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Research paper

Amplification of a calcium channel subunit *CACNG4* increases breast cancer metastasis

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A B S T R A C T

Background: Previously, we found that amplification of chromosome 17q24.1-24.2 is associated with lymph node metastasis, tumour size, and lymphovascular invasion in invasive ductal carcinoma. A gene within this amplicon, *CACNG4,* an L-type voltage-gated calcium channel gamma subunit, is elevated in breast cancers with poor prognosis. Calcium homeostasis is achieved by maintaining low intracellular calcium levels. Altering calcium influx/efflux mechanisms allows tumour cells to maintain homeostasis despite high serum calcium levels often associated with advanced cancer (hypercalcemia) and aberrant calcium signaling.
Methods: In vitro 2-D and 3-D assays, and intracellular calcium influx assays were utilized to measure tumourigenic activity in response to altered CANCG4 levels and calcium channel blockers. A chick-CAM model and mouse model for metastasis confirmed these results *in vivo*.
Findings: CACNG4 alters cell motility *in vitro*, induces malignant transformation in 3-dimensional culture, and increases lung-specific metastasis *in vivo*. CACNG4 functions by closing the channel pore, inhibiting calcium influx, and altering calcium signaling events involving key survival and metastatic pathway genes (*AKT2, HDAC3, RASA1* and *PKCζ*).
Interpretation: CACNG4 may promote homeostasis, thus increasing the survival and metastatic ability of tumour cells in breast cancer. Our findings suggest an underlying pathway for tumour growth and dissemination regulated by *CACNG4* that is significant with respect to developing treatments that target these channels in tumours with aberrant calcium signaling.
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1. Introduction

Metastatic spread occurs via the blood and lymphatic circulatory systems. The sentinel lymph node (SLN) is the first node that receives lymphatic drainage from primary breast tumours, and contains populations of malignant cells with the earliest necessary genomic changes to allow metastasis. In a previous study, we used aCGH to compare the genomes of primary breast invasive ductal carcinomas (IDCs), their sentinel and more distant lymph node metastases; to IDCs without any nodal metastases [6]. A significant correlation was found between gain of chromosome 17q24.1-24.2 and poor prognosis markers such as the presence of sentinel and more distant lymph node (LN) metastases, larger tumour size, and more frequent peritumoural lymphovascular

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Research in context

Evidence before this study

A significant correlation was found between gain of chromosome 17q24.1-24.2, including the *CACNG4* gene, and poor prognosis markers such as lymph node metastases, larger tumour size, and more frequent peritumoural lymphovascular invasion. Voltage gated calcium channels (T, L, N, P/Q, and R- type VGCCs) have been associated with several cancers [1]. They play a role in cell motility, and are thought to be linked to calcium dependent mitogenic signals from epidermal growth factor [2–4]. In breast cancer, only T-type VGCCs have been found to be overexpressed in the HER2-positive SKBR3 cell line that acquired resistance to trastuzumab, as well as in luminal versus basal breast cancers [5]. The functions of L-type VGCCs and *CACNG4* in these non-excitable tissues, especially breast, are unknown.

Added value of this study

We have shown that L-type VGCCs are expressed and regulate calcium signaling in non-excitable breast cancer cells. We have elucidated a role for the gamma subunit, *CACNG4,* in aggressive tumour cell and metastatic behavior using 2-D and 3-D *in vitro*, as well as *in vivo* models. We have also validated our previous findings by showing that higher expression of *CACNG4* is significantly associated with LN metastasis ($n = 1661$). CACNG4 modulates VGCCs in a closed state, maintaining low intracellular calcium levels, promoting homeostasis and metastatic abilities such as cell survival, adhesion, motility, and dissemination. These findings are functionally significant with respect to developing treatments that target these channels in tumours with aberrant calcium signaling. VGCCs function in parallel to cell surface receptors involved in calcium signaling, for example, EGFR. Therapeutic combinations of anti-CACNG4 and anti-EGFR agents could improve targeted inhibition of cancer cells overexpressing *CACNG4*, or of cells that have developed resistance to other inhibitors.

Implications of all the available evidence

Down regulation of *CACNG4* modulates the channels to preferentially remain in their active or open state resulting in higher intracellular Ca^{2+} levels. Elevated intracellular calcium destabilises conditions of homeostasis, and could thus result in the decreased tumourigenic functions we observed. By disrupting the effects of CACNG4, dissemination of cancer cells to lymph nodes could be blocked, therefore preventing deaths from breast cancer metastasis. *CACNG4* provides a novel and targetable pathway that cancer cells use to progress to aggressive disease beyond the breast.

invasion. Candidate driver genes of the amplicon, including L-type voltage gated calcium channel gamma subunit 4 (*CACNG4*), were further examined by qPCR and IHC in invasive breast tumours. Tumours with gains of the 17q amplicon, and tumours with nodal metastases were associated with higher protein expression of CACNG4 compared to those without gains or metastases [6].

Calcium ions are crucial second messengers involved in the regulation of multiple signaling pathways. Calcium homeostasis is tightly regulated by influx/efflux mechanisms *via* ion channels, ATPase pumps and exchangers [1]. All cell types maintain low intracellular calcium levels to achieve homeostasis [7]. Altering the calcium infux/efflux mechanisms allow cells to buffer calcium in conditions of high serum calcium that can be brought on by advanced cancer (hypercalcemia) and aberrant calcium signaling [8–11]. Voltage gated calcium channels (T, L, N, P/Q, and R- type VGCCs) have been associated with prostate, colon, and pancreatic cancers, melanomas and gliomas [1]. They play a role in cell motility, and are thought to be linked to calcium dependent mitogenic signals from epidermal growth factor [2–4]. Calcium channel blockers that target VGCCs, are among the most widely prescribed drugs used to treat high blood pressure by reducing intracellular calcium, thus relaxing and widening blood vessel walls. Recently however, a meta-analysis of 17 studies including almost 150,000 women, found a significant association of increased breast cancer risk after long term use ($>$ 10 years) of these drugs [12]. Although this association has been refuted, a link between VGCCs and breast cancer has not previously been investigated [13–15].

The VGCC complex is composed of α, β, δ, and γ subunits with several isoforms. The $\alpha 1$ subunit forms the point of entry for Ca^{2+} ions, and offers unique binding sites for toxins, drugs, and determinants of activation and inactivation. The accessory $\alpha 2$, β, and δ subunits modulate channel activity, expression and trafficking of the other subunits to the plasma membrane [16,17]. The γ subunits, with 8 isoforms (CACNG1-8) are unique to L-type channels and are thought to be involved in the activation and inactivation of the channel itself by inhibiting Ca^{2+} currents (Fig. 1(a)) [17–21]. *CACNG4* was found to be one of 10 genes significantly over-expressed in glioblastoma cell lines resistant to erlotinib treatment [22]. It was also ranked as the 10th most overexpressed gene out of 45 other genes up-regulated by androgens in human prostate cancer xenografts in mice [23]. In breast cancer, only T-type VGCCs have been found to be overexpressed in the HER2-positive SKBR3 cell line that acquired resistance to trastuzumab, as well as in luminal versus basal breast cancers [5]. They have also been associated with an increase in cell proliferation of the luminal breast cancer cell line MCF7 compared to non-malignant MCF10A breast epithelial cells, which could be curbed with calcium channel blockers [24, 25]. T-type calcium channels have been widely studied in several cancer types including breast, prostate and ovarian cancers, where inhibition of these channels has been shown to lower proliferation rates as well as induce apoptosis of tumour cells *in vitro* and *in vivo* [25–28]. There is also literature on the role of other calcium channel subunits in cancer, such as *CACNA2D3* as a tumour suppressor in metastatic breast cancer, lung cancer, esophageal cancer, and neuroblastoma [29].The functions of L-type VGCCs and *CACNG4* in these non-excitable tissues, especially breast, are yet to be elucidated. In the present study, we sought to characterize *CACNG4* in breast cancer, with two particular objectives: to determine if intracellular calcium levels are modulated through L-type VGCCs and *CACNG4*, as well as elucidate its role in metastasis-related functions.

2. Materials and methods

All experimental procedures carried out as a part of this study were approved by the Research Ethics Board at the Princess Margaret Cancer Center.

2.1. CACNG4 RNAi vectors and antibodies

Stable and transient knockdown was achieved using lentiviral shRNA and siRNA interference in MCF7 and MDA-MB-231 invasive breast cancer cell lines (Genecopoeia and Sigma-Aldrich). CACNG4 over-expressing transfectants with GFP reporters were also established using lentiviral cDNA vectors (System Biosciences). The vector was modified for compatibility with gateway cloning technology for efficient cDNA transfer into the dual promoter vector system of choice (Life Technologies). Antibodies against CACNG4 were purchased from Aviva Systems Biology and used at a dilution of 1:5000 for western blotting. For immunofluorescence and IHC staining, the CACNG4 antibody was purchased from Novus Biologicals and used at a dilution of 1:50 and 1:800, respectively. The CACNAE1 antibody was purchased from Alomone Labs, Ltd. and used at a dilution of 1:500. Antibodies against p-PKC-zeta, AKT2 and p-AKT were

