Cellular Signalling 26 (2014) 2621–2632

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

The monoamine oxidase-A inhibitor clorgyline promotes a mesenchymal-to-epithelial transition in the MDA-MB-231 breast cancer cell line

Tamara Satram-Maharaj [a], Jennifer N.K. Nyarko [b], Kelly Kuski [a], Kelsey Fehr [a], Paul R. Pennington [a], Luke Truitt [c], Andrew Freywald [c], Kiven Erique Lukong [b], Deborah H. Anderson [b], Darrell D. Mousseau [a,*]

[a] Cell Signalling Laboratory, Department of Psychiatry, University of Saskatchewan, Canada
[b] Department of Biochemistry, University of Saskatchewan, Canada
[c] Department of Pathology, University of Saskatchewan, Canada

ARTICLE INFO

Article history:
Received 4 May 2014
Received in revised form 29 July 2014
Accepted 15 August 2014
Available online 22 August 2014

Keywords:
Monoamine oxidase
Depression
Breast cancer
Mesenchymal-to-epithelial transition
E-cadherin
Metastasis

ABSTRACT

Monoamine oxidase-A (MAO-A) dysfunction has been historically associated with depression. Recently, depression as well as altered MAO-A expression have both been associated with a poor prognosis in cancers, although the mechanism involved remains ambiguous. For example, MAO-A mRNA is repressed across cancers, yet MAO-A protein and levels of serotonin, a substrate of MAO-A implicated in depression, are paradoxically increased in malignancies, including breast cancer.

The effect of clorgyline (CLG), a selective inhibitor of MAO-A, on malignant behaviour, expression of transitional markers, and biochemical correlates was examined in two human breast carcinoma cell lines, i.e. the epithelial, oestrogen receptor (ER)-positive MCF-7 cell line and the post-EMT (mesenchymal), ER-negative MDA-MB-231 cell line.

CLG exerted little effect on malignant behaviour in MCF-7 cells, but inhibited proliferation and anchorage-independent growth, and increased invasiveness and active migration of MDA-MB-231 cells. CLG induced the expression of the mesenchymal marker vimentin in MCF-7 cells, but not in MDA-MB-231 cells. In contrast, CLG induced the epithelial protein marker E-cadherin in both cell lines, with a more robust effect in MDA-MB-231 cells (where a nuclear E-cadherin signal was also detected). This effect appears to be independent of any canonical Snai1-mediated regulation of E-cadherin mRNA expression. CLG interfered with the β-catenin/[phospho]GSK-3β complex as well as the E-cadherin/β-catenin complex in both cell lines cells, but, again, the effect was more robust in MDA-MB-231 cells. Parallel studies revealed a general lack of effect of CLG on the ER-negative, epithelial Au565 breast cancer cell line. Thus, any effect of CLG on metastatic behaviours appears to rely on the cell's EMT status rather than on the cell's ER status.

These data suggest that inactivation of MAO-A triggers a mesenchymal-to-epithelial transition in MDA-MB-231 cells via a non-canonical mechanism. This potentially implicates an MAO-A-sensitive step in advanced breast cancer and should be borne in mind when considering pharmacological treatment options for co-morbid depression in breast cancer patients.

1. Introduction

Serotonin, a neurotransmitter whose levels are often reduced during the course of clinical depression, has also been implicated in the regulation of breast epithelial physiology [1]. Epidemiological studies support an association between the risk of cancer development and the use of serotoninergic antidepressants, with the patient's hormone [oestrogen/progesterone] receptor status potentially contributing a modest influence [2,3].

Depression has been associated historically with monoamine oxidase-A (MAO-A), the enzyme that degrades serotonin, and inhibitors of MAO-A were the first antidepressant drugs to be used clinically. Although

* Corresponding author at: Cell Signalling Laboratory, GB41 Health Science Building, 107 Wiggins Avenue, University of Saskatchewan, Saskatoon S7N 5E5, SK, Canada. Tel.: +1 306 966 8824.
E-mail addresses: tsatram@gmail.com (T. Satram-Maharaj), jnn270@mail.usask.ca (J.N.K. Nyarko), kellykuski@gmail.com (K. Kuski), kdf044@mail.usask.ca (K. Fehr), prp676@mail.usask.ca (P.R. Pennington), luke.truitt@usask.ca (L. Truitt), andrew.freywald@usask.ca (A. Freywald), kel232@mail.usask.ca (K.E. Lukong), deborah.anderson@saskcancer.ca (D.H. Anderson), darrell.mousseau@usask.ca (D.D. Mousseau).

http://dx.doi.org/10.1016/j.cellsig.2014.08.005
0898-6568/

inhibitors of MAO-A can induce severe side effects, which limit the use of these drugs in the clinic, they continue to be used when all other antidepressants fail to provide therapeutic effect. Alterations in MAO-A expression might represent a possible biomarker in cancers [4], yet the exact mechanism involved is not clear. Indeed, there is indication that the inhibition of MAO-A would be beneficial. For example, MAO-A activity is increased in experimental breast cancer [5] and MAO-A protein expression is increased in advanced stages of prostate cancer [6], while inhibition of MAO-A with clorgyline exerts anticancer properties towards cultured prostate cancer cells [7] and resveratrol, a potent inhibitor of MAO-A isolated from red grapes [8], induces MCF-7 breast cancer cell death [9]. Yet in apparent contradiction, MAO-A mRNA is downregulated in 94% of cancers screened, including breast and prostate cancers [4], and epidemiological studies associate usage of antidepressants, including inhibitors of MAO-A, with an increased risk for cancer [10,11]. Interestingly, we have demonstrated that overexpression of a catalytically *dead* MAO-A variant increases proliferation and *de novo* DNA synthesis in neuronal cells [12], which introduces the potential for a role for the MAO-A protein beyond simple substrate catabolism. This also introduces the possibility that active and inactive pools of MAO-A might exist, and that at any given time a cell's phenotype would be influenced by the predominant pool. This simple notion could certainly be contributing to the ambiguity in the cancer literature regarding risk associated with MAO-A protein expression and paradoxical substrate accumulation [13].

Epithelial-to-mesenchymal transition (EMT) during tumour progression involves loss of epithelial cell adhesion following a reduction in E-cadherin expression [14] that reflects a GSK-3-dependent, Snai1-mediated transcriptional regulation [15]. The oestrogen receptor (ER)-negative, mesenchymal-like MDA-MB-231 cells are a model of a post-EMT cells that are characterized as 'E-cadherin-null' as well as by reduced adhesion and increased cell mobility. In contrast, the ER-positive, epithelial-like MCF-7 cells are far less aggressive, express E-cadherin and are often contrasted experimentally with the MDA-MB-231 cell line [16].

We have examined the influence of clorgyline (CLG) — a selective inhibitor of MAO-A — on malignant/metastatic behaviour, on the expression of transitional markers, and on biochemical correlates in MDA-MB-231 and MCF-7 breast cancer cells. Our study strongly supports a role for MAO-A inhibition in triggering a mesenchymal-to-epithelial transition in the MDA-MB-231 breast cancer cell line. The absence of a similar pattern of effects in the ER-negative, epithelial Au565 breast cancer cell line suggests that the effect of MAO-A inhibition in this context is not reliant as much on the cell's ER status as it is on the cell's EMT status.

2. Materials and methods

2.1. Reagents and antibodies

Serotonin, 5-bromo-2′-deoxyuridine (BrdU), and the BrdU and β-actin antibodies were purchased from Sigma-Aldrich Co. [^{14}C]-Serotonin (NEC-225) was purchased from PerkinElmer Life Sciences. The MAO-A (H-70) antibody as well as the GSK-3β (phospho-Ser9 and total) and vimentin antibodies were purchased from Santa Cruz Biotechnology. The E-cadherin and β-catenin antibodies were purchased from Cell Signalling Technology. The AlexaFluor-594 and AlexaFluor-488 labelled donkey anti-mouse/anti-rabbit IgGs, respectively, and IgG-HRP conjugates were obtained from Cedarlane Laboratories Ltd. Protein-A/G sepharose and the enhanced chemiluminescence kit were obtained from GE Healthcare Bio-Sciences Inc.

2.2. Cell culture and transfection

MCF-7 cells (ATCC: HTB-22) were cultured in α-Minimum Essential Medium supplemented with 10% Fetal Bovine Serum (FBS), 1% L-glutamine, 1% Pen-strep and 10 μg/μL bovine insulin. MDA-MB-231 cells (ATCC: HTB-26) were cultured in RPMI-1640 supplemented with 10% FBS and 1% Pen-Strep. Cells in log-phase (5×10^5 cells/well of a 6-well plate) were transfected (i.e. 1–2 μg plasmid/well) using Lipofectamine® 2000 (Life Technologies Inc.). Cells were routinely tested 24 h post-transfection. Treatment with CLG was kept at 1 μM (this dose is routinely used to selectively inhibit MAO-A activity [17]).

2.3. MAO-A catalytic activity

MAO-A activity was estimated using 250 μM [^{14}C]-serotonin and 100 μg total cell protein (homogenate) suspended in oxygenated potassium buffer [18]. The 10-minute reaction (37 °C) was terminated by acidification, and the radiolabeled metabolites were extracted into water-saturated ethyl acetate/toluene (1:1, v/v) and quantitated using scintillation spectrometry. Activity experiments ($n \geq 6$) were performed in triplicate-quintuplicate.

2.4. Cell proliferation assays

Three indices of proliferation were used. (a) Cells were seeded into 12-well culture plates at a density of 1×10^3 cells/well. Individual wells were harvested and the cells manually counted using a haemocytometer over a period of six days. (b) Cells were assayed for the mitochondrial conversion of MTT (a reaction that is proportional to cell number) ($n = 4$–9, each based on four to six replicates). (c) Quantification of BrdU incorporation into newly synthesized DNA relied on experimental means of three to four experiments, based on counts of 500+ cells per experimental condition.

2.5. Anchorage-independent growth

A 4×10^6/ml cell suspension (in 0.36% soft agar solution) was layered onto a solid nutrient-based 0.61% agar layer and allowed to grow for four weeks. A top agar solution supplemented with vehicle or CLG was added to each plate every seven days.

2.6. In vitro migration and invasion assays

The migration/Boyden chamber assay [19] was performed in 48-well chemotaxis chamber supplied with a polycarbonate membrane of 8-μm pore size (Neuroprobe). Chemotaxis assays (5×10^4 cells per well) were performed at 37 °C for 4 h (vehicle or CLG treated). Cells were removed from the upper side of the membrane with a cotton swab and cells attached to the lower side were stained with crystal violet. The migration index for each experiment was calculated as the mean difference in the number of cells that migrated toward the medium in the bottom chamber containing 10% FBS versus the number of cells that migrated toward the medium that contained 0.1% FBS.

In vitro invasion was also assessed using the Boyden chamber, but with the polycarbonate membrane covered with Matrigel (a model of the basement membrane: BD Biosciences). Cells (4×10^4) were plated in the upper chamber in DMEM/5% FBS. The inserts were incubated at 37 °C in the presence or absence of CLG, and the number of cells that had invaded the lower surface of the membrane was determined as above.

In these migration and invasion experiments, cells in five random fields were counted by two observers in a blinded fashion using a BX71 Olympus microscope (20× magnification).

2.7. Adhesion to extracellular matrix proteins

Plates (96-well) were coated with either rat tail collagen I (GIBCO) or fibronectin (Calbiochem) (50 μg/mL, 1 h, 37 °C), rinsed and then blocked (0.1% BSA in PBS, 1 h, 37 °C). Cells were treated with CLG (24 h), and then harvested and incubated for 30 min at a density of 20,000 cells *per* coated well. Unattached cells were removed by washing and attached cells were fixed (4% paraformaldehyde in PBS, RT), rinsed and then stained with

Crystal violet (10 min). The stain was solubilized with 2% SDS and absorbance was read at 550 nm. Wells devoid of cells were used as background. Data from adhesion assays represent 30–36 wells *per* group.

2.8. Indirect immunofluorescence microscopy

CLG (24 h)-treated cells cultured on chambered slides were fixed with 4% formaldehyde (30 min), permeabilized with 0.5% Triton-100/PBS for 15 min, and mounted with Pro-Long Gold (Life Technologies Inc.). Following incubation (4 °C) overnight with either anti-vimentin or anti-E-cadherin antibody, cells were extensively washed and incubated with an AlexaFluor 594 secondary antibody with excitation/emission maxima of 590/617 nm or with the AlexaFluor 488 secondary antibody with excitation/emission maxima of 488/519 nm. Images were captured under an Olympus BX 71 fluorescence microscope. The relative quantity of proteins in the nucleus/cytoplasm along a bisecting line centred on the nucleus ('50') and extending beyond the edge of the cell was determined using ImageJ 1.32j (http://rsb.info.nih.gov/ij/). The length of the

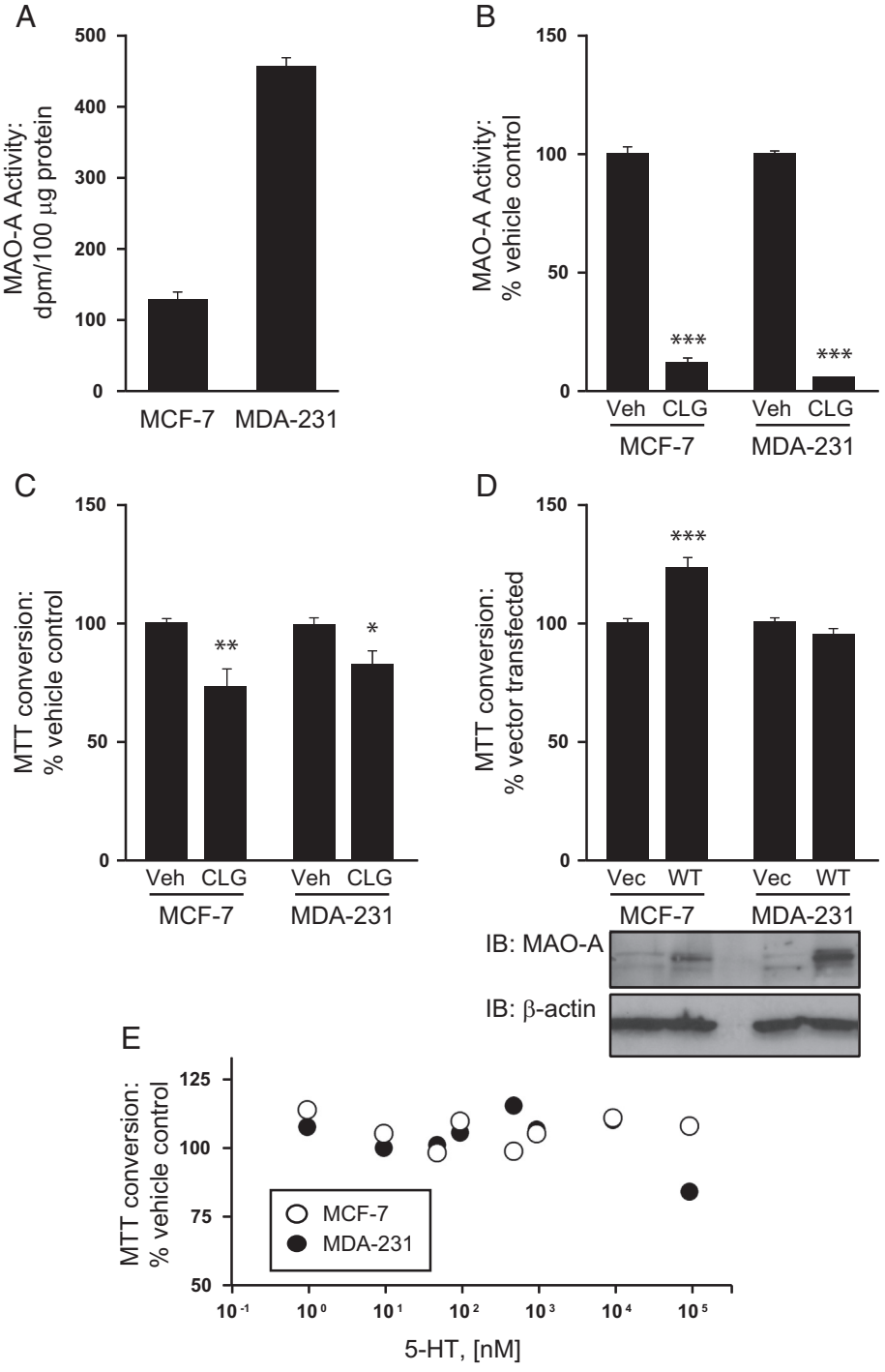

Fig. 1. MCF-7 and MDA-MB-231 cells respond to the MAO-A inhibitor CLG, but not serotonin. (A) MAO-A catalytic activity was determined in MCF-7 and MDA-MB-231 cells. (B) MAO-A activity is inhibited by the MAO-A-selective inhibitor, CLG (1 µM, 24 h) in both cell lines. MTT conversion was then determined (C) following treatment with CLG, (D) following the overexpression of MAO-A, and (E) in the presence of increasing concentrations of the MAO-A substrate, serotonin (n = 3). For panels A–D, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus the respective control condition (n = 6–9).

bisecting line (e.g. 0–50) was consistent through all analyses, thus permitting the averaging of pixel intensities across numerous cells (n = 166–257 cells per group).

As differences due to sample preparation, fixation, staining and even the lighting and imaging system used can all introduce variability, we have attempted to avoid artefact by preparing all samples in the same manner (e.g. each series of slides was prepared on the same day) and image capture relied on a single microscope/CCD-camera-based system. Furthermore, lighting, contrast, gain, and magnification parameters were all kept constant.

2.9. Quantitative real-time PCR

10 ng of cDNA, e.g. reverse-transcribed mRNA (RNeasy® Mini Kit: Qiagen), was assayed for E-cadherin (*CDH1*: Integrated DNA Technologies) or MAO-A (Taqman®, Invitrogen Canada Inc.). All comparisons [n = 3–5, in triplicate] were normalized to the vehicle-treated group. Amplification efficiency was required to be between 90 and 110% for both the gene-specific and housekeeping gene primers sets, and data were normalized to vehicle-treated groups.

2.10. Statistical analyses

Significance was set at $P < 0.05$ and comparisons between experimental means were assessed either by unpaired *t*-tests (and two-tailed *P*-values) or by ANOVA with *post hoc* analyses relying on Bonferroni's adjustment for multiple comparisons (GraphPad Prism v3.01). Data are presented as mean ± standard error of the mean (SEM) and *P*-values are provided as summary statistics.

3. Results

3.1. MCF-7 and MDA-MB-231 cell lines have different innate MAO-A activities

MCF-7 cells have a significantly lower level of MAO-A catalytic activity than MDA-MB-231 cells (Fig. 1A). The dose-time course of CLG treatment (1 μM, 24 h) effectively inhibited MAO-A activity in MCF-7 cells [$P < 0.001$] and in MDA-MB-231 cells [$P < 0.001$] (Fig. 1B).

3.2. Inhibition of MAO-A regulates breast cancer cell proliferation

Treatment with CLG reduced MTT conversion in MCF-7 cells [$P = 0.003$] as well as in MDA-MB-231 cells [$P = 0.029$] (Fig. 1C). MTT conversion was increased by the overexpression of MAO-A in MCF-7 cells [$P < 0.001$], but not in MDA-MB-231 cultures [$P = 0.074$] (Fig. 1D). Treatment (24 h) with increasing concentrations of serotonin, a major substrate of MAO-A thought to promote cancer cell proliferation [4], did not exert any effect on MTT conversion in either cell line (Fig. 1E). ANOVA suggests a significant effect of 5-HT on MDA-MB-231 cells (e.g. $P = 0.0051$); however, *post hoc* analysis reveals that this significance is driven primarily by differences between the samples treated with 500 nM and 100 μM ($P < 0.01$). Any (patho)physiological relevance of this statistic is questionable.

MCF-7 cell proliferation was significant over a 6-day period [$P < 0.001$], but remained unaffected by CLG treatment [$P = 0.0691$] (Fig. 2A). MDA-MB-231 cell proliferation was also significant over the 6-day period [$P < 0.001$] and was slowed by day 6 of CLG treatment [$P < 0.001$] (Fig. 2B).

Proliferation is assumed to be proportional to total *de novo* DNA synthesis, which can be measured using BrdU labelling as an index. A baseline index of 37.4 ± 0.1% for MCF-7 cells and an index of 29.7 ± 2.3% for MDA-MB-231 cells are in keeping with published reports [20,21]. There was no effect of CLG on BrdU staining after the sixth day of treatment in MCF-7 cells [$P = 0.5325$]. In contrast, there was a marginal — and paradoxical — increase in BrdU staining in MDA-MB-231 cells [$P = 0.0487$] (Fig. 2C).

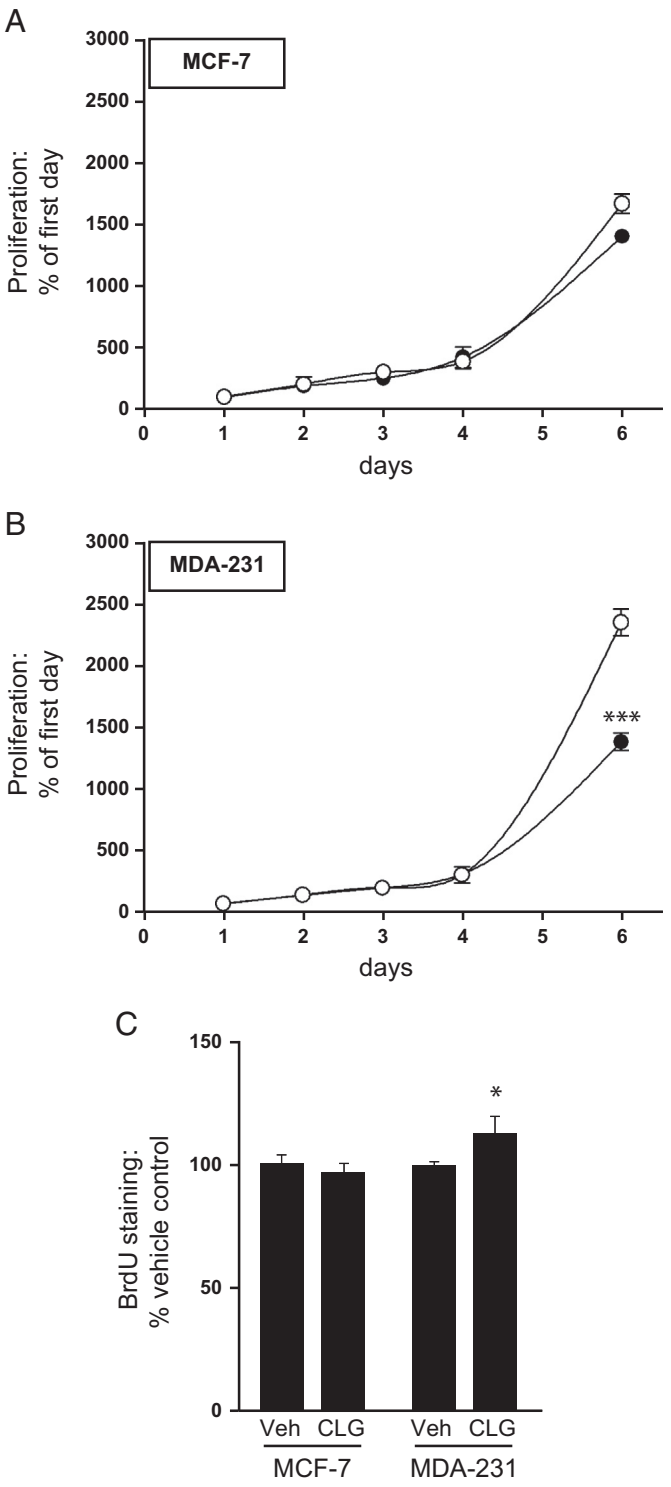

Fig. 2. CLG inhibits proliferation, but increases *de novo* DNA synthesis, in MDA-MB-231 cells. (A) MCF-7 and (B) MDA-MB-231 cell numbers were counted on successive days of treatment with either CLG (●) or the PBS vehicle (Veh: ○). ***$P < 0.001$ versus the vehicle control (n = 4). (C) The effect of CLG on BrdU incorporation was examined in both cell lines. *$P < 0.05$ versus the vehicle (Veh) control (n = 7–8).

While the effect of CLG on the rate of proliferation and BrdU staining was not as straightforward as anticipated, these data do reveal that CLG can exert effects in these cell lines. We next examined the influence of

MAO-A inhibition on other indices of tumorigenicity such as anchorage-independent growth, migration and invasion.

3.3. Inhibition of MAO-A alters anchorage-independent breast cancer cell growth, migration, invasion and adhesion

Anchorage-independent growth of MCF-7 cells was not affected by CLG treatment [$P = 0.9170$]. In contrast, a significant reduction in the number of MDA-MB-231 colonies following treatment with CLG was observed [$P < 0.001$] (Fig. 3A).

MDA-MB-231 cells are a more aggressive lineage and, not unexpectedly, migrated through the supporting membrane in Boyden chambers far more readily than did MCF-7 cells [$P < 0.001$] (Fig. 3B). Treatment with CLG did not change the migration rate of MCF-7 cells [$P = 0.984$], but it did increase that of MDA-MB-231 cells [$P = 0.001$] (Fig. 3C). CLG treatment also enhanced the invasive capacity (through Matrigel) of MDA-MB-231 cells [$P = 0.040$] (Fig. 3D).

The general absence of extracellular matrix, such as collagen and fibronectin, can alter a tumour cell's capacity for migration and invasion in vivo. CLG enhanced the adhesion of MCF-7 cells to both a fibronectin [$P < 0.001$] and a collagen [$P < 0.001$] matrix (Fig. 3E & F). In contrast, it did not alter the adhesion of MDA-MB-231 cells to either fibronectin [$P = 0.504$] (Fig. 3E) or collagen [$P = 0.276$] (Fig. 3F).

Given the effect of CLG on malignant behaviour in these cell lines, we chose to determine its ability to alter the expression of transitional markers, namely vimentin and E-cadherin.

3.4. Inhibition of MAO-A induces the epithelial marker, E-cadherin

Semi-quantitative densitometry (i.e. line profile plots) revealed that vimentin is diffusely distributed in MCF-7 cells, including within the nucleus (although it appears to be excluded from the nucleoli) and a modest increase was observed in CLG-treated cultures (Fig. 4A & B). E-cadherin in MCF-7 cells is detected in proximity of the plasma membrane and areas of cell–cell contact, and was significantly increased by CLG treatment (Fig. 4A & C). In MDA-MB-231 cells, CLG did not affect vimentin expression (Fig. 4D & E), but it did enhance E-cadherin immunofluorescence, particularly within the nuclear compartment (Fig. 4D & F).

Western blot analysis and densitometry revealed an increase in vimentin expression in CLG-treated MCF-7 cells ($P = 0.0217$) and further revealed that the effect of CLG on E-cadherin immunodetection in MCF-7 cells reflected an increase in proteolytic cleavage fragments, e.g. bands in the 55–80 kDa range, although this was not significant ($P = 0.5838$) (Fig. 5A & C). In contrast, vimentin expression was unaffected by CLG treatment in MDA-MB-231 cells ($P = 0.9931$), whereas the increase in E-cadherin immunofluorescence observed in these cells (see Fig. 4) reflected increases in the inactive E-cadherin precursor protein (135 kDa) and two other species at the very limit of detection, e.g. the mature 120 kDa form ($P = 0.0327$) as well as the 55 kDa proteolytic cleavage fragment ($P = 0.0453$) (Fig. 5B & D). Expression levels of β-catenin, which, in conjunction with E-cadherin, are important for normal intercellular adhesion [22], were unchanged in both CLG-treated MCF-7 cells ($P = 0.1772$) and MDA-MB-231 cells ($P = 0.1577$).

β-catenin co-immunoprecipitated with both the inactive E-cadherin precursor protein (135 kDa) [23] and its mature form (120 kDa) [24] in MCF-7 cells; the interaction with the mature form was modestly affected by CLG treatment ($P = 0.0375$), whereas the interaction with the 55 kDa E-cadherin species was significantly enhanced ($P = 0.0256$) (Fig. 6A & C). Interestingly, a strong immunodetectable E-cadherin band (at 130 kDa) and an almost imperceptible band at 120 kDa co-immunoprecipitated with β-catenin in vehicle-treated MDA-MB-231 cells; the interaction between β-catenin and this 120 kDa species was not affected by CLG ($P = 0.9482$: Fig. 6B & D). However, CLG did enhance the co-immunoprecipitation of β-catenin with the weaker E-cadherin immunodetectable species at 55 kDa ($P < 0.0001$). Immunoprecipitating for E-cadherin and probing for β-catenin revealed a loss of the β-catenin-E-cadherin complex in MCF-7 cells ($P = 0.0013$: Fig. 6A & C) as well as in MDA-MB-231 cells ($P = 0.0006$: Fig. 6B & D).

It is known that the degradation of β-catenin relies, in part, on its incorporation into a complex with GSK-3β [25]. Co-immunoprecipitation experiments revealed that CLG disrupted the interaction between β-catenin and phosphoSer9-GSK-3β in MCF-7 cells ($P = 0.0059$) and tended to decrease the interaction with total GSK-3β, although this was not significant ($P = 0.1960$). In MDA-MB-231 cells, CLG disrupted the interaction between β-catenin and GSK-3β regardless of its phosphorylation status (GSK-3β: $P = 0.0022$, pGSK-3β: $P = 0.0004$: Fig. 7). Neither the phosphorylation of GSK-3β (Fig. 7), nor that of Akt, the upstream kinase of GSK-3β (data not shown), was altered by CLG.

3.5. The induction of E-cadherin by CLG is independent of changes in transcription

We questioned whether the change in E-cadherin expression in MDA-MB-231 cells reflected changes in E-cadherin transcription. Quantitative real time-PCR supports the MDA-MB-231 cell line as being E-cadherin-deficient ($3.08e-4 \pm 7.55e-6$ arbitrary units (AU) versus MCF-7: 15.59 ± 0.62 AU). Treatment with CLG did not affect the expression of E-cadherin mRNA in either MCF-7 cells [veh: $100 \pm 4.0\%$; CLG: $97.4 \pm 0.4\%$; $P = 0.332$] or MDA-MB-231 cells [veh: $100 \pm 2.5\%$; CLG: $97.4 \pm 5.0\%$; $P = 0.342$]. Snai1 and its interaction with GSK-3β are integral for the regulation of E-cadherin transcription [26], but their interaction was not affected by CLG treatment (Fig. 8), thereby precluding any influence of canonical Snai1-mediated transcription in the current model.

In parallel experiments, basal MAO-A mRNA expression was significantly different between MCF-7 (0.547 ± 0.60 AU) and MB-MDA-231 (0.886 ± 0.09 AU) [$P = 0.0029$] and reflected the difference in MAO-A protein and activity in these cell lines (see Fig. 1). MAO-A mRNA was not affected by CLG treatment in either MCF-7 cells [veh: $100 \pm 11.0\%$; CLG: $96.8 \pm 2.5\%$; $P = 0.325$] or MDA-MB-231 cells [veh: $100 \pm 10.3\%$; CLG: $95.5 \pm .8.9\%$; $P = 0.599$].

3.6. The effects of CLG are not reliant on the cell's oestrogen receptor (ER) status

It is well documented that the MCF-7 cell line is ER-positive and that the MDA-MB-231 cell line is ER-negative. To address the influence of ER status in the response of MDA-MB-231 cells to CLG, we examined the effect of CLG on the Au565 ER-negative breast cancer cell line. Aside from an effect on anchorage-independent growth, CLG did not exert any appreciable effect on any of the malignant behaviours, transitional markers, and biochemical correlates assayed using Au565 cells (summarized in Fig. 9). Thus, the much broader range of effects of CLG on MDA-MB-231 cells is unlikely due to the ER-negative status of these cells.

4. Discussion

The high incidence of depression in patients with breast cancer — with rates of co-morbidity reaching almost 40% [27–29] — certainly implicates a role for monoaminergic dysfunction and for MAO-A given the historical association of both factors with experimental and clinical depressive phenotypes [13]. This is supported by the increase in MAO-A activity linked to malignancy in experimental breast cancer [5] and by the increase in the risk of breast cancer associated with antidepressants that target the monoaminergic systems [10,11]. Yet, in apparent contradiction, treatment with CLG, an MAO inhibitor with antidepressant potential, improves prognosis in prostate cancer [6], whereas treatment with Selective Serotonin Reuptake Inhibitor (SSRI) antidepressants reduces the risk of colorectal cancer [30], but has no effect on risk of breast cancer [31]. Clearly, the increased risk of breast cancer associated with antidepressant usage does not rely solely on increased functional

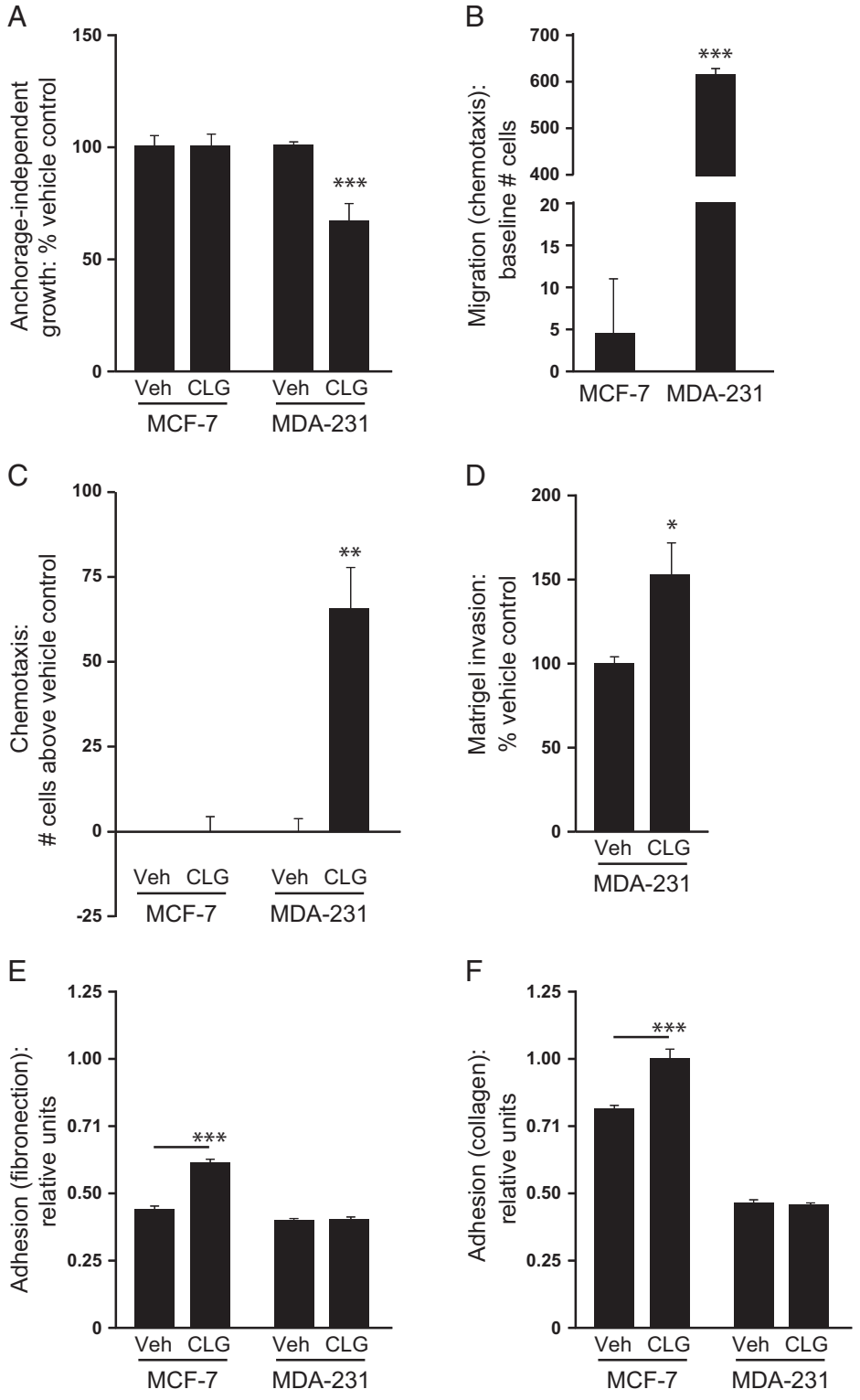

Fig. 3. CLG suppresses malignant properties of MDA-MB-231 cells. (A) The effect of CLG on anchorage-independent growth was determined in MCF-7 and MDA-MB-231 cells (n = 6–9). (B) MDA-MB-231 cells have a far greater migratory potential (chemotaxis: 10% FBS) than did MCF-7. (C) Chemotaxis was tested in the absence or presence of CLG in both cell lines (n = 3–4). (D) CLG increases MDA-MB-231 cell invasion (through Matrigel: n = 4). Note that MCF-7 cells were not tested as they were not able to migrate through the Matrigel. The effect of CLG on cell-substrate adhesion was tested on both (E) fibronectin and (F) collagen (n = 30–39 wells, each condition). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus the vehicle (Veh) control.

availability of molecules such as serotonin, which would be expected following treatment with either SSRIs or MAO-A inhibitors (e.g. CLG). Off-target effects of either class of drug could certainly be involved and, in the case of MAO-A, which can exist in both catalytically active and inactive states (both of which influence distinct phenotypic changes), a cell's fate could be influenced by the predominating state of MAO-A at any given time [12,13].

Escape of tumour cells from the primary mass, local invasion, and intravasation into the circulation for transport to distal sites are pivotal steps of metastasis. Yet it is the subsequent escape of tumour cells from

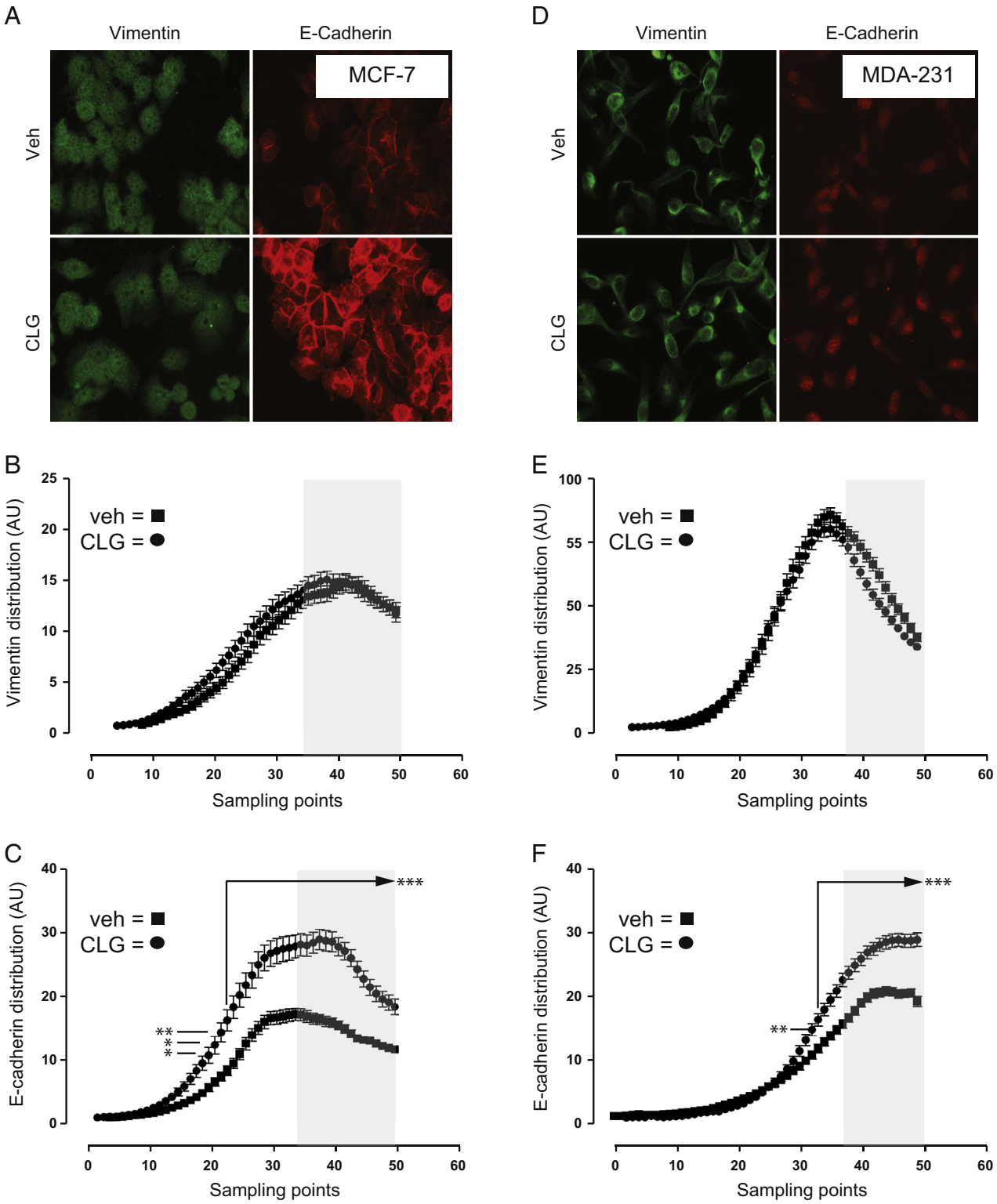

Fig. 4. CLG induces E-cadherin immunoreactivity in MCF-7 and MDA-MB-231 cultures. (A) MCF-7 cells were probed for Vimentin and E-cadherin expression using indirect immunofluorescence. The distribution of (B) Vimentin (VEH: n = 171 cells; CLG: n = 166 cells) and (C) E-cadherin (VEH: n = 202 cells; CLG: n = 169 cells) in MCF-7 cells was quantitated using densitometry, with the nucleus centred at '50' on the X-axis. (D) MDA-MB-231 cells were probed for Vimentin and E-cadherin expression and the distribution of (E) Vimentin (VEH: n = 173 cells; CLG: n = 199 cells) and (F) E-cadherin (VEH: n = 225 cells; CLG: n = 257 cells) in MBA-MD-231 cells was presented as plot profiles. The grey bars represent the average radius of (B, C) MCF-7 cells and (E, F) MDA-MB-231 cells. The plasma membrane in MCF-7 cells is situated between '10' and '20' on the X-axis, and in MDA-MB-231 cells is situated at approximately '20' along the X-axis. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus corresponding sampling point in the vehicle (Veh)-treated cultures.

the circulation (extravasation) that is a major rate-limiting step in metastasis. While EMT is required for cancer cell intravasation, the extravasation phase and establishment at the secondary site requires reverting to the epithelial phenotype. In advanced breast cancer, this mesenchymal-to-epithelial (reverting) transition (ME(r)T) reflects the re-induction of E-cadherin and other factors back to their 'normal' levels [32]. Invasiveness and migration are critical for both intravasation and extravasation. However, the MDA-MB-231 cell line is a post-EMT cell

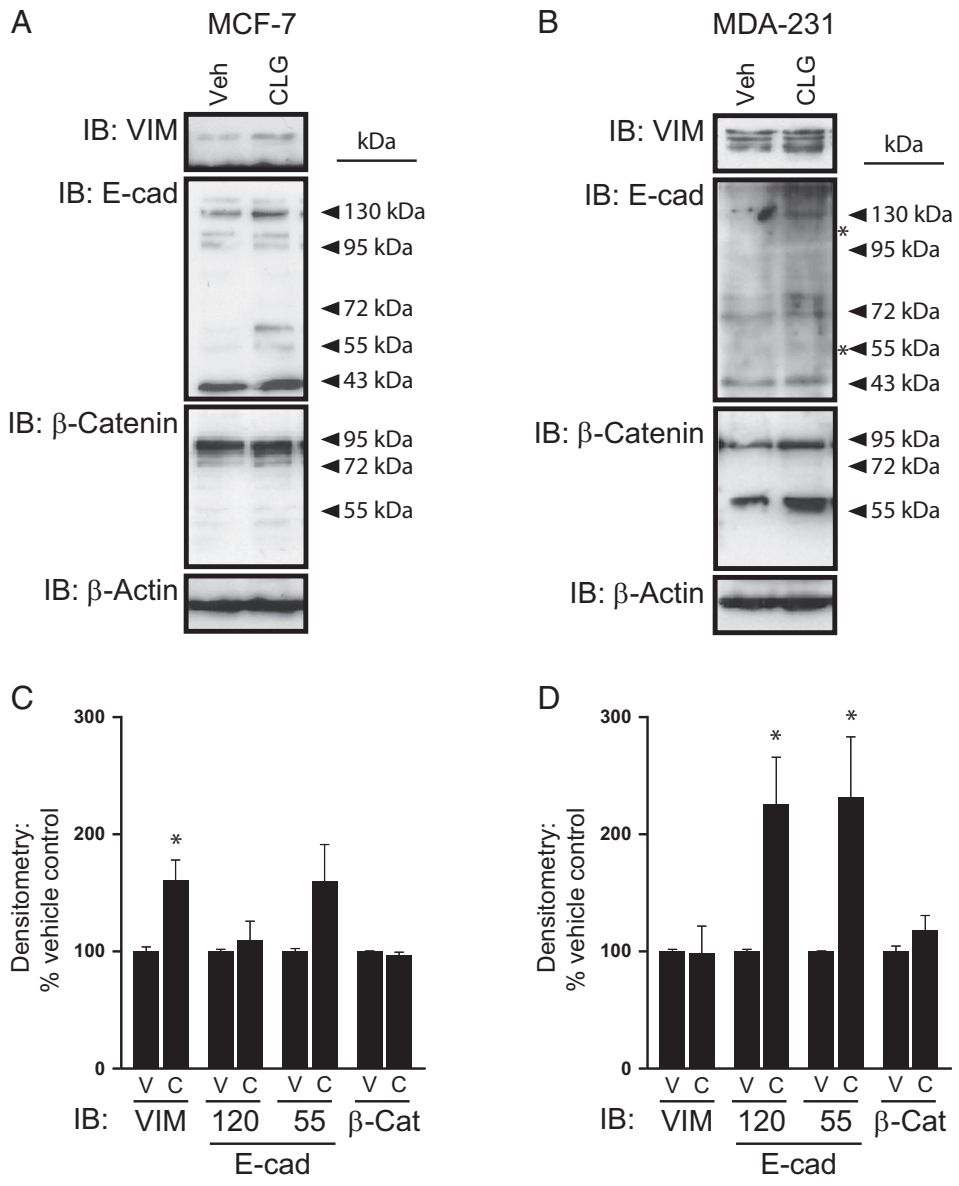

Fig. 5. CLG affects the expression of transitional markers in MCF-7 and MDA-MB-231 cells. (A) MCF-7 and (B) MDA-MB-231 cell extracts were probed for Vimentin (VIM), E-cadherin (E-cad) and β-catenin (β-Cat). E-Cad was detected as the precursor protein (130 kDa) and a putative proteolytic fragment at 55 kDa. Densitometry was used to quantitate target protein expression in (C) MCF-7 (n = 3–6) and (D) MDA-MB-231 (n = 3–7) cell extracts (C) There was a significant increase in VIM immunodetection in CLG-treated MCF-7 cells. The increased detection of E-cad in these same lysates was not significant. (D) In contrast, CLG had no effect on VIM detection — but exerted a significant effect on E—cadherin (120 kDa and 55 kDa species) detection — in MDA-MB-231 cell extracts. *$P < 0.05$ versus the vehicle ('V')-treated control. 'C': CLG-treated. The asterisks in panel (B) indicate the position of the barely detectable 120 and 55 kDa E-cad species.

line [33], which strongly suggests that the decrease of anchorage-independent growth and increased invasiveness following CLG-induced MAO-A inhibition are behaviours which would be more likely associated with extravasation. This is supported by the significant induction of E-cadherin in CLG-treated MDA-MB-231 cultures and corroborates the increased expression of *cadherin* genes in CLG-treated prostate cancer cells [34].

Our ability to detect smaller fragments (i.e. 55 kDa) in CLG-treated MDA-MB-231 cells could potentially be implicating matrix metalloproteinase and/or γ-secretase-mediated proteolysis [35], both of which have been linked to E-cadherin shedding [36] as well as to E-cadherin fragment-induced cancer cell invasion [37]. CLG did not affect either the phosphorylation of GSK-3β(Ser9), a signalling molecule known to stimulate the transcription factor Snai1 and to repress E-cadherin mRNA expression [15], or the interaction between GSK-3β and Snai1, which strongly suggests that any change in E-cadherin expression in CLG-treated cells does not result from a canonical Snai1-mediated transcriptional mechanism.

Levels of β-catenin, a known interactor of E-cadherin [38], were not affected by CLG-treatment. The β-Catenin-GSK-3β complex facilitates the phosphorylation of β-catenin and its degradation via the ubiquitin-proteosomal pathway [39]. A release of β-catenin from this complex (as we've observed herein) would promote β-catenin stability [22] and stabilization of an E-cadherin-β-catenin complex would be necessary for binding to the cytoskeleton, for maintaining cell adhesiveness and for diminishing invasive potential; this could explain the poor invasiveness of MCF-7 cells. We noticed that CLG treatment promoted a significant cleavage of E-cadherin and also promoted an association of a smaller 55 kDa E-cadherin species with β-catenin. This would presumably alter the normal course of E-cadherin-β-catenin signalling and could potentially facilitate oncogenic signalling, as suggested elsewhere [35,40]. Thus, the post-EMT status of the MDA-MB-231 cell line in

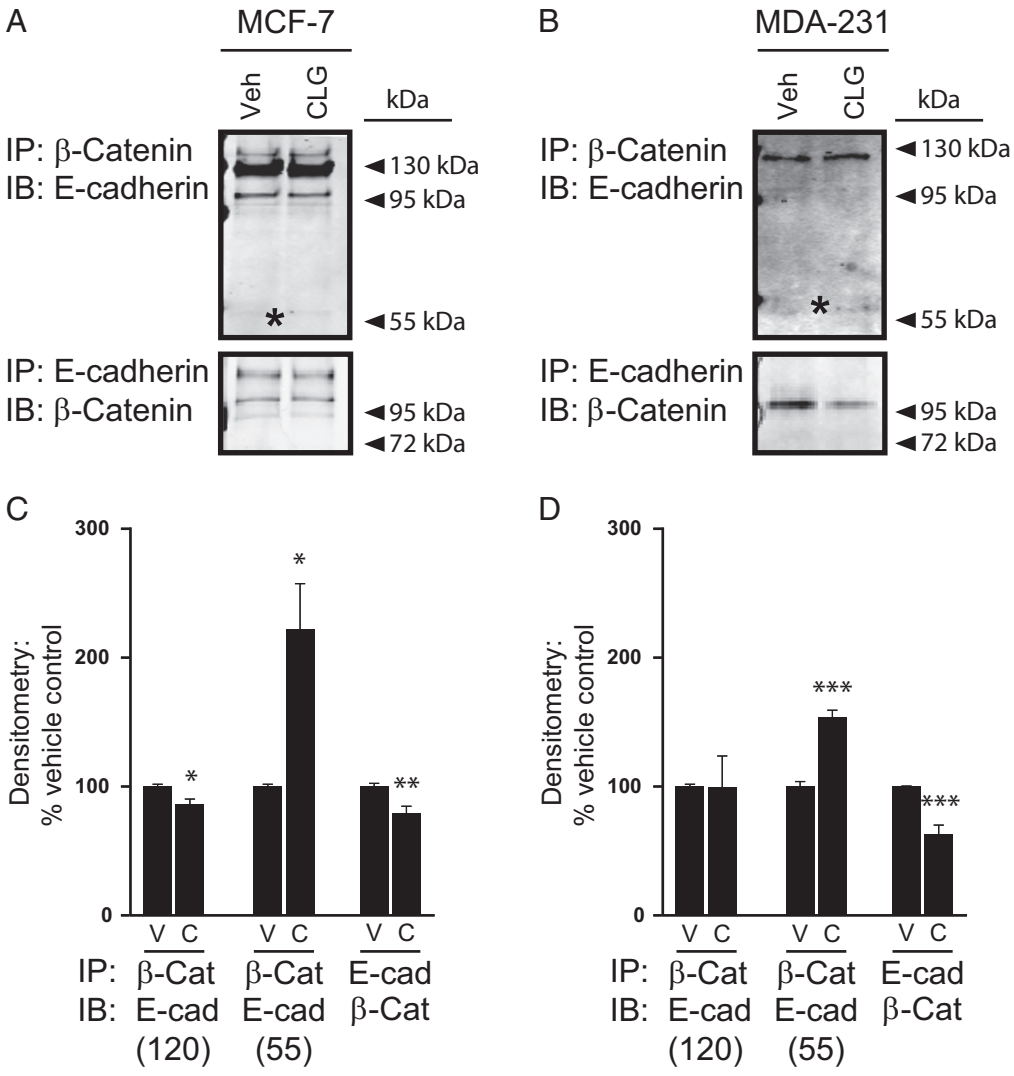

Fig. 6. CLG affects β-catenin/E-cadherin co-immunoprecipitation in MCF-7 and MDA-MB-231 cells. (A) MCF-7 and (B) MDA-MB-231 cell extracts were immunoprecipitated (IP) for β-catenin and immunoblotted (IB) for E-cadherin. The complementary experiments, i.e. IP for E-cadherin and probing for β-catenin, were also performed. Densitometry was used to quantitate protein expression in (C) MCF-7 (n = 3–7) and (D) MDA-MB-231 (n = 5–7) cell extracts, and revealed that CLG exerted subtly different effects on the β-catenin/E-cadherin complex in extracts from the two cell lines. Some of these differences might reflect the increased interaction between the smaller 55 kDa E-cadherin species and β-catenin in both cell lines. The asterisk '*' indicates a faint lower (~55 kDa) E-cadherin immunoreactive band (in panels A and B) used for the densitometric analyses summarized in panels C and D. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus the vehicle (Veh) control. 'V': vehicle-treated; 'C': CLG-treated.

combination with the potential of a CLG-induced loss of a complex between E-cadherin (120 kDa) and β-catenin, or the enhanced interaction between E-cadherin (55 kDa) and β-catenin, could have bearing on their high metastatic potential.

The role of GSK-3β in cancer progression is complicated as it can exert effects independently of any canonical change in phosphorylation status. Indeed, it may function as a "tumour suppressor" in some cases, but promote growth and development in others [25]. Clearly, CLG/MAO-A has a non-canonical influence on GSK-3β since the phosphorylation of both GSK-3β and Akt are unaltered, yet CLG had an unpredictable effect on β-catenin expression and its ability to interact with E-cadherin. While the nature of CLG's effects in the current context remains unclear, it has been shown to induce the transcription of oncogenic genes, including β-catenin in prostate cancer cells [7].

In MDA-MB-231 cells, we observed a very faint 120 kDa E-cadherin immunoreactive band in complex with β-catenin, which seems to have been replaced with a 55 kDa E-cadherin band (*note*, both of these E-cadherin species were at the very limit of detection in these cells). In corresponding cells, E-cadherin immunofluorescence appears to have increased primarily in the nucleus. Such an observation is not unique. Indeed, the proteolysis (cleavage) of E-cadherin is known to promote its nuclear localization, but work using colorectal as well as breast cancer cells reveals that nuclear E-cadherin is not necessarily associated with the anticipated metastatic phenotypes, e.g. reduced invasiveness, foci formation and proliferation [41,42]. The functional implication(s) of nuclear E-cadherin expression in primary tumours and metastases remains unclear, but it has been suggested that any associated phenotype undoubtedly reflects the proportion of pools of full-length versus cleaved E-cadherin species, and contributions to non-canonical environment-dependent regulation of malignant behaviour [41].

Aside from the (post)translational regulation of signalling complexes discussed above, another mechanism contributing to our observations might reside with a role for MAO-A which is independent of its catalytic activity. Indeed, we have reported that a catalytic-dead variant of MAO-A affects BrdU incorporation and cell proliferation in neuronal cultures [12]. Furthermore, a related amine oxidase, e.g. the vascular adhesion protein-1, also has a dual function depending on its localization. For example, within the cytoplasm it acts as a catalytic enzyme, but upon relocation to the cell surface it promotes endothelial adhesion and the spread of cancer [43,44]. Preliminary cross-linking studies

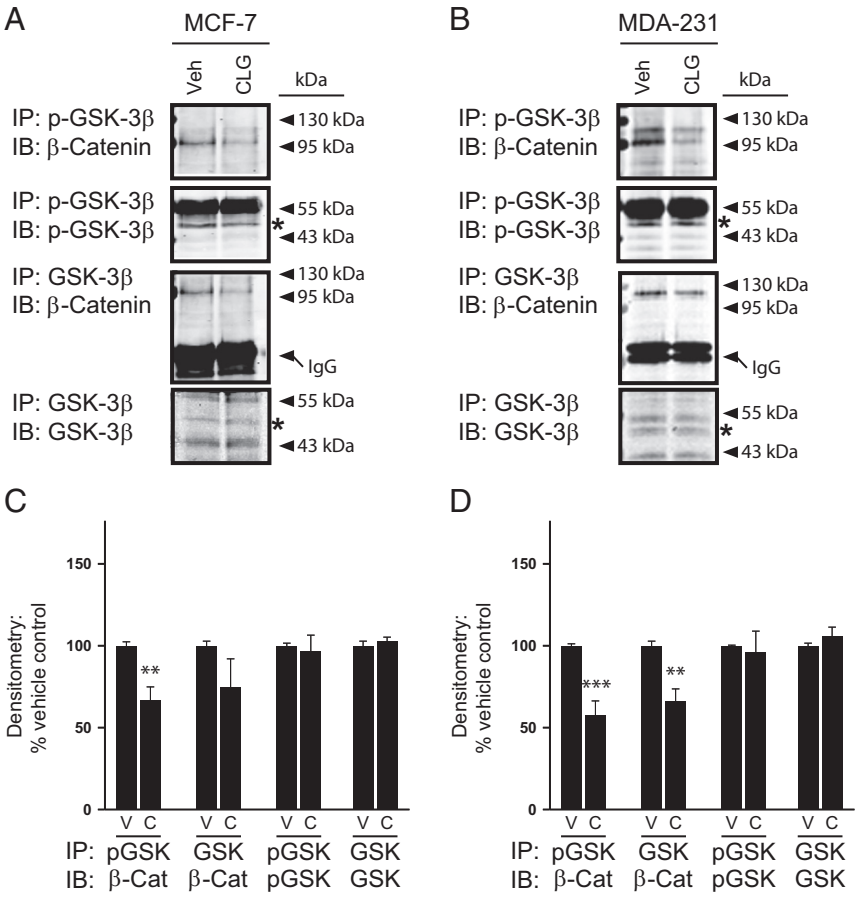

Fig. 7. CLG affects the GSK-3β/β-catenin co-immunoprecipitation in MCF-7 and MDA-MB-231 cells. (A) MCF-7 and (B) MDA-MB-231 cell extracts were immunoprecipitated (IP) for GSK-3β and immunoblotted (IB) for β-catenin. Densitometry was used to quantitate protein expression in (C) MCF-7 (n = 3–5) and (D) MDA-MB-231 (n = 7) cell extracts. CLG disrupts the phospho-Ser9 GSK-3β (p-GSK-3β)/β-catenin complex in both cell lines. The asterisk '*' in A and B indicates phosho-GSK-3β and total GSK-3β species in corresponding immunoblots. $^{**}P < 0.01$; $^{***}P < 0.001$ versus the vehicle (Veh) control. 'V': vehicle-treated; 'C': CLG-treated.

reveal the existence of a significant pool of active MAO-A on the surface of MCF-7 as well as MDA-MB-231 cells, with a greater proportion of the cell's MAO-A protein being expressed on the surface of MBA-MD-231 cells. Perhaps different stages of cancer progression (extravasation?) are influenced by the proportion of mitochondrial versus surface-expressed pools of MAO-A? Perhaps there are EMT- or MET-specific roles for amine oxidases such as MAO-A or the vascular adhesion protein-1 discussed above? This is not an unreasonable notion as

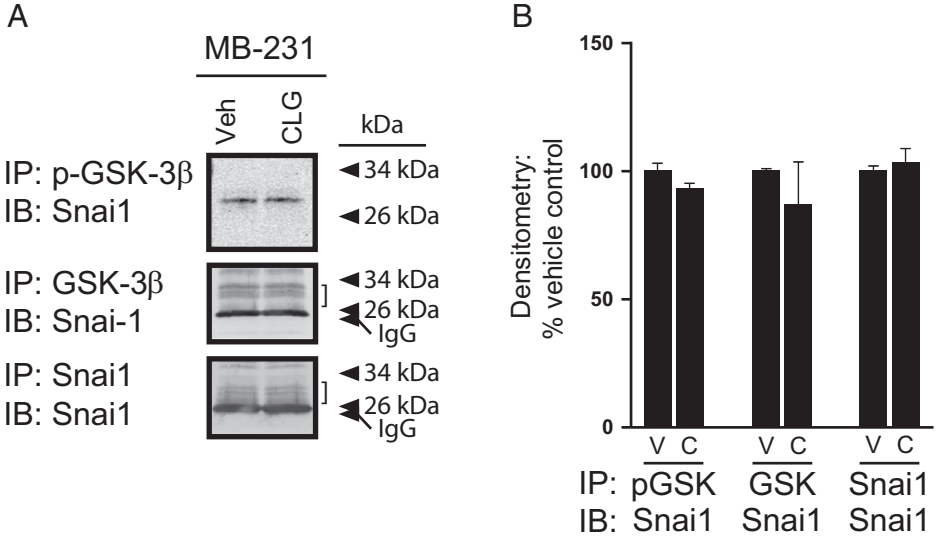

Fig. 8. CLG does not affect the interaction between GSK-3β and Snai1 in MDA-MB-231 cells. (A) MDA-MB-231 cell extracts were immunoprecipitated (IP) for phosphoSer9-GSK-3β (p-GSK-3β) or for total GSK-3β, and immunoblotted (IB) for Snai1. (B) Densitometry (n = 4–6) confirms that CLG does not affect the interaction between GSK-3β and Snai1, or the expression of Snai1 itself. Square brackets indicate the Snai1 immunoreactive bands. 'V': vehicle-treated; 'C': CLG-treated.

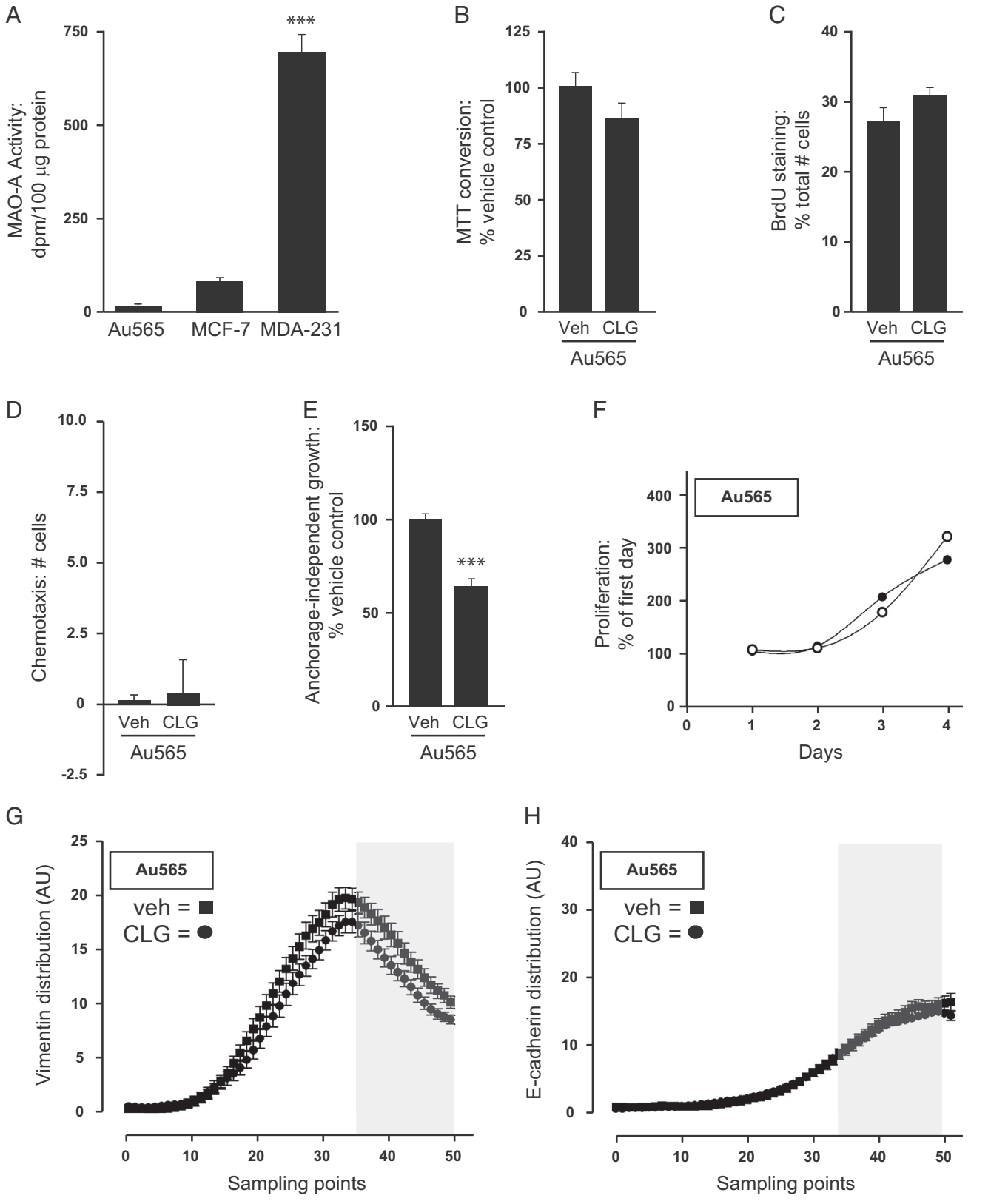

Fig. 9. CLG exerts a limited effect on the ER-negative Au565 human breast carcinoma cell line. (A) MAO-A activity is appreciably lower in Au565 cells in comparison to MCF-7 and MDA-MB-231 cells ($P < 0.001$). CLG (1 µM, 24 h) has no significant effect on (B) MTT conversion ($P = 0.215$), (C) *de novo* DNA synthesis/BrdU staining ($P = 0.154$), or (D) active migration of Au565 cells ($P = 0.251$). (E) CLG significantly impacts anchorage-independent growth (soft agar) ($P < 0.001$), but does not exert any effect on (F) Au565 proliferation over four days ($P = 0.764$). The expression levels/distribution (densitometry plot profiles) of both (G) Vimentin and (H) E-cadherin are also unaffected by treatment of AU565 cell cultures with CLG.

Snai1-dependent repression of E-cadherin and the induction of an EMT in MCF-7 cells has recently been associated with overexpression of lysyl oxidase-like 2, a copper-dependent amine oxidase [45].

Our investigation did reveal a reduction in proliferation and a concurrent increase in BrdU staining in CLG-treated MDA-MB-231 cells. While seemingly paradoxical, the only explanation we can provide comes from a recent report that found BrdU uptake in pancreatic β-cells can indicate *de novo* DNA synthesis as well as a DNA damage response, albeit in a context-dependent manner [46].

Finally, the lack of similarity in the response of MDA-MB-231 and Au565 cells (both being ER-negative) to treatment with CLG strongly suggests that the cell's ER status might not be a primary factor in our observations. However, an interaction between CLG/MAO-A and ER status in breast carcinomas cannot be discounted absolutely and is certainly worthy of further examination.

5. Conclusions

While it is unclear whether manipulation of the MAO-A system (depression-related or otherwise) could be predisposing to breast cancer metastasis in the clinic, our observations certainly do support this troubling possibility. Comparing the broad range of effects of CLG on MDA-MB-231 (ER-negative, mesenchymal) cells to the limited effects of CLG on MCF-7 (ER-positive, epithelial) and Au-565 (ER-negative, epithelial) cells leads us to propose that inhibition of MAO-A on breast cancer progression is dependent more on the cell's EMT status than on its ER status. This notion could help in resolving some of the ambiguity surrounding the fundamental role of MAO-A in malignant behaviour — with a subtle role for MAO-A in extravasation — and could provide fundamental insight into new strategies for diagnosis, prognosis, and prevention of metastatic cancers.

List of abbreviations

MAO-A	monoamine oxidase-A
CLG	clorgyline
EMT	epithelial-to-mesenchymal transition
ME(r)T	mesenchymal-to-epithelial (reverting) transition
GSK-3β	glycogen synthase kinase-3β
PBS	phosphate-buffered saline
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
BrdU	5-bromo-2′-deoxyuridine
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Authors' contributions

Conceived and designed the experiments: DDM TSM KK JNKN AF. Performed the experiments: TSM KK JNKN KF PRP LT. Analyzed the data: DDM TSM. Contributed reagents/materials/analysis tools: DDM EL AF DHA. Wrote the paper: DDM TSM. Revised the manuscript for critical content: DDM TSM JNKN PRP AF EL DHA. All authors approved the final manuscript.

Competing interests

There are no competing interests.

Acknowledgements

Part of this work was presented at the 2010 Amine Oxidase Workshop (Edmonton, Alberta, Canada). This work was funded by grants from the Canadian Breast Cancer Foundation–Prairies/NWT Region and the Canada-Saskatchewan Western Economic Partnership Agreement (project# 000011361) (both to DDM). We also gratefully acknowledge funding in the form of a University of Saskatchewan (UofS) College of Graduate Studies and Research M.Sc. Scholarship (TSM), and by a UofS Graduate Teaching Fellowship and a UofS College of Medicine Scholarship (JNKN).

References

[1] V.P. Pai, A.M. Marshall, L.L. Hernandez, A.R. Buckley, N.D. Horseman, Breast Cancer Res. 11 (2009) R81.
[2] S. Bahl, M. Cotterchio, N. Kreiger, Psychother. Psychosom. 72 (2003) 185–194.
[3] M. Cotterchio, N. Kreiger, B. Theis, M. Sloan, S. Bahl, Cancer Epidemiol. Biomarkers Prev. 12 (2003) 1053–1060.
[4] L.A. Rybaczyk, M.J. Bashaw, D.R. Pathak, K. Huang, BMC Genomics 9 (2008) 134.
[5] J.M. Lizcano, E. Escrich, T. Ribalta, J. Muntane, M. Unzeta, Biochem. Pharmacol. 42 (1991) 263–269.
[6] D.M. Peehl, M. Coram, H. Khine, S. Reese, R. Nolley, et al., J. Urol. 180 (2008) 2206–2211.
[7] H. Zhao, V. Flamand, D.M. Peehl, BMC Med. Genomics 2 (2009) 55.
[8] M. Yanez, N. Fraiz, E. Cano, F. Orallo, Biochem. Biophys. Res. Commun. 344 (2006) 688–695.
[9] G. Filomeni, I. Graziani, G. Rotilio, M.R. Ciriolo, Genes Nutr. 2 (2007) 295–305.
[10] R.B. Wallace, B.M. Sherman, J.A. Bean, Oncology 39 (1982) 279–283.
[11] M. Cotterchio, N. Kreiger, G. Darlington, A. Steingart, Am. J. Epidemiol. 151 (2000) 951–957.
[12] Z. Wei, T. Satram-Maharaj, B. Chaharyn, K. Kuski, P.R. Pennington, et al., J. Neural Transm. 119 (2012) 1285–1294.
[13] D.D. Mousseau, G.B. Baker, Curr. Top. Med. Chem. 12 (2012) 2163–2176.
[14] G. Berx, K.F. Becker, H. Hofler, F. van Roy, Hum. Mutat. 12 (1998) 226–237.
[15] R.E. Bachelder, S.O. Yoon, C. Franci, A.G. de Herreros, A.M. Mercurio, J. Cell Biol. 168 (2005) 29–33.
[16] M.J. Hendrix, E.A. Seftor, D.A. Kirschmann, R.E. Seftor, Breast Cancer Res. 2 (2000) 417–422.
[17] X. Cao, L. Rui, P.R. Pennington, J. Chlan-Fourney, Z. Jiang, et al., J. Neurochem. 111 (2009) 101–110.
[18] X. Cao, Z. Wei, G.G. Gabriel, X. Li, D.D. Mousseau, BMC Neurosci. 8 (2007) 73.
[19] H.C. Chen, Methods Mol. Biol. 294 (2005) 15–22.
[20] J. Van Heusden, P. de Jong, F. Ramaekers, H. Bruwiere, M. Borgers, et al., J. Histochem. Cytochem. 45 (1997) 315–319.
[21] H. Hernandez-Vargas, J. Palacios, G. Moreno-Bueno, Oncogene 26 (2007) 2902–2913.
[22] B.P. Wijnhoven, W.N. Dinjens, M. Pignatelli, Br. J. Surg. 87 (2000) 992–1005.
[23] J. Masterson, S. O'Dea, Cells Tissues Organs 185 (2007) 175–179.
[24] P.L. Ryan, A.F. Valentine, C.A. Bagnell, Biol. Reprod. 55 (1996) 1091–1097.
[25] J. Luo, Cancer Lett. 273 (2009) 194–200.
[26] B.W. Doble, J.R. Woodgett, Cells Tissues Organs 185 (2007) 73–84.
[27] J.R. Fann, A.M. Thomas-Rich, W.J. Katon, D. Cowley, M. Pepping, et al., Gen. Hosp. Psychiatry 30 (2008) 112–126.
[28] M.J. Massie, J. Natl. Cancer Inst. Monogr. 57–71 (2004).
[29] S. Williams, J. Dale, Br. J. Cancer 94 (2006) 372–390.
[30] P.F. Coogan, B.L. Strom, L. Rosenberg, Pharmacoepidemiol. Drug Saf. 18 (2009) 1111–1114.
[31] P.F. Coogan, B.L. Strom, L. Rosenberg, Breast Cancer Res. Treat. 109 (2008) 527–531.
[32] Y.L. Chao, C.R. Shepard, A. Wells, Mol. Cancer 9 (2010) 179.
[33] M.J. Voss, M.F. Moller, D.G. Powe, B. Niggemann, K.S. Zanker, et al., BMC Cancer 11 (2011) 158.
[34] V. Flamand, H. Zhao, D.M. Peehl, J. Cancer Res, Clin. Oncol. 136 (2010) 1761–1771.
[35] P. Marambaud, J. Shioi, G. Serban, A. Georgakopoulos, S. Sarner, et al., EMBO J. 21 (2002) 1948–1956.
[36] K. Bauer, A. Dowejko, A.K. Bosserhoff, T.E. Reichert, R.J. Bauer, Carcinogenesis 30 (2009) 1781–1788.
[37] R. Kuefer, M.D. Hofer, J.E. Gschwend, K.J. Pienta, M.G. Sanda, et al., Clin. Cancer Res. 9 (2003) 6447–6452.
[38] M. Conacci-Sorrell, J. Zhurinsky, A. Ben-Ze'ev, J. Clin. Invest. 109 (2002) 987–991.
[39] S. Kishida, H. Yamamoto, S. Ikeda, M. Kishida, I. Sakamoto, et al., J. Biol. Chem. 273 (1998) 10823–10826.
[40] K. Ito, I. Okamoto, N. Araki, Y. Kawano, M. Nakao, et al., Oncogene 18 (1999) 7080–7090.
[41] M.V. Cespedes, M.J. Larriba, M.A. Pavon, P. Alamo, I. Casanova, et al., Am. J. Pathol. 177 (2010) 2067–2079.
[42] S. Elloul, M.B. Elstrand, J.M. Nesland, C.G. Trope, G. Kvalheim, et al., Cancer 103 (2005) 1631–1643.
[43] M. Salmi, S. Jalkanen, FEBS Lett. 585 (2011) 1543–1550.
[44] P. Bono, M. Salmi, D.J. Smith, