 \times 10^3$  cells/well) or MDA-MB-468 ( $1.5 \times 10^4$  cells/well) cells were seeded in a 96-well CellBIND plate (Corning Life Sciences, Corning, NY, USA) in antibiotic-free DMEM containing L-glutamine (4 mM) and 10% FBS (MDA-MB-468 cells were seeded at a higher density due to their slower proliferation rate). At 24 h post-plating, the FBS concentration was decreased to 8%. At 72 h post-plating,  $\text{Ca}^{2+}$  assays were performed using a fluorescence imaging plate reader, FLIPR<sup>TETRA</sup> (Molecular Devices, LLC, Sunnyvale, CA, USA) and 4  $\mu\text{M}$  Fluo-4 AM dye (Molecular Probes; Thermo Fisher Scientific, Inc.) in physiological salt solution, as previously described (24). Cells were excited at 470–495 nm and emission was assessed at 515–575 nm over an 800 sec period. Relative cytoplasmic (CYT)  $[\text{Ca}^{2+}]_{\text{CYT}}$  was determined in the presence of 300 and 100 ng/ml recombinant human CXCL12 (R&D Systems, Inc., Minneapolis, MN, USA) or 100  $\mu\text{M}$  adenosine 5'-triphosphate (ATP; Sigma-Aldrich; Merck KGaA). Data were acquired using ScreenWorks<sup>TM</sup> software (v2.0.0.27, Molecular Devices, LLC) and are presented as the response over baseline, which is a measure of relative  $[\text{Ca}^{2+}]_{\text{CYT}}$ .

**RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was isolated using the RNeasy<sup>®</sup> Plus Mini kit (Qiagen GmbH, Hilden, Germany). RT reactions were performed using an Omniscript Reverse Transcriptase kit (Qiagen GmbH) with random primers and RNase inhibitor (Promega Corporation), according to the manufacturer's protocol. qPCR was conducted using Applied Biosystems TaqMan gene expression assays and TaqMan Universal PCR Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). Assays included CD24 (Assay ID: Hs02379687\_s1), CXCR4 (Assay ID: Hs00237052\_m1), CXCR7 (Assay ID: Hs00664172\_s1), HIF2 $\alpha$  (Assay ID: Hs01026149\_m1) and the endogenous control 18S ribosomal RNA (4319413E). All amplifications were performed using universal cycling conditions [20 sec at 95°C (holding stage)], followed by 40 cycles of denaturation for 1 sec at 95°C and combined annealing and extension steps for 20 sec at 60°C) in a StepOnePlus<sup>TM</sup> Real-Time PCR System Thermal Cycling Block (Applied Biosystems; Thermo Fisher Scientific, Inc.). Data were normalised to 18S ribosomal RNA and analysed using the comparative  $C_q$  method as previously described (25).

**Epidermal growth factor (EGF)-induced EMT.** For assessment of EGF-induced EMT, MDA-MB-468 cells were plated

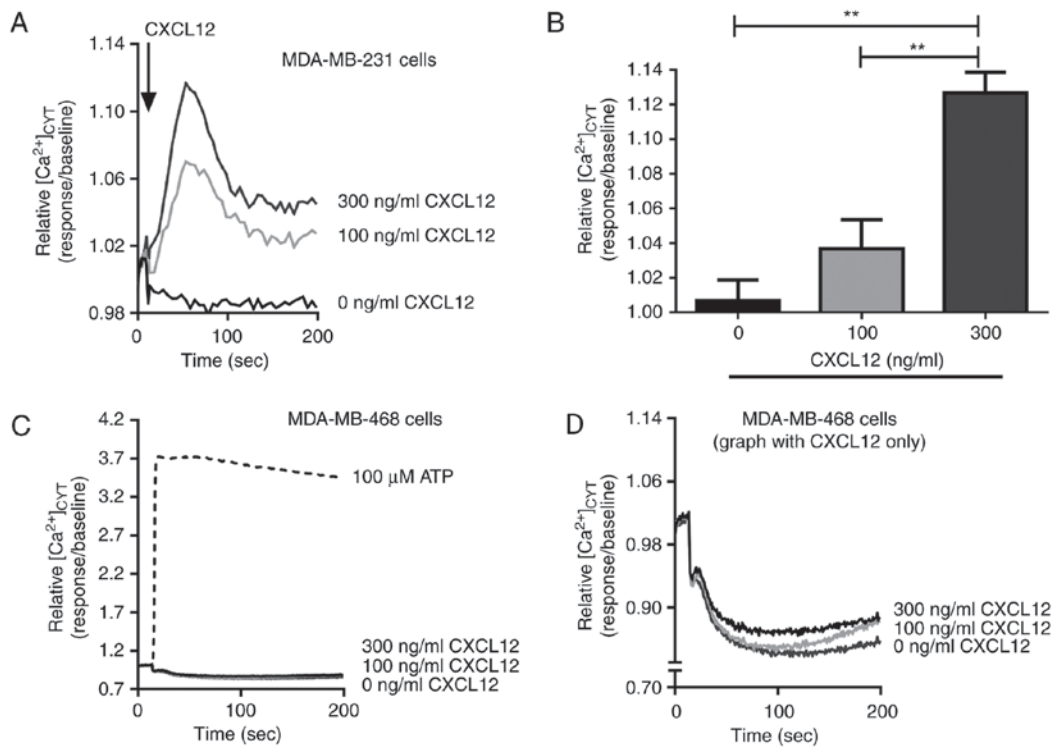


Figure 1. Effect of CXCL12 on  $[Ca^{2+}]_{cyt}$  in MDA-MB-231 and MDA-MB-468 breast cancer cells. (A) Representative  $[Ca^{2+}]_{cyt}$  transients following treatment with 100 and 300 ng/ml CXCL12 in MDA-MB-231 cells. (B) Each bar represents the response (peak relative  $[Ca^{2+}]_{cyt}$ ) to either 100 ng/ml CXCL12 or 300 ng/ml CXCL12 in MDA-MB-231 cells ( $n=3$ ; mean  $\pm$  standard deviation). (C)  $[Ca^{2+}]_{cyt}$  levels following treatment with 100 and 300 ng/ml CXCL12 in MDA-MB-468 cells. Stimulation with 100  $\mu$ M ATP was used as a positive control for this assay (representative of three independent experiments). (D) Presents only the CXCL12 response using the same maximum Y-axis scale that was used for MDA-MB-231 cells. Statistical analysis was performed using one-way analysis of variance with Tukey's multiple comparison post-hoc test. \*\* $P<0.01$ . CXCL, CXC ligand; CYT, cytoplasmic;  $[Ca^{2+}]$ , intracellular calcium concentration.

at a density of  $2 \times 10^4$  into a 96-well plate and serum-starved (0.5% FBS) for 24 h prior to treatment with 50 ng/ml EGF (Sigma-Aldrich; Merck KGaA), as previously described (26). Total RNA was isolated at 24 h post-EGF treatment and subjected to RT-qPCR following the aforementioned protocol to assess the changes in CXCR4, CXCR7, CD24 and HIF2 $\alpha$  expression.

**Hypoxia-induced EMT.** For hypoxia-induced EMT, MDA-MB-468 cells were seeded at a density of  $2 \times 10^4$  in a 96-well plate and serum-deprived (0.5% FBS) for 24 h. Cells were then exposed to hypoxic conditions (1%  $O_2$ ) in a Sanyo MCO-18M multi-gas incubator (Sanyo Electric Co., Ltd., Tokyo, Japan). Normoxic control MDA-MB-468 cells were incubated in a humidified incubator (37°C, 5%  $CO_2$ ) with normal atmosphere (21%  $O_2$ ). Total RNA was isolated at 24 h following normoxic or hypoxic conditions to assess changes in CXCR4, CXCR7, CD24 and HIF2 $\alpha$  expression, following the aforementioned protocol.

**Analysis of CXCR4 and CXCR7 expression in breast tumours.** Breast tumour gene expression data were sourced from the METABRIC breast cancer data (www.cbioportal.org, last accessed April 3, 2017) (27-29). This dataset comprises gene expression profiles from 1,860 tumours from six molecular subtypes including 198 basal-like (Basal), 182 Claudin-low (C-low), 218 human epidermal growth factor receptor 2-enriched (HER2), 673 luminal A (LumA), 454 luminal B (LumB) and 135 normal-like (N-Like).

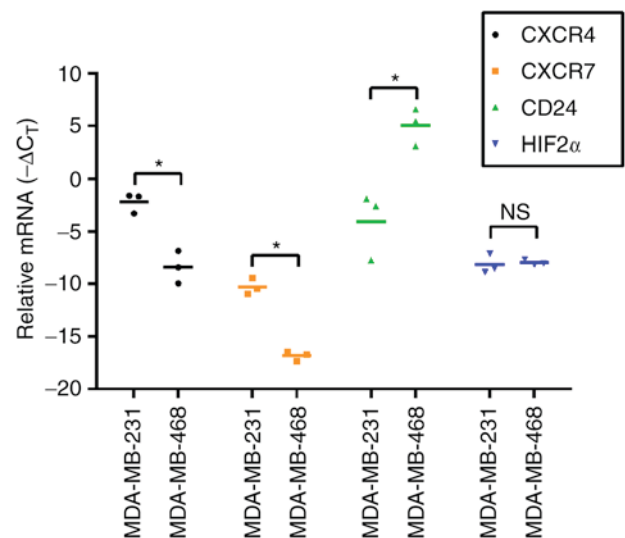


Figure 2. CXCR4, CXCR7, CD24 and HIF2 $\alpha$  mRNA levels in MDA-MB-231 and MDA-MB-468 breast cancer cells normalised to 18S ribosomal RNA ( $-\Delta C_q$ ). Results were taken from three independent experiments and the horizontal line represents the mean ( $n=3$ ). Statistical analysis was performed using an unpaired t-test. \* $P<0.05$ . NS, not significant; CXCR, CXC chemokine receptor; CD, cluster of differentiation; HIF, hypoxia-inducible factor.

**Statistical analysis.** Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA). Results are expressed as the mean  $\pm$  standard deviation from the specified number of independent experiments.

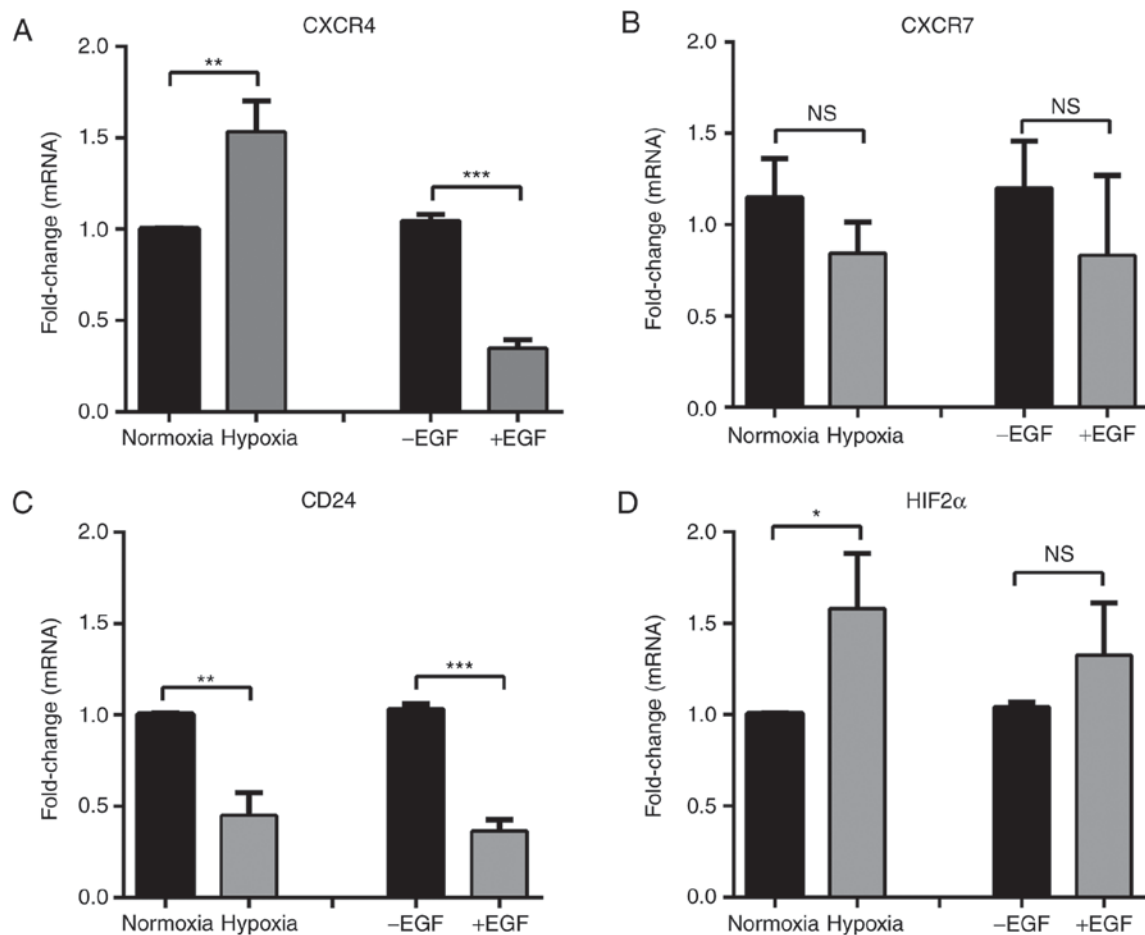


Figure 3. mRNA levels of CXCR4, CXCR7, CD24 and HIF2 $\alpha$  mRNA following hypoxia- and EGF-induced EMT in MDA-MB-468 breast cancer cells expressed as fold change. (A) CXCR4, (B) CXCR7, (C) CD24, and (D) HIF2 $\alpha$  mRNA levels in MDA-MB-468 cells incubated under normoxic or hypoxic (24 h) conditions or stimulated with EGF (24 h) to induce EMT. Results are presented as the mean  $\pm$  standard deviation (n=3). Statistical analysis was performed using an unpaired t-test; \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001. NS, not significant; CXCR, CXC chemokine receptor; CD, cluster of differentiation; HIF, hypoxia-inducible factor; EMT, epithelial-mesenchymal transition; EGF, epidermal growth factor.

The statistical tests used are stated in each figure legend, and included one-way analysis of variance followed by Tukey's multiple comparison and an unpaired t-test, where appropriate.

## Results

**CXCL12-induced intracellular free  $\text{Ca}^{2+}$  increases in breast cancer cells.** The effects of CXCL12 on intracellular  $\text{Ca}^{2+}$  concentrations were assessed in two basal-like breast cancer cell lines, MDA-MB-231 (basal B) and MDA-MB-468 (basal A). A significant concentration-dependent increase in  $[\text{Ca}^{2+}]_{\text{CYT}}$  was observed following CXCL12 treatment in MDA-MB-231 breast cancer cells ( $P$ <0.01; Fig. 1A and B) compared with the untreated control. However, no significant increase in  $[\text{Ca}^{2+}]_{\text{CYT}}$  was observed in MDA-MB-468 cells treated with CXCL12 compared with the untreated control, despite a pronounced elevation in intracellular  $\text{Ca}^{2+}$  levels observed during stimulation with the purinergic receptor activator ATP (Fig. 1C and D).

**Levels of CXCR4, CXCR7, CD24 and HIF2 $\alpha$  mRNA in MDA-MB-231 and MDA-MB-468 breast cancer cells.** To explore the potential reasons for the lack of CXCL12-induced  $[\text{Ca}^{2+}]_{\text{CYT}}$  in MDA-MB-468 basal A breast cancer cells,

compared with the significant increases observed in more metastatic MDA-MB-231 basal B breast cancer cells, mRNA expression of the potential regulators of CXCL12 responses were measured. Levels of mRNA for the CXCL12 receptor CXCR4 were significantly increased in MDA-MB-231 cells compared with MDA-MB-468 cells ( $P$ <0.05; Fig. 2). Similarly, levels of CXCR7, another receptor for CXCL12 (6), were significantly increased in MDA-MB-231 cells compared with MDA-MB-468 cells ( $P$ <0.05; Fig. 2). CD24 is a negative regulator of CXCL12 responses in MDA-MB-231 cells (13) and in the present study, it was revealed that there were significantly higher levels of CD24 mRNA in MDA-MB-468 cells ( $P$ <0.05; Fig. 2), which were not responsive to CXCL12 as assessed by increases in  $[\text{Ca}^{2+}]_{\text{CYT}}$  (Fig. 1C), compared with MDA-MB-231 cells. Given that an association between HIF2 $\alpha$  and CXCR4 expression has been identified (14), HIF2 $\alpha$  levels were also assessed in the two cell lines in the present study; however, no significant difference was observed (Fig. 2).

**Assessment of CXCR4, CXCR7, CD24 and HIF2 $\alpha$  during hypoxia- and EGF-induced EMT in MDA-MB-468 breast cancer cells.** MDA-MB-231 is a basal B cell line and exhibits mesenchymal features, including vimentin expression and a lack of E-cadherin expression (30). Given that CXCL12-mediated



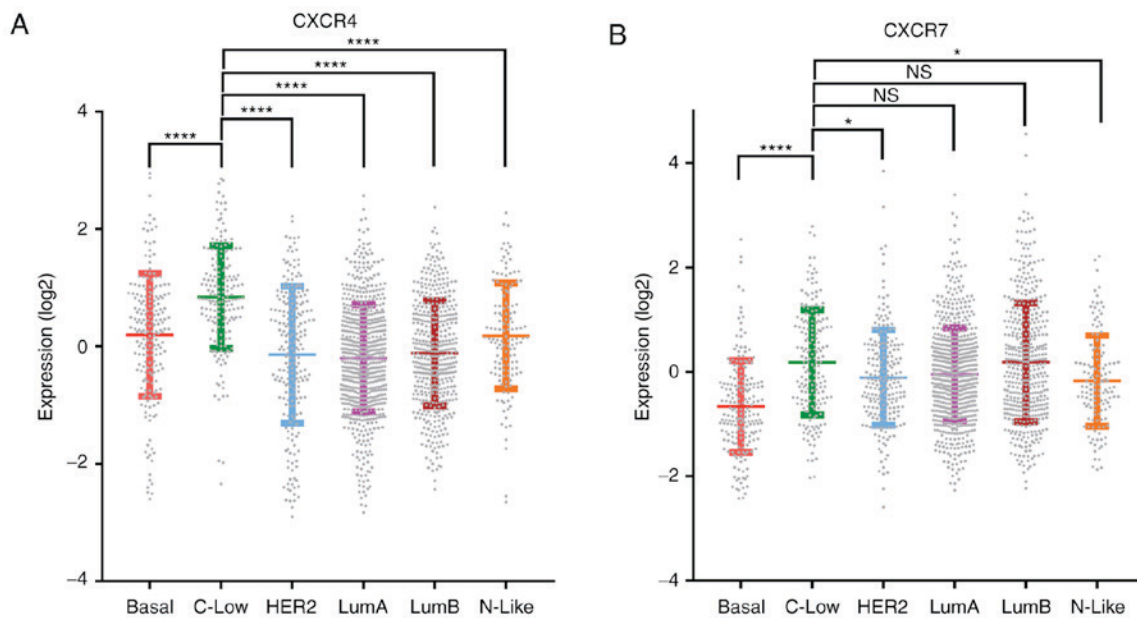


Figure 4. The expression of CXCR4 and CXCR7 is enriched in the C-Low molecular subtype of breast tumours compared with the basal molecular subtype. Log2 expression values of (A) CXCR4 and (B) CXCR7 in 6 subtypes of breast tumours, including Basal, C-Low, HER2, LumA, LumB and N-Like. Data were sourced from the METABRIC breast cancer data (29) and analysed using [www.cbioportal.org](http://www.cbioportal.org) (27,28). Statistical analysis was performed using one-way analysis of variance followed by Tukey's multiple comparisons post-hoc test. \* $P < 0.05$ , \*\*\*\* $P < 0.0001$ . NS, not significant; Basal, Basal-Like; C-Low, Claudin-Low; HER2, human epidermal growth factor receptor 2-enriched; LumA, Luminal A; LumB, Luminal B; N-Like, Normal-like; CXCR, CXC chemokine receptor.

calcium signalling may be influenced by CXCR4, CXCR7 and CD24 (7,13), and that their expression differed between the more epithelial MDA-MB-468 (basal A) and the more mesenchymal MDA-MB-231 (basal B) breast cancer cell lines, the effect of EMT induction on CXCR4, CXCR7, CD24 and HIF2 $\alpha$  expression in MDA-MB-468 cells was assessed. In the present study EMT induction with two distinct inducers was assessed to define the changes associated with EMT rather than the stimuli. Our previous studies of EMT in EGF and hypoxia models in this cell line produced increases in levels of the mesenchymal markers N-cadherin, zinc finger protein SNAI1, zinc finger E-box binding homeobox 1, CD44, twist-related protein and vimentin, downregulation of the epithelial markers E-cadherin and Claudin-4, as well as CD24, and changes to a more spindle morphology (31-33). In the present study, EGF and hypoxia produced opposing effects on CXCR4 levels and did not affect CXCR7 levels (Fig. 3A and B). Only the mRNA level of the known EMT marker CD24 was significantly decreased following induction of the EMT by the two inducers ( $P < 0.01$ ; Fig. 3C). HIF2 $\alpha$  mRNA levels significantly increased following hypoxia ( $P < 0.05$ ), however they were unaffected by EGF (Fig. 3D).

*CXCR4 and CXCR7 expression is enriched in breast tumours with mesenchymal features.* Assessment of gene expression profiles in breast tumours classified based on the differential expression of 50 genes (PAM50) (29), demonstrated that CXCR4 and CXCR7 levels were higher in the C-Low subtype compared with the other subtypes (Fig. 4). The C-Low subtype is markedly associated with the EMT and stem cell-like features (34).

## Discussion

The present study identified distinct CXCL12-induced  $\text{Ca}^{2+}$  responses between two basal-like breast cancer

cell lines, MDA-MB-231 (basal B) and MDA-MB-468 (basal A). CXCL12-mediated  $\text{Ca}^{2+}$  responses were observed in MDA-MB-231 cells but not in MDA-MB-468 cells. It has been demonstrated that CXCL12/CXCR4-mediated  $\text{Ca}^{2+}$  signalling is enhanced in invasive breast cancer cell lines, such as MDA-MB-231 and BT-549, compared with non-metastatic cell lines, due to differences at the level of G protein subunit coupling that may prevent the activation of CXCR4 in less metastatic cell lines (35). The results of the present study expand upon these findings, as it was demonstrated that CXCL12-induced increases in  $[\text{Ca}^{2+}]_{\text{CYT}}$  were more pronounced in the more metastatic MDA-MB-231 cell line compared with less metastatic MDA-MB-468 cells.

The potential cause of differential CXCL12-induced  $\text{Ca}^{2+}$  signalling between MDA-MB-231 and MDA-MB-468 cells was explored by assessing the expression of the CXCL12 receptors CXCR4 and CXCR7, as well as potential regulators of CXCL12 responses, specifically CD24 and HIF2 $\alpha$  (13,14). The results of the present study demonstrate that the expression of CXCR4 and CXCR7 is increased in MDA-MB-231 cells compared with MDA-MB-468 cells. The presence of CXCR4 and CXCR7 in the two breast cancer cell lines supports the results of previous studies which state that CXCR4 and CXCR7 are expressed in a variety of human malignancies, including in breast and lung cancer (36,37). The expression of CXCR4 and CXCR7 suggests that these receptors may contribute to the CXCL12-induced  $\text{Ca}^{2+}$  responses in MDA-MB-231 cells, which were observed in the present study. The non-responsiveness of MDA-MB-468 cells to CXCL12 stimulation (as measured by increases in  $\text{Ca}^{2+}$  levels) may be due in part to the decreased expression of CXCR4 and CXCR7 in this cell line compared with MDA-MB-231 cells. Analysis of CD24 mRNA expression indicated that CD24 was abundantly expressed in non-responsive MDA-MB-468 cells

compared with MDA-MB-231 cells, a known feature of basal A cell lines compared with basal B (38). The differential CD24 expression detected in the present study is also consistent with the results of a previous study by Schindelmann *et al* (39); although this study did not assess MDA-MB-468 cells, it was reported that CD24 mRNA levels are generally increased in non-invasive breast cancer cell lines compared with invasive cell lines. The significant upregulation of CD24 observed in MDA-MB-468 cells may have also contributed to the attenuation of CXCL12-mediated  $\text{Ca}^{2+}$  signalling in this cell line, given that CD24 interferes with CXCL12/CXCR4-mediated cell migration and tumour growth in pre-B lymphocytes and breast cancer cells (13). By contrast, levels of HIF2 $\alpha$ , which has been demonstrated to modulate CXCR4 expression (14), did not differ significantly between the two cell lines, therefore it is unlikely to have contributed to any differences in CXCL12-mediated  $\text{Ca}^{2+}$  signalling.

Having revealed that CXCR4, CXCR7 and CD24 were differentially expressed in more mesenchymal MDA-MB-231 cells (40) compared with MDA-MB-468 cells in their more epithelial state (17), the expression of these targets were investigated in MDA-MB-468 cells following EGF- and hypoxia-induced EMT. Induction of EMT with EGF in MDA-MB-468 cells decreased CXCR4 mRNA levels while the hypoxia-induced EMT was associated with a significant increase in CXCR4 levels. This suggests that the induction of the EMT via EGF and hypoxia differentially affects CXCR4 expression in MDA-MB-468 cells. Bertran *et al* (41) previously demonstrated an increase in CXCR4 expression in rat hepatoma cells treated with transforming proliferation factor- $\beta$  to induce the EMT. Hence, transcriptional regulation of CXCR4 may differ depending on the EMT stimuli and may not be a fundamental characteristic of a more mesenchymal state. In the present study, CXCR7 levels were unaltered during hypoxia- and EGF-induced EMT in MDA-MB-468 cells. Hypoxia- and EGF-induced EMT in MDA-MB-468 cells produced a significant decrease in levels of CD24, consistent with its known association as a marker of the more epithelial state (31). CXCR4 and CXCR7 levels were enriched in the C-Low molecular subtype of breast tumours compared with the basal molecular subtype. The C-Low subtype is highly associated with metaplastic, EMT and stem cell-like features (34,42). Hence, increased levels of CXCR4 and CXCR7 in C-Low sub-types of breast cancer is consistent with the increased levels of these two receptors in the basal B MDA-MB-231 breast cancer cell line, which usually exhibits increased levels of mesenchymal markers (30) compared with the less mesenchymal basal A MDA-MB-468 cell line (30). Hence, significant differences in CXCL12-induced  $\text{Ca}^{2+}$  signalling may also be a feature of different types of breast cancer and influence their invasive/metastatic properties. This should be the primary focus of future *in vivo* studies as methods of assessing  $\text{Ca}^{2+}$  signalling in xenografts continue to progress. In conclusion, these studies have defined distinct differences in CXCL12-mediated  $\text{Ca}^{2+}$  signalling between MDA-MB-468 and MDA-MB-231 breast cancer cells. The present study also provides evidence for the occasional differential remodelling of potential  $\text{Ca}^{2+}$  signalling regulators as a consequence of EGF and hypoxia in MDA-MB-468 breast cancer cells. It also provides evidence that CXCR4 and

CXCR7 expression is enriched in breast tumours with mesenchymal features.

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