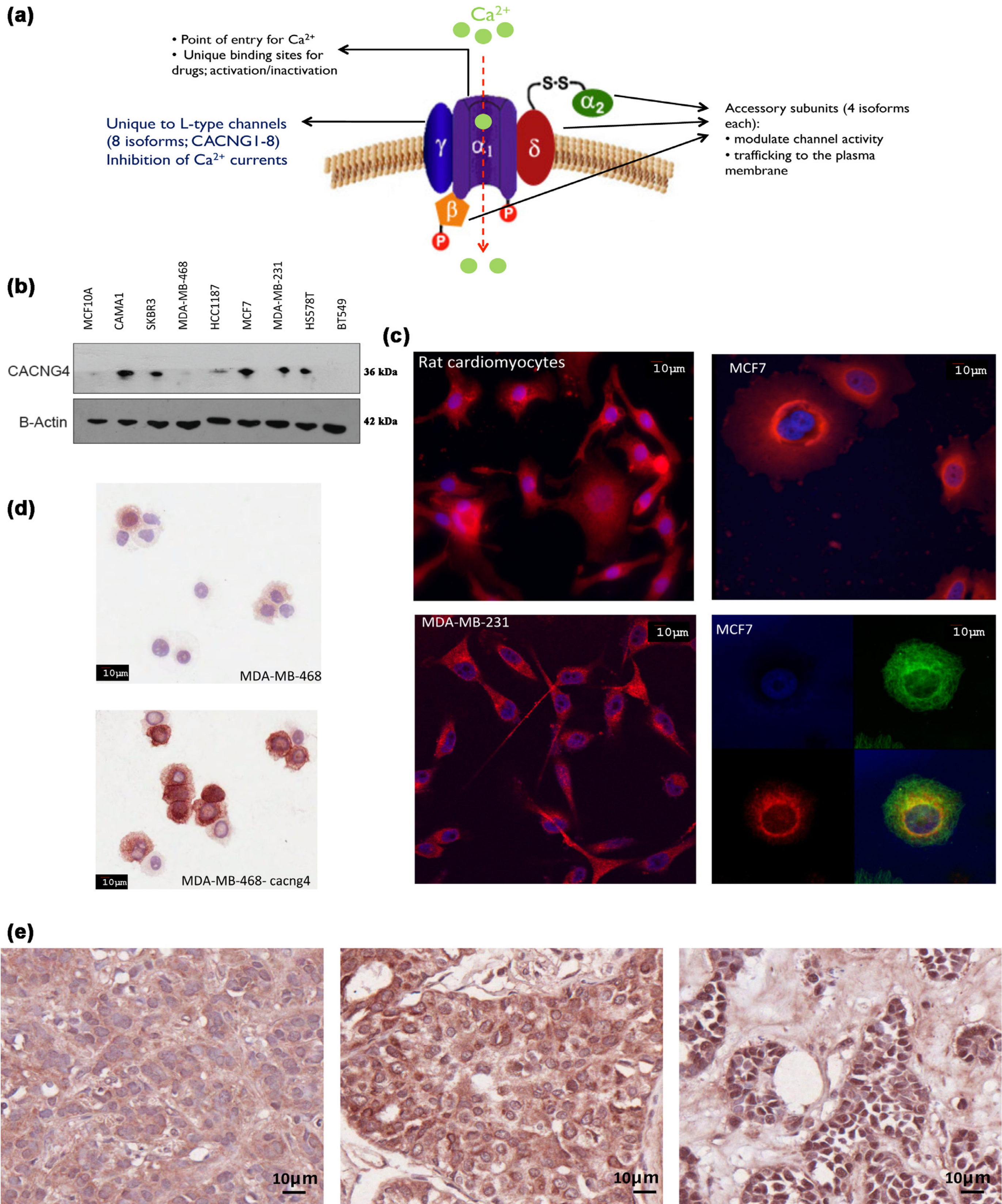

Fig. 1. Gamma subunit CACNG4 of L-type VGCCs is variably expressed in breast cancer cells (a) VGCCs and their subunits; gamma subunits are unique to L-type VGCCs (b) Protein expression of CACNG4 relative to beta-actin in 8 breast cancer cell lines of various invasive potentials, and a non-malignant breast epithelial cell line (MCF10A), single band is observed at 36kDa (c) Immunofluorescence staining of rat cardiomyocytes and breast cancer cell lines MCF7 and MDA-MB-231 show cytoplasmic/perinuclear and some membrane localization of CACNG4 protein (d) Immunohistochemical staining of the breast cancer cell line MDA-MB-468 (wildtype versus overexpressing CACNG4) show cytoplasmic/perinuclear and membrane localization (e) IHC staining of primary breast tumours shows a range of low, intermediate and high expression of CACNG4 protein in the cytoplasm and membrane of tumour cells.

purchased from Cell Signaling and used at a dilution of 1:1000. The HDAC3 antibody was purchased from Abcam and used at a dilution of 1:1000. The beta-actin antibody was purchased from Sigma-Aldrich and used at a dilution of 1:100,000.

2.2. Cell culture and transfections

MDA-MB-231 and MCF7 cell lines (American Type Culture Collection) were maintained in DMEM (+Pen/Strep) supplemented with 10% FBS (Sigma-Aldrich). MCF10A cells were grown in DMEM (+Pen/Strep) supplemented with 5% horse serum (Life Technologies), EGF (20ng/ml), hydrocortisone (0.5ug/ml), cholera toxin (100ng/ml), and insulin (10ug/ml). Cells were transfected using Endofectin (Genecopoeia) or the pPACK high titer lentivirus packaging system (System Biosciences). HEK293t cells were transfected with 2μg/4mL of lentiviral vectors and packaging plasmids and selected with 2.5mg/mL neomycin G418 or 0.5μg/ml puromycin for over-expression and knockdown cell lines, respectively. Control cells were transfected with scrambled lentiviral vectors in the same manner. For transient RNAi, 3 individual siRNA clones for *CACNG4* and a scrambled control were transfected with Lipofectamine RNAiMAX (Life Technologies) into breast cancer cell lines at 100pmol/2mL.

2.3. Cell proliferation assay

Cell proliferation was measured in 96 well plates by MTT assay (Promega) between days 1−7 as absorbance measurements (490 nm) using the Flexstation 3 (Molecular Devices). Results are expressed as percentage of absorbance measurements of untreated control cells, and are provided as an average of experiments performed in triplicate (Students *t*-test, $p<0.05$ for significance).

2.4. Transwell migration and invasion assay

24-Transwell plates (8-um pore size; BD Biosciences) coated with Matrigel™ or without a coating were used for the invasion and migration assays, respectively. 10% FBS was used as a chemo-attractant in the lower chamber to initiate cell migration or invasion (through Matrigel™). The total number of migrating or invasive cells was counted in the entire field of each insert using scanned images (20× magnification). Results are expressed as percentage of control cells, and are provided as an average of experiments performed in triplicate (Students *t*-test, $p<0.05$ for significance).

2.5. Adhesion/aggregation assay

Cell aggregation was analyzed by hanging drop assays. 1.5×10^4 trypsinised single cells were suspended in a 35 μL drop of culture medium in the lid of a culture dish, inverted and incubated overnight to allow cell-cell aggregates to form. Aggregate size was analyzed with the Positive-Pixel algorithm as a measurement of the number of pixels in the area selected (ImageScope).

2.6. Western blotting

Cells cultured under normal conditions were lysed in CytoBuster protein extraction reagent (Novagen) containing kinase and phosphatase inhibitors. $10-20\mu$g total protein was loaded onto 4−12% SDS-NuPage gradient gels (Life Technologies). Images were scanned with a Bio-Rad scanner and quantified by Quantity One software (Bio-Rad).

2.7. Immunofluorescence

For immunofluorescence studies, cells were grown on collagen IV coated coverslips and imaged using confocal microscopy (BD Biosciences). Cells were fixed with room temperature 4% paraformaldehyde. This was followed by blocking with 1% BSA-PBST. Primary antibodies were diluted to 1:50 in the same solution for an overnight incubation at 4 °C. Secondary antibodies conjugated to FITC or Cy3 against rabbit or mouse, respectively were diluted to 1:1000 and incubated for 1 h at room temperature. After the final wash, DAPI (Life Technologies, 1mg/ml) diluted 1:50,000 in water, was applied to the cells for 30 s. Coverslips were then mounted in Vectashield (Vector Labs) for microscopy.

2.8. Calcium influx assays

MDA-MB-231 cells were treated with increasing concentrations of calcium channel antagonists amlodipine and verapamil (5μM−100μM) for 4 h. Cells treated with the EGFR specific inhibitor Tryphostin AG178 were incubated at increasing concentrations for 1 h (5−100 μM). Intracellular calcium influx response to addition of 20 μl FBS, was measured at 2 s intervals for a period of 200 s at 37 °C using the Fluo-4NW calcium indicator dye (Molecular Probes, Invitrogen) on the Flexstation 3 (Molecular Devices). Measurements are given as Relative Fluorescent Units (RFU) based on the level of calcium indicator fluorescence (Excitation/Emission: 488/530 nm). Protocols were followed as per manufacturer's instructions.

2.9. Gene expression

EGFR and intracellular calcium signaling gene expression analysis was carried out using TaqMan® Array Plates and validated with SYBR green RT-PCR (Applied Biosystems) using primers designed in-house.

CACNAC1/ Ca_v1.2: F GCAGGAGTACAAGAACTGTGAGC, R CGAAGTAGGTGGAGTTGA CCAC
RAS: F CTGCTGGTGTTCGCCATTAACG, R GATCTGCCTTGTTCCCGACCAA
HDAC3: F GAGTTCTGCTCGCGTTACACAG, R CGTTGACATAGCAGAAGCCA GAG
ATP2A1: F ATTCACCCTGGAGTTCTCCCGA, R CTCGCACATAGTTACAGCG GTC
AKT1: F TGGACTACCTGCACTCGGAGAA, R GTGCCGCAAAAGGTCTTCATGG
AKT2: F CATCCTCATGGAAGAGATCCGC, R GAGGAAGAACCTGTGCTCCATG
AKT3: F CGGAAAGATTGTGTACCGTGATC, R CTTCATGGTGGCTGCATCT GTG
EGFR: F AACACCCTGGTCTGGAAGTACG, R TCGTTGGACAGCCTTCAAG ACC
ELK1: F GCTGCCTCCTAGCATTCACTTC, R CCACGCTGATAGAAGGGAT GTG
HRAS: F ACGCACTGTGGAATCTCGGCAG, R TCACGCACCAACGTGTAGA AGG
MUC1: F CCTACCATCCTATGAGCGAGTAC, R GCTGGGTTTGTGTAAGAGA GGC
PIK3R2: F ATGGCACCTTCCTAGTCCGAGA, R CTCTGAGAAGCCATAGTG CCCA
RAF1: F TCAGGAATGAGGTGGCTGTTCTG, R CTCGCACCACTGGGTCAC AATT
RHOB: F ACATTGAGGTGGACGGCAAGCA, R CTGTCCACCGAGAAGCAC ATGA
RHOD: F CCGAAGAAACGGATTGGAGCCT, R AAGACGGCGTGGACGTTG TCAT
VAV1: F TCAGTGCGTGAACGAGGTCAAG, R CCATAGTGAGCCAGAGAC TGGT
CBL: F GCACGTTCAGTCTGGATACCTC, R GCAGTTTTGGCACAGGAAGAGG
PDPK1: F CGTTCTTCGAGTCCGTCACGTG, R CAGCCAAACTGGCTCAGGAGAT
RASA1: F GGGACATCCAATAAACGCCTTCG, R TTTGCTACTTGGACACTATT CAGG

2.10. In vivo chick chorioallantoic membrane (CAM) assays for metastasis

The avian embryo model of spontaneous metastasis was used as described previously [30]. Briefly, control and CACNG4 over-expressing HT1080 fibrosarcoma cells (8×10^4 cells in PBS) were applied topically onto the CAM (chorioallantoic membrane) of 10 day old chicken embryos *in ovo* through a small window in the eggshell near the chorioallantoic vein. Seven days after tumour cell application, the primary tumours were harvested, weighed, and processed. To quantify the extent of tumour cell metastasis genomic DNA was isolated from the lower CAM, lung and liver tissues. The amount of human *Alu* sequences was measured by quantitative RT-PCR relative to chicken *GAPDH*, as an internal control to normalize the host tissue contribution. The metastatic potential of both control and CACNG4 overexpressing groups was calculated with the ΔCT method. Significance was determined using the Students *t*-test ($p<0.05$ for significance).

2.11. Animal study

PyMT RhoC-/- cells were obtained from PyMT RhoC knockout mice [31]. A lentiviral construct for *CACNG4* was generated by cloning the full-length cDNA of human *CACNG4* into the plenti-C-Myc-DDK-IRES-Puro vector system purchased from ORIGENE. PyMT-Rho null cells were transfected using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's protocol. To obtain transfectants stably over-expressing CACNG4, the transfected cells were selected in the presence of puromycin at a concentration of 2 μg/ml. Before injection, the cells were washed with PBS, harvested by trypsinisation, resuspended and kept on ice until injection. Precise cell counts were obtained from samples of the cell suspensions. Five-week-old female NOD/SCID mice were injected intravenously with 1×10^6 tumour cells resuspended in a volume of 500 μl PBS via the lateral tail vein. The mice were maintained in the animal facility at the Princess Margaret Cancer Center/Ontario Cancer Institute in accordance with the established ethical care regulations of the Canadian Council on Animal Care.

2.12. Three-dimensional culture in Matrigel ™

Protocols were followed as per previously described [32]. MCF10A cells transduced with the empty lentiviral vector and a *CACNG4* cDNA lentiviral expression vector (System Biosciences) were mixed with growth medium+ 4% Matrigel™ and plated on top of the solidified Matrigel™ in each well of the chamber slide for a final count of 5000 cells per well. Acinar structures were imaged and harvested on day 6, 10 and 15. For harvesting, cells were fixed in 4% paraformaldehyde followed by 2% formalin for 2 h in the wells themselves. Washes were performed with PBS/Glycine. The contents of each well were then scooped into tissue molds and embedded in Histo-Clear (National Diagnostics) followed by paraffin. FFPE blocks were sectioned at 3.5 μm thickness for immunofluorescence staining. Size of acini was determined using images obtained from light microscopy and the ImageScope software, measured in pixels/mm^2. Significance was determined using the Students *t*-test ($p<0.05$ for significance).

3. Results

3.1. L-type channels and subunit CACNG4 are expressed in breast cancer

CACNG4 showed variable expression across a panel of breast cancer cell lines with different invasive potentials (Fig. 1(b)). Specificity of the antibody was tested with a blocking peptide incubation that targeted the expected 36kDa protein band for CACNG4 only (see Fig. S1(a)). CACNG4 expression is high in ER positive cell lines (MCF7, MDA-MB-361, CAMA1) and lower in EGFR-high cell lines (MDA-MB468, MDA MB-231) (see Fig. S1(c)). The non-tumourigenic breast cancer cell line MCF10A did not show expression of CACNG4. *CACNG4* was amplified in 7–9% of cases within TCGA (n = 816) and METABRIC (n = 2173) copy number datasets [33,34]. The NKI (Netherlands Cancer Institute) microarray gene expression dataset of 295 breast tumours showed that higher *CACNG4* expression was directly correlated with LN metastasis, ER positivity, and AR positivity, and negatively correlated with triple negative breast cancer (see Fig. S2 (a)) [35]. The IPC (Institut Paoli-Calmettes) breast cancer dataset of 266 patients showed significant correlation of higher *CACNG4* expression with LN involvement, increased tumour grade, ER, and AR positivity (see Fig. S2(a)) [36]. A TCGA (The Cancer Genome Atlas) dataset of breast cancers (n = 1100) showed positive association of overexpression of *CACNG4* with LN metastasis and ER, AR, and HER2 positivity [34] (Fig. S2(b)). There was no significant effect on progression-free or disease-free survival of patients over-expressing *CACNG4* (TCGA and METABRIC datasets). Taken together, in accordance with our previous findings, increased *CACNG4* expression shows significant association with LN metastasis in three of three datasets (n= 1661 breast tumours).

With the WolfPsort protein structure prediction software, it was determined that the CACNG4 protein is 327a.a long, with three tentative transmembrane domains, suggesting a high likelihood of plasma membrane localisation. By confocal microscopy we showed that CACNG4 protein is localised in the cytoplasm of MCF7 and MDA-MB-231 cells, in endocytic vesicles or the endoplasmic reticulum due to the strong perinuclear concentration (Fig. 1(c)). This is concordant with previous reports of the channel alpha and beta subunits that also show localisation in the perinuclear region. There was evidence of membrane staining that was most clearly seen in the MDA-MB-468 cell line transduced to over-express *CACNG4* (Fig. 1(d)). Highest intensity of CACNG4 was seen at cell-cell contact regions. Expression in primary breast tumours showed cytoplasmic and membrane localisation (Fig. 1(e)). As a positive control, we stained rat cardiomyocytes that are known to express *CACNG4* and other subunits of the L-type calcium channels that are involved in cardiac muscle contraction. These cells also showed similar cytoplasmic and membrane staining (Fig. 1(c)). We have also shown that the localisation of $Ca_v1.2$ (ubiquitously expressed alpha/pore subunit) is similar to that of CACNG4 in MCF7 and MDA-MB-231 cells (see Fig. S1(b)). Thus, we concluded that L-type channels are expressed in non-excitable breast cancer cells both in the plasma membrane as well as in the cytoplasm with strong perinuclear localisation.

3.2. Altered expression of CACNG4 affects tumourigenic functions of breast cancer cells in 2-D culture

3.2.1. Proliferation, migration, and invasion

We created stable lentiviral *CACNG4* knockdown cell lines with 21–28% of CACNG4 protein expression compared to scrambled controls and parental MCF7 cell lines. We were also able to transiently knockdown *CACNG4* in both MCF7 and MDA-MB-231 breast cancer cells (see Fig. S3(a)). Knockdown of *CACNG4* reduced the proliferation of MCF7 breast cancer cells significantly at the 48 h time point (Student's *t*-test, $p<0.05$) over a period of 120 h (5 days) (Student's *t*-test, $p<0.005$) (Fig. 2(a)). The migration and invasion ability of breast cancer cells was decreased upon down regulation of *CACNG4*. In the MDA-MB-231 cell line, migration was reduced to 63% compared to controls (Student's *t*-test, $p<0.005$), and in the MCF7 cell line, migration and invasion were reduced up to 7% and 42%, respectively compared to controls (Student's *t*-test, $p<0.001$)) (Fig. 2(b)–(d)). We also observed that cell viability was unaffected upon knockdown of *CACNG4* over a period of 96 h in culture (see Fig. S3(b)). Thus, the assay results were a direct result of compromised cellular function and not cell death due to reduced expression of *CACNG4*.

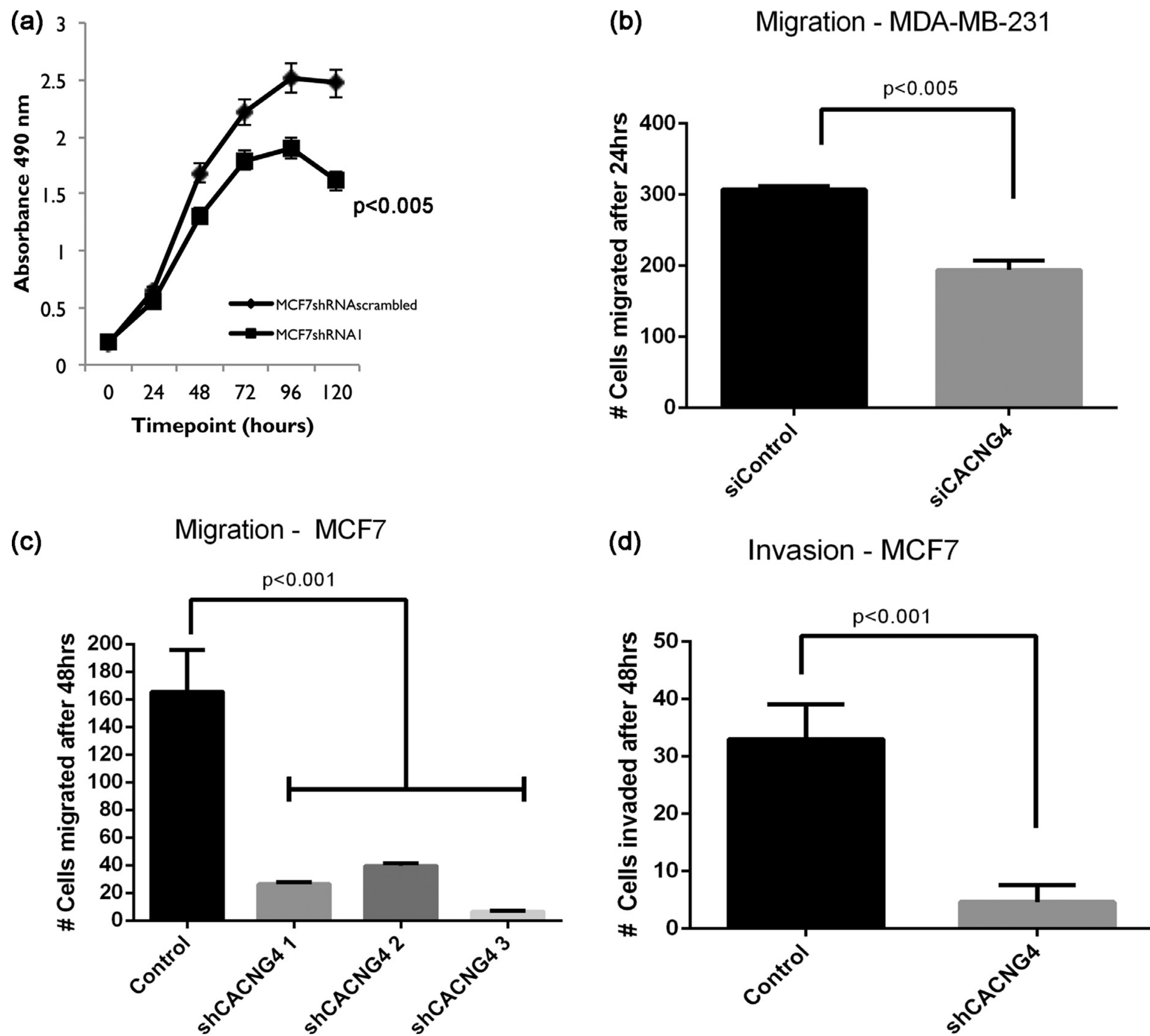

Fig. 2. Down regulation of *CACNG4 via* shRNA or siRNA results in decreased proliferation, motility and cell adhesion of breast cancer cells *in vitro* (a) A decrease in cell proliferation over 5 days in shCACNG4 MCF7 breast cancer cells versus scrambled control (b), (c) A decrease in cell migration through Boyden chamber inserts in shCACNG4 MCF7 and siCACNG4 MDA-MB-231 breast cancer cells versus scrambled controls (d) A decrease in cell invasion through Matrigel^{TM} coated Boyden chamber inserts in shCACNG4 MCF7 breast cancer cells versus scrambled control.

3.2.2. Cell-cell aggregation

In a hanging drop assay, knockdown of *CACNG4* resulted in larger sized cell aggregates indicative of a higher degree of cell-cell adhesion in MDA-MB-231 cells compared to control cells which were visibly suspended as single cells or small clusters (Student's *t*-test, $p = 0.0001$) (Figs. 3(a) and S3(c)). Therefore, knockdown of CACNG4 results in reversion of phenotypes of motile, non-aggregate cells which is suggestive of novel roles for *CACNG4* in known calcium dependent activities such as cell movement during dissemination.

3.3. Benign breast epithelial cells show malignant transformation upon over-expression of CACNG4

Upon stable lentiviral over-expression of *CACNG4* in non-malignant MCF10A cells, between days 4–8 we observed that the size of acinar structures was larger in MCF10A-CACNG4 cells compared to controls. At day 10 the average size of MCF10A-CACNG4 cells was 5033 pixels/mm^2 compared to controls at 795 pixels/mm^2; for a size differential of 6.3-fold (Fig. 4(a)). At day 15 this phenotype was maintained with MCF10A-CACNG4 cells showing an average size of 7585 pixels/mm^2 compared to controls at 1369 pixels/mm^2; for a size differential of 5.5-fold (Fig. 4(a)). Some structures had developed an irregular multi-acinar morphology indicative of increased proliferative/invasive potential as well as loss of polarisation as a result of malignant transformation (Fig. 4(b)). The acini were sectioned and stained for the apoptosis marker cleaved-Caspase 3, and E-cadherin. Indeed, acini of CACNG4 over-expressing cells between day 10–16 had filled lumens with non-apoptotic cells and irregular E-cadherin staining, which are both malignant phenotypes (Fig. 4(c) and (d)). We were thus able to show that CACNG4 plays a role in the proliferative/invasive potential and breakdown of cell-cell organisation, which are the precursors to cell dissemination. Knockdown of CACNG4 in our 2-D in vitro assays shows reduced proliferation and invasion, and conversely, over-expression of CACNG4 in our 3-D assay shows increased proliferation and cell disorganisation.

3.4. Over-expression of CACNG4 results in increased metastatic potential of tumour cells in vivo

We next validated our 2-D and 3-D *in vitro* evidence of aggressive tumour cell behavior, in an *in vivo* functional model that more closely resembles the complex metastatic cascade. The chick embryo

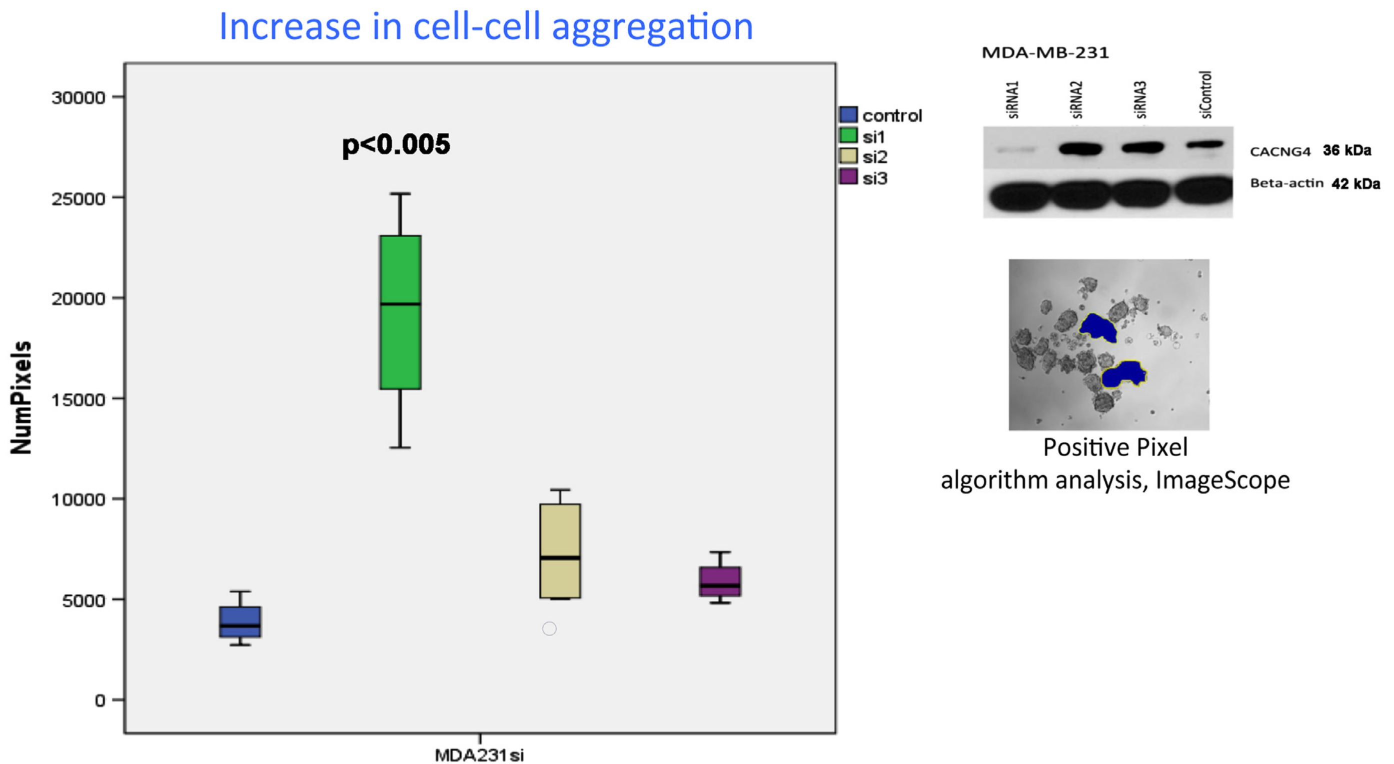

Fig. 3. Cell−cell interactions are altered upon regulation of CACNG4 in breast cancer cells. An increase in MDA-MB-231 cell-cell aggregation in the siRNA1 clone, only (down regulation of CACNG4 was observed in siRNA1 but not siRNA 2 or 3), as measured by aggregate size (number of positive pixels in area selected).

chorioallantoic membrane (CAM) in the *ex ovo* model is a large, translucent organ that is naturally immunodeficient and supports the growth of primary human tumours [30,37−41]. Once vascularized, tumour cells are able to invade the chorionic epithelium and mesenchyme, intravasate the blood vessels, survive in microcirculation, arrest in the vasculature of a secondary organ, extravasate and proliferate to form micrometastatic tumours at distant sites (Fig. 5(a)). Using this model we produced primary tumours on the chick CAM from a weakly metastatic fibrosarcoma cell line HT1080 transduced with control and *CACNG4* over-expressing lentivirus. There was no significant difference in the size of primary tumours produced in the model (60.9 mg for controls versus 63.7 mg for *CACNG4* overexpressing cells) (Fig. 5(b)). There was a significant increase in the dissemination and metastatic potential of *CACNG4* over-expressing cells specifically to the lungs compared to controls (Student's *t*-test, $p<0.05$; $n = 13, 16$ respectively) as assessed by ΔCT measurements of human *Alu* relative to chicken GAPDH (average of 9.48 control versus 75.94 *CACNG4* over-expressing metastasised cells in lungs) (Fig. 5(c)). There was no significant increase in metastatic potential to the liver and lower CAM (see Fig. S4). We next obtained non-metastatic PyMT RhoC-/- cells from PyMT RhoC knockout mice and introduced the human *CACNG4* gene into these cells to create a stable over-expressing cell line [31]. Following a tail vein injection, *CACNG4* over-expressing cells were capable of metastasising to the NOD-SCID mouse lung. As Kaplan Meier analysis illustrates, 4 of 4 mice were positive for lung metastases, and 3 of 4 mice died on day 45 post-injection (1 of 4 mice was euthanized on day 46 post-injection), compared to controls that did not develop metastasis and survived past 100 days (Fig. 5(d)). Thus we concluded that *CACNG4* increases metastatic behavior of tumours *in vivo*, with selectivity for the lungs in a chick model, and while it also significantly reduces survival in mice, we cannot definitely say this was due to increased metastatic burden or other off-target factors affecting survival.

3.5. Voltage-gated calcium channels are active in breast cancer cells and modulated by CACNG4

To determine if L-type VGCCs are targetable in breast cancer, we studied the effects of two L-type specific calcium channel antagonists on tumour cell growth. Verapamil belongs to the phenylalkylamine class of drugs; and amlodipine belongs to the dihydropyridine class of drugs. Verapamil binds an intracellular domain of the channel, while amlodipine directly binds the channel pore's extracellular domain shifting the channel into the inactive or closed state. We showed that L-type specific calcium channel antagonists inhibit cell proliferation in MCF7 (luminal) and MDA-MB-231 (triple negative) breast cancer cells in a dosage dependent manner with varying sensitivity for each cell line (Fig. 6(a) and (b)). The non-malignant MCF10A cell line that does not express CACNG4, was resistant to any effect on cell proliferation compared to breast cancer cell lines, even at the highest dosage tested for both antagonists (20 μM) (see Fig. S5). We tested a panel of breast cancer cell lines and observed a range of IC_{50} concentrations for both drugs; 50 − >100 μM for verapamil (prescribed dosage of 40−120mg /day); and 10−40 μM for amlodipine (prescribed dosage of 2.5−10mg/day) (Fig. 6(a), (b); S5). Cell lines were more tolerant to verapamil ($IC_{50} = 80$ μM for MCF7, >100 μM for MDA-MB-231) and were significantly more sensitive to treatment with amlodipine ($IC_{50} = 15$ μM for MCF7, 40 μM for MDA-MB-231). For subsequent experiments we treated cells with amlodipine due to their measurable response at lower dosages. Thus, we have shown that L-type VGCCs are targetable with calcium channel antagonists, in a dosage dependent manner, decreasing proliferation in breast cancer cells.

Next, we tested whether the channel antagonists were affecting calcium currents within the cell, leading to altered growth of tumour cells. Serum induced Ca^{2+} influx was abrogated by amlodipine in a dosage dependent manner in MDA-MB-231, MDA-MB-468, and BT549 cells (observed as a diminished peak of extracellular Ca^{2+}

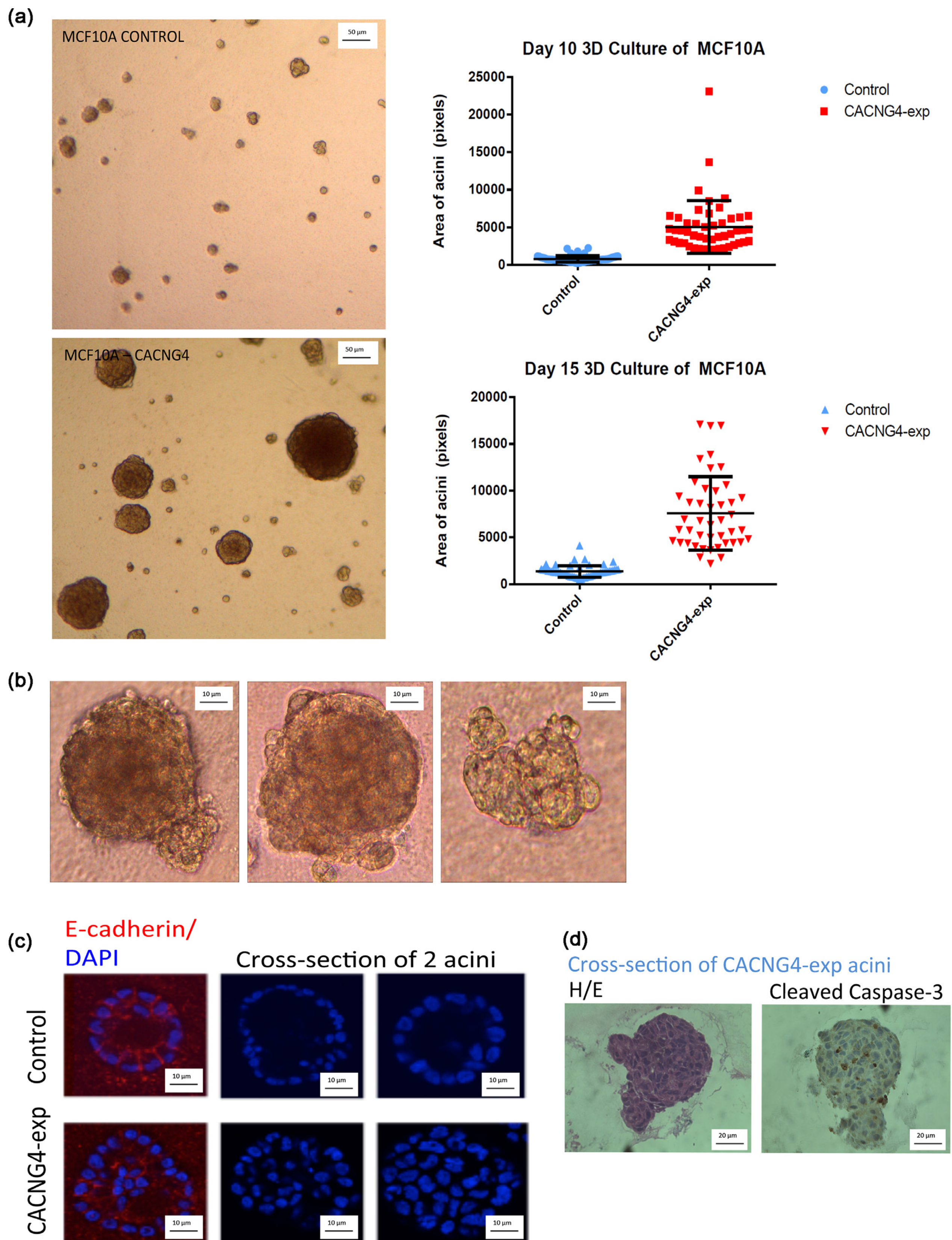

Fig. 4. 3D culture of MCF10A non-malignant breast epithelial cells shows tumourigenic morphogenesis upon over-expression of CACNG4 (a) A significant increase in size was observed in cells over-expressing CACNG4 compared to controls at day 10 and 15 (increase in size of acini were measured as number of pixels in selected area, 4x magnification) (b) Multiacinar structures which are a feature of increased proliferation and loss of polarity in MCF10A cells over-expressing CACNG4 (day 10, 10× magnification) (c) Immunofluorescence staining of MCF10A acini showing a cross-section with filled lumens and disorganisation of cells (loss of E-cadherin: red) in MCF10A cells over-expressing CACNG4 (bottom panel) compared to controls (top panel) between day 10–16 (d) IHC staining for H/E and cleaved Caspase-3 marker for apoptosis in a section of MCF10A acini grown in 3D culture shows mostly non-apoptotic cells filling the lumen of acini between day 10–16.

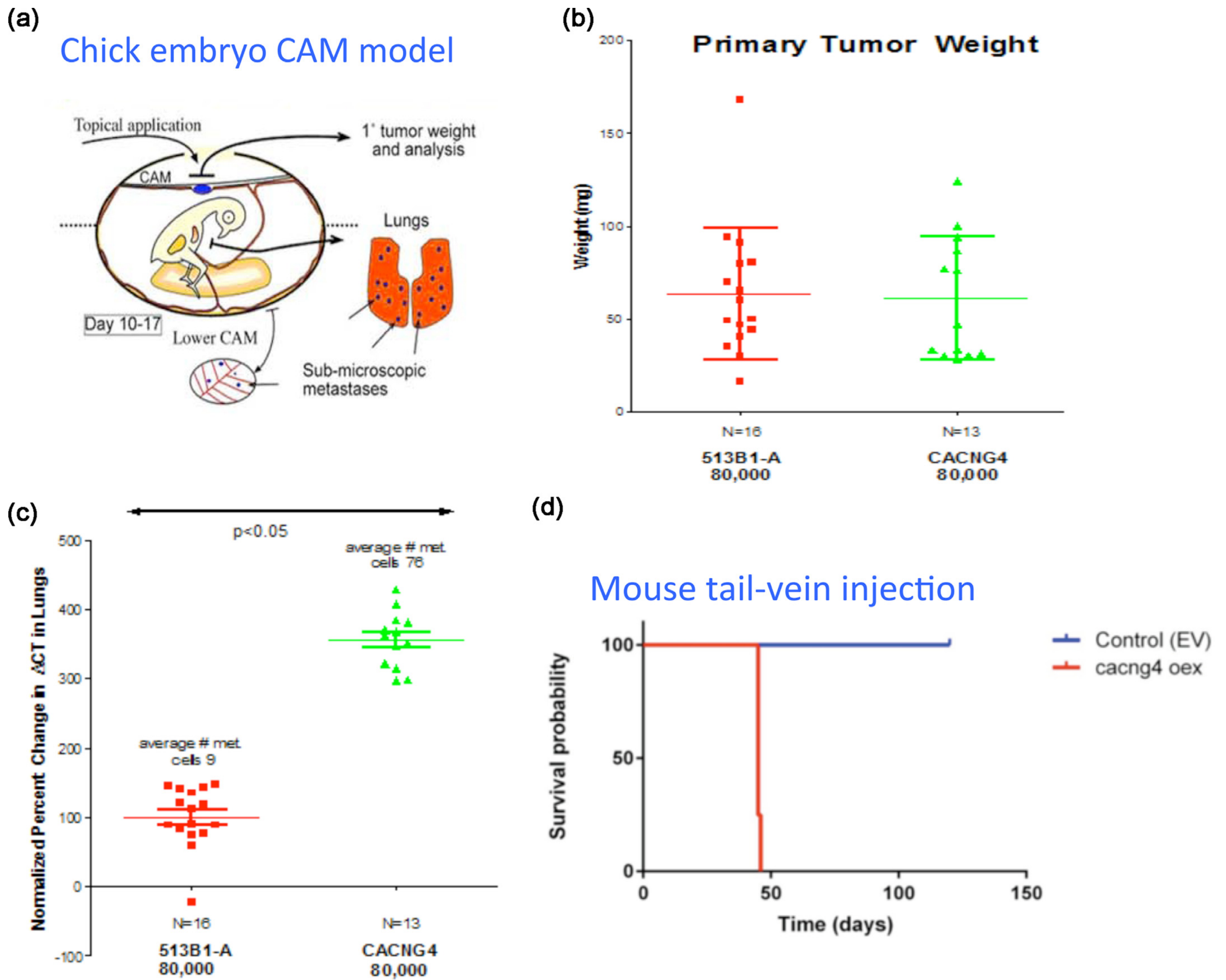

Fig. 5. Over-expression of CACNG4 leads to increased metastasis to lungs *in vivo* (a) Schematic of chick embryo CAM model for metastasis. Micrometastases that form in the chick embryo lungs, liver or lower CAM are analysed for changes in metastatic potential of primary tumours produced from human tumour cells (b) There was no significant change in primary tumour size between CACNG4 over-expressing cells and controls (weighed on day 20) (c) Average number of cells that metastasise to the chick embryo lungs is higher in the metastasis assays that over-express CACNG4 in primary tumours compared to controls. Data are represented as mean ± SEM; Control labeled 513B1-A (d) Non-metastatic PyMT RhoC-/- cells were transduced with *CACNG4* over-expression vectors (cacng4 oex) compared to empty vector controls (EV). Kaplan Meier data illustrates, 4 of 4 mice were positive for lung metastases, and 3 of 4 mice died on day 45 post injection compared to controls.

influx from the plasma membrane as well as decreased height of the plateau phase from intracellular store release) (see Fig. S6). Notably, the MCF7 breast cancer cell line had a very low response to serum induced calcium influx, which could be completely blocked at 20 μM (see Fig. S6). The MCF7 cell line has the highest copy number of the 17q amplicon, and highest expression of CACNG4 compared to other breast cancer cell lines tested [42]. It has been predicted that structurally, the gamma subunits interact with the channel pore maintaining it in an inactive or closed state [18,19,21]. It is possible that the channel pores in MCF7 cells are much more tightly maintained in the closed state as a result. Our results indicate that L-type VGCCs when targeted, result in altered calcium influx in tumour cells of the breast.

We next assessed what effect *CACNG4* has on the modulation of calcium influx through the L-type VGCCs [43,44]. We hypothesised that down regulation of *CACNG4* would work towards modulation of the channel to an active and open state (leading to higher intracellular calcium). The first observation was that *CACNG4* down-regulation significantly rescued the previously described inhibition of proliferation in amlodipine treated MCF7 cells (Fig. 6(c)). Secondly, the baseline Ca^{2+} levels (external and internal stores) was increased upon knockdown of *CACNG4* in untreated MCF7 and MDA-MB-231 cells (+0.25; +0.27 Relative Fluorescent Units (RFU) and +0.45; +0.03 RFU for internal stores; and external influx in each cell line, respectively) (Fig. 6(d)). Lastly, upon knockdown of CACNG4, cells were resistant to previously Ca^{2+} influx inhibiting concentrations of amlodipine (Fig. 6(d)). At the highest antagonist concentration in *CACNG4* knockdown cells (40μM), Ca^{2+} influx exceeded levels of untreated control cells, highlighting the magnitude of the effect that CACNG4 knockdown has on modulating channels in an active open state. These observations suggest that, down regulation of *CACNG4* modulates the channels to preferentially remain in their active or open state resulting in higher intracellular Ca^{2+} levels. Elevated intracellular calcium destabilises conditions of homeostasis, and could thus result in the decreased tumourigenic functions we observed. CACNG4 may also play a role in the dynamics of drug binding to the channel pore, based on the observation that upon knockdown cells showed resistance to amlodipine induced inhibition of calcium influx; and rescue of amlodipine induced cell growth inhibition.

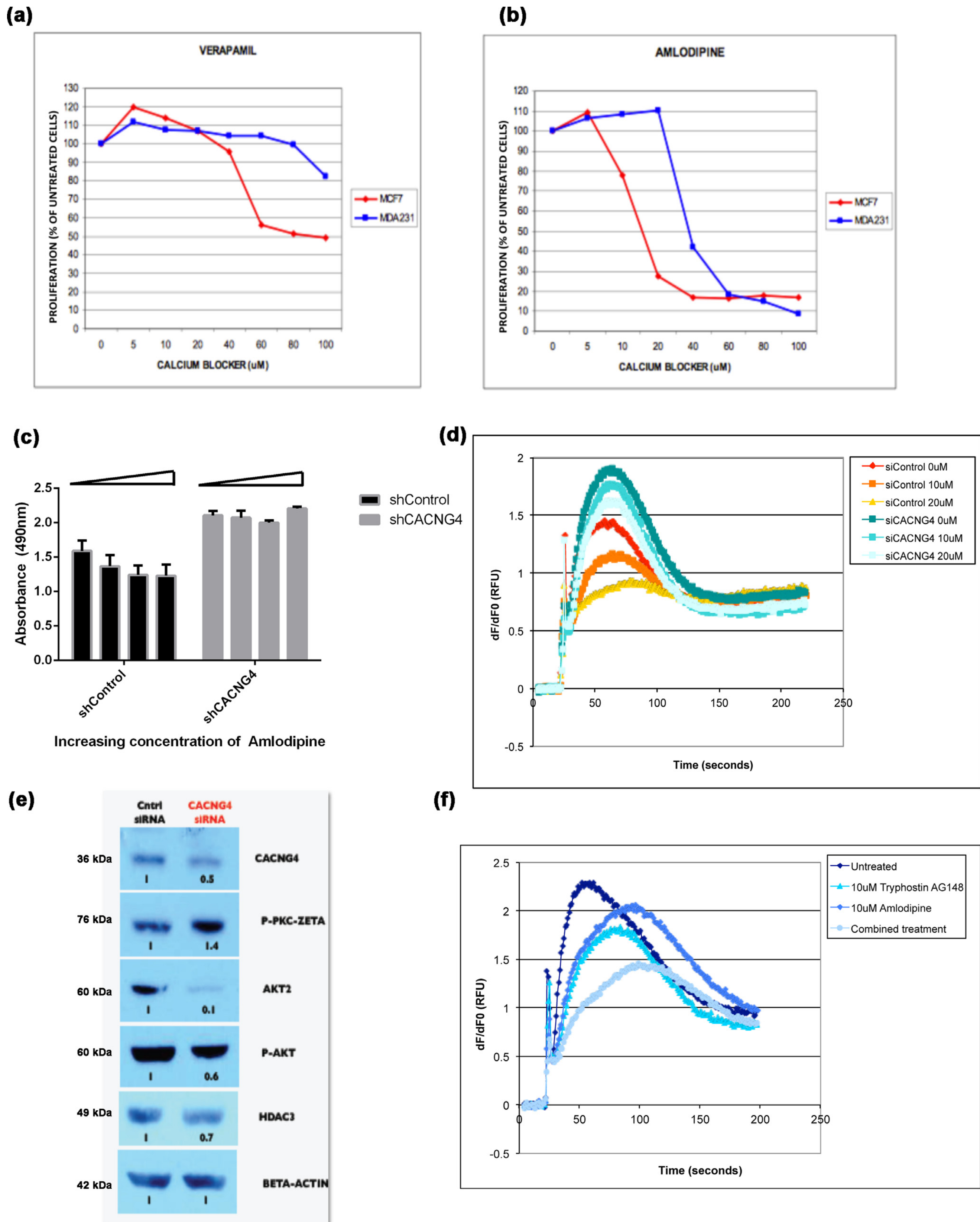

Fig. 6. CACNG4 modulates calcium influx and VGCC antagonist binding kinetics (a) and (b) L-type calcium channel antagonists verapamil and amlodipine inhibit MCF7 and MDA-MB-231 breast cancer cell proliferation in a dosage dependent manner at 48hrs. Data are represented as mean of triplicate values (c) shRNA knockdown of *CACNG4* results in rescue of inhibition of proliferation by amlodipine (0,1,5 and 10μM) in MCF7 breast cancer cells (d) Knockdown of CACNG4 increases serum induced Ca2+ influx and also leads to resistance to influx inhibiting concentrations of amlodipine in MDA-MB-231 breast cancer cells. Data for c and d are represented as mean ± SEM for triplicate values. (e) Down regulation of CACNG4 in breast cancer cells affects EGFR and calcium signaling pathways; AKT2, pAKT, and HDAC3 are down regulated while p-PKCZ is up regulated as confirmed by protein expression levels in MCF7 cells validating the mRNA results (f) Serum induced calcium influx in MDA-MB-231 cells is reduced when cells are treated individually with EGFR specific inhibitor Tryphostin AG148 or L-type VGCC antagonist amlodipine. The level of calcium influx is most strongly reduced when cells are treated with both calcium influx antagonists combined.

3.6. CACNG4, L-type VGCCs affect regulation of EGFR and calcium signaling pathways

Activation of EGFR by ligand binding leads to opening of calcium channels and calcium influx from the plasma membrane [45,46]. This increase in cytosolic calcium concentration in turn results in the release of calcium from internal stores in the ER (via ryanodine receptors or inositol triphosphate receptors) via PLCγ production of IP3. Calcium released from ER stores in turn activates PKCα also capable of activating EGFR *via* phosphorylation, creating a positive feedback loop. Depending on the cascade of calcium signaling within the cell, regulation of proliferation, survival, cytoskeletal reorganisation, adhesion, or cell migration is achieved. We tested the effect of *CACNG4* on a total of 188 genes involved in EGFR signaling and calcium signaling *via* transcriptional regulation at the mRNA level (see Fig. S7). Upon *CACNG4* knockdown, we identified significant transcriptional down regulation of *AKT2* and *PI3KR2* suggesting the involvement of PI3K/AKT signaling affecting the survival and metastatic behavior of cells. *RhoD* (involved in motility) and *HDAC3* (frequently overexpressed in various cancers) were also down regulated (see Fig. S8(a)) [47]. Conversely, *RASA1/p120 GAP*, a potent inhibitor of the MAPK pathway, and *PKC*ζ (zeta), involved in tight junction formation and cell polarity were up regulated [48] (see Fig. S8(b)). We validated these results by showing the corresponding changes in protein and phosphorylated protein levels (Fig. 6(e)). We also validated that the over-expression of *CACNG4* resulted in the inverse of these results, with lower *PKC*ζ and *RASA1* mRNA levels in MCF10A cells (see Fig. S8(b)). Of note, mRNA levels of the calcium channel alpha/pore unit *CACNAC1*/$Ca_v1.2$ was increased upon knockdown of *CACNG4*. This result provides evidence of a role for CACNG4 in inactivating the channel *via* transcriptional down-regulation of the alpha/pore subunit itself. It potentially explains increased calcium influx upon *CACNG4* knockdown as a result of not just open pore kinetics, but also due to up-regulation of the pore subunit.

We also performed a high throughput drug screen of libraries of molecules that have pharmacological, biological and biochemical relevance with FDA approval or clinical trial status: Tocris Bioscience collection (n = 700). We identified several compounds with different cytotoxic activity against cells over-expressing CACNG4 in relation to the parental MCF10A cell line that does not express CACNG4 [49–51]. EGFR inhibitors, PI3-kinase inhibitors and Protein kinase C inhibitors were identified as potential agents for targeting CACNG4 over-expressing tumour cells, again highlighting a parallel role for these pathways with VGCCs in breast cancer cells (see Table S1).

To determine the role of EGFR in the observed calcium influx, we treated cells with EGFR specific tyrosine kinase inhibitor Tryphostin AG148 (Fig. 6(f)). The peak phase was specifically abrogated (−0.24 RFU) showing inhibition of calcium release from internal stores. The plateau phase was also slightly diminished (−0.13 RFU) showing reduced calcium influx of extracellular calcium, in the presence of an EGFR blockade. Blocking of the calcium channels alone by the antagonist amlodipine showed a diminished peak, and an elongated plateau phase (25 s longer) suggesting inhibition of calcium influx from internal stores (−0.47 RFU) to be the main point of blockade. This inhibitory effect of the peak phase was most strongly diminished by the synergistic action of Tryphostin AG148 and amlodipine (−0.86 RFU), however the plateau phase showed similar levels as the EGFR inhibitor alone. It appears that EGFR activation is the more potent regulator of calcium levels in the plasma membrane, and VGCCs are the more potent regulator of calcium levels from internal stores. This result clarifies the independent paths of calcium influx from EGFR activation and VGCCs, while also highlighting their parallel functioning in regulating calcium levels within a cell. There may be potential clinical utility of therapeutic combinations of anti-EGFR and anti-CACNG4 agents which could improve targeted growth inhibition in cells over-expressing CACNG4, or in cells that have developed resistance to EGFR inhibition.

3.7. CACNG4 in metastatic breast cancer

We further examined the protein expression of CACNG4 in primary breast invasive tumours with matched lymph node metastases, as well as a series of metastatic tumours from distant sites and a local breast recurrence (Fig. 7(a)). We observed low to moderate, heterogeneous expression of CACNG4 in spatially separate areas of the primary tumours (B1 and B2). As expected, highest expression of CACNG4 was observed in matched lymph node metastases, concordant with our own studies as well as gene expression database associations. Interestingly, distant sites (liver) as well as a local breast recurrence showed low to even negative expression of CACNG4, comparable to expression levels in normal breast tissue (Fig. 7(b)). This result is also concordant with gene expression database associations that did not show significant increases in distant metastasis or death. Taken together, our results indicate a plausible role for CACNG4 in early metastatic events from primary tumours to lymph nodes. It however, may not play a critical role in metastasis beyond lymph nodes to distant organs. Larger numbers of metastatic samples, including lung metastases, would need to be evaluated to establish its role in distant metastasis.

4. Discussion

We have shown that L-type VGCCs are expressed and regulate calcium signaling in non-excitable breast cancer cells. We have elucidated a role for the gamma subunit, *CACNG4,* in aggressive tumour cell and metastatic behavior using 2-D and 3-D *in vitro*, as well as *in vivo* models. We have also validated our previous findings by showing that higher expression of *CACNG4* is significantly associated with LN metastasis (n = 1661). CACNG4 modulates VGCCs in a closed state, thus affecting intracellular calcium levels, which in turn regulates homeostasis and metastatic abilities such as cell survival, adhesion, motility, and dissemination. These findings are functionally significant with respect to developing treatments that target these channels in tumours with aberrant calcium signaling.

Calcium channel antagonists have anti-proliferative effects on prostate cancer cells, retinal pigment cells, and vascular cell types [52–56]. Ion channels are already a major class of drug targets. Calcium channel blockers such as verapamil and amlodipine are extensively studied and have been used to treat angina, hypertension and arrhythmias for several decades. Verapamil has been used *in vitro* and in combined chemotherapy treatments for its ability to reverse drug resistance in tumours expressing the multi-drug resistance receptor ABCB1 [57]. The association of this class of drugs with breast cancer however, has been controversial and conflicting, with recent evidence that long term use is significantly linked to increased risk for breast cancer [12,58]. Our results show that amplified *CACNG4* acts much like a calcium channel blocker, by blocking the channel, inhibiting calcium influx, and thus affecting calcium signaling events. It is known that calcium homeostasis is achieved by maintenance of low intracellular calcium in all cell types. In 20–30% of cancer patients, hypercalcemia, or high blood serum calcium, develops as a result of abnormalities in bone resorption and impaired renal clearance [8]. Patients with breast cancer, lung cancer, and myeloma are most commonly affected by hypercalcemia, which is associated with poor prognosis and disseminated disease [9]. Calcium is released by tumour cells locally (increased parathyroid hormone-related protein in early breast cancer) or at the sites of metastases (osteolysis of bone) [11]. Higher serum calcium has also been associated with fatal prostate cancer [10]. In response to high serum calcium levels, tumour cells develop mechanisms to buffer intracellular calcium, and thrive compared to non-malignant cells [7]. It has been shown that the mortal breast epithelial cell line MCF-10M is less tolerant to high levels of calcium compared to its immortal and oncogenic sub-line MCF10A (spontaneously transformed) and MCF10AneoT (H-Ras

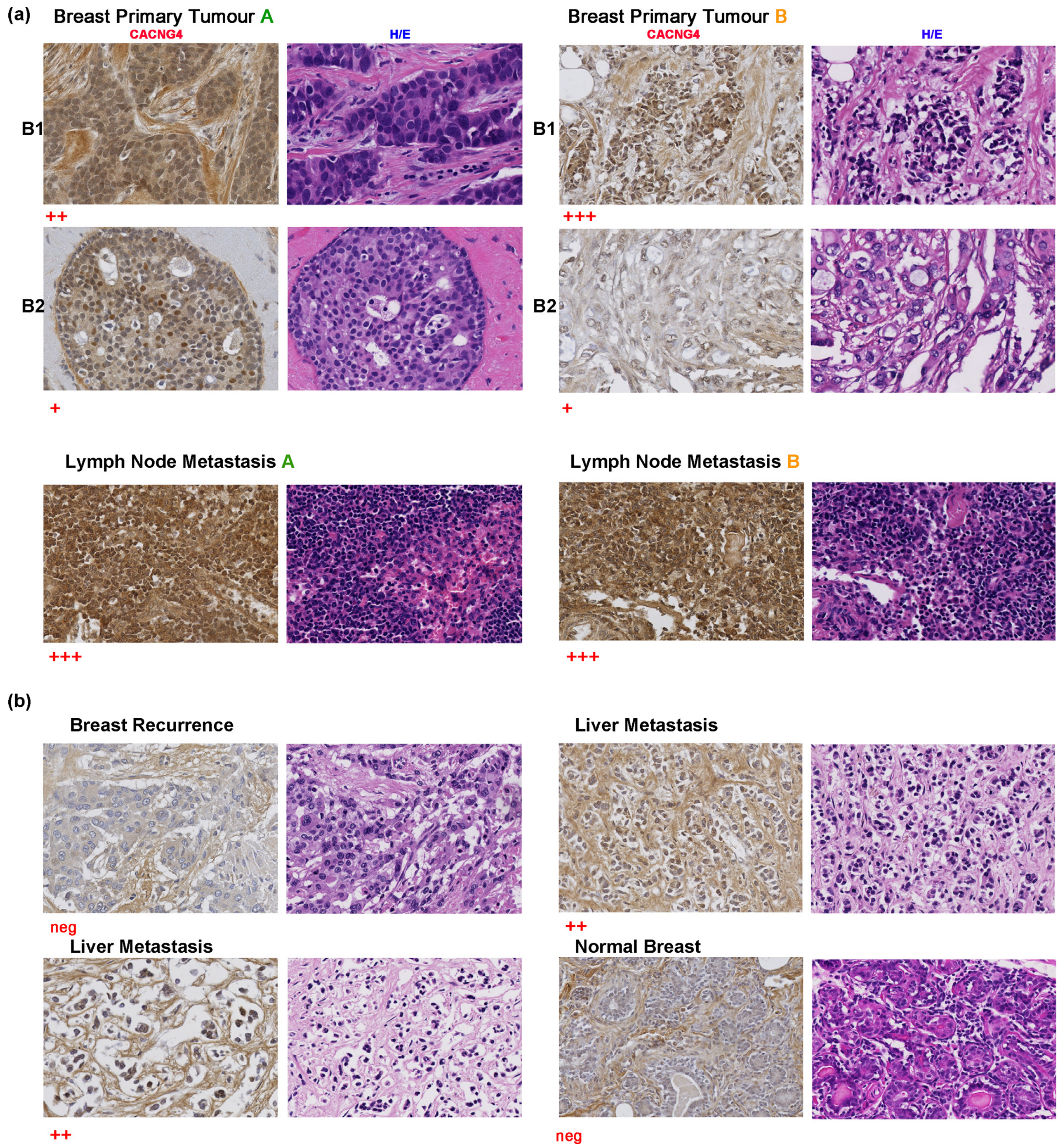

Fig. 7. CACNG4 expression in metastatic breast cancer. (a) Two spatially separate blocks of primary tumours A and B (B1 and B2) show heterogeneous staining of CACNG4. Matched lymph node metastases show highest expression of CACNG4 (b) distant metastases to liver and a local breast recurrence show absent to moderate expression of CACNG4, comparable to normal breast tissue that does not show expression. (low = +, moderate = ++, high = +++, absent = neg).

transformed) [7,59]. This is because mortal cells are unable to efficiently buffer against elevated intracellular calcium. A 2-3 fold increase in levels results in negative effects on cell growth and programmed cell death. It is likely that amplification of *CACNG4* is a mechanistic response of tumour cells to block calcium influx, and thus maintain low intracellular calcium, sustaining tumour growth. It may be that these tumours are consequently more adept at survival and metastasis.

In conclusion, our findings suggest an underlying pathway for tumour growth and dissemination regulated by *CACNG4* (Fig. 8). Subsequent analyses on associations of gene expression with clinical datasets on distant metastases and resistance to specific treatments, particularly EGFR inhibitors, would be informative. As this gene is frequently amplified in breast cancer, targeting of *CACNG4* to indirectly modulate the L-type VGCCs is an attractive therapeutic approach to disrupt growth and metastasis. The observation that channel blockers

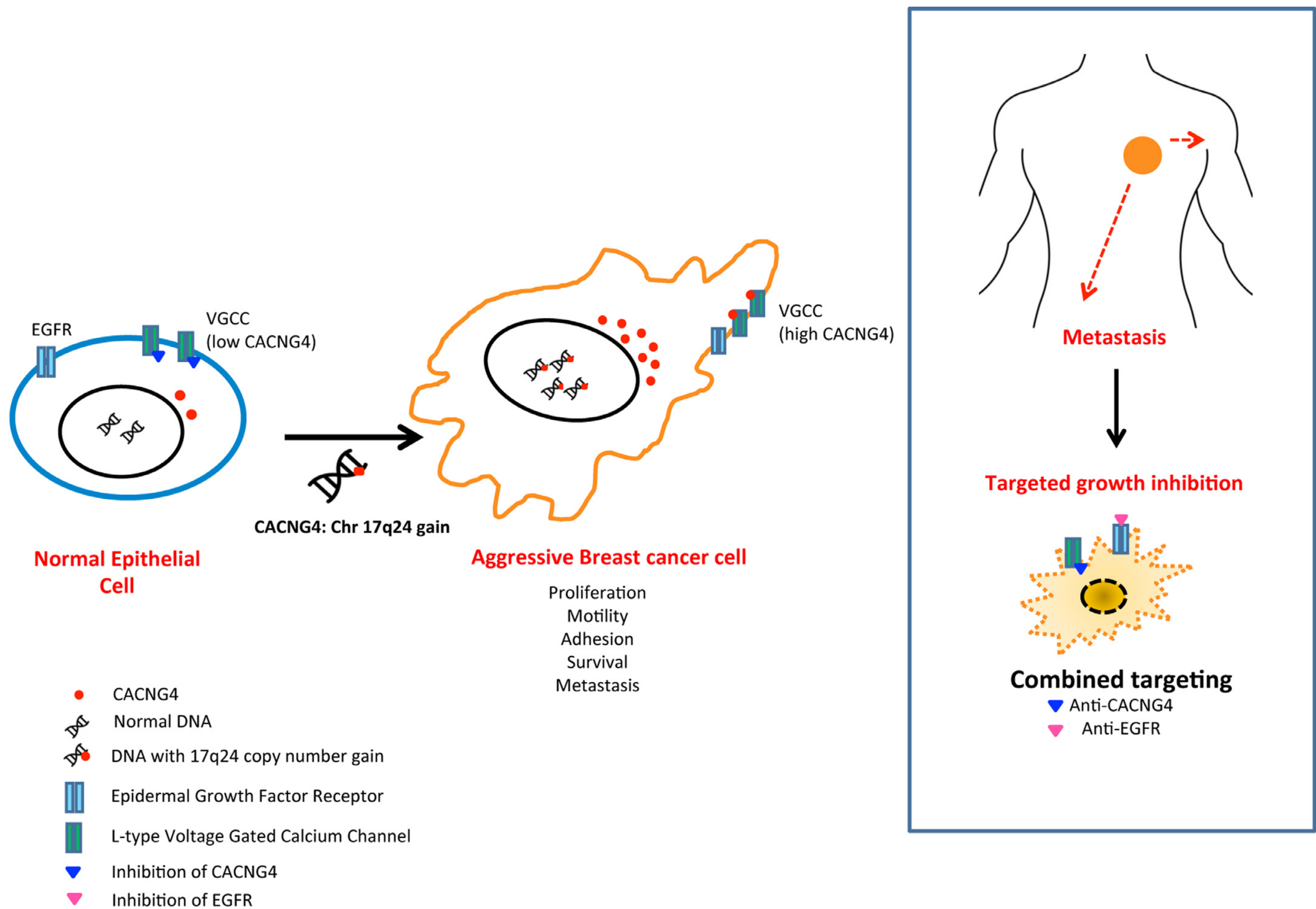

Fig. 8. Model for tumour growth and dissemination regulated by *CACNG4*. Under normal conditions of CACNG4 expression, VGCCs are regulated in an open/closed state, to maintain low intracellular calcium conditions for a state of homeostasis. In breast cancer, tumour cells with gains of chromosome region 17q24 (encompassing the CACNG4 gene), have an increased expression of CACNG4 which results in closed VGCCs and a preferential low intracellular calcium level for tumour cells which allows for survival and other aggressive functions leading to dissemination and metastasis. Targeting CACNG4 would keep VGCCs in an open state, therefore increasing intracellular calcium levels, leading to decreased tumour growth and metastasis as we have demonstrated. Alternatively, anti-CACNG4 is a combinatory option to overcome acquired drug resistance of parallel signaling pathways such as EGFR.

and EGFR tyrosine kinase inhibitors act synergistically is indicative of a parallel role of EGFR activation and VGCCs. It has been reported that *CACNG4* is up-regulated in EGFR inhibitor (erlotinib) resistant tumour cell lines [22]. This further supports the possibility that the L-type VGCCs are functioning in parallel to EGFR. T-type VGCCs were recently found to be overexpressed in a trastuzumab resistant HER2+ breast cancer cell line [5]. Therapeutically, the regulation of VGCCs to manipulate calcium levels in tumour cells could be an alternative or combinatory option to overcome acquired drug resistance. This, as well as a role for *CACNG4* in the regulation of oncogenes such as *AKT2* and *HDAC3*, and tumour suppressors such as *RASA1*, suggests exciting new treatment possibilities for invasive breast cancer.

Declaration of Competing Interest

The authors have no conflicts of interest to disclose.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2020.102646.

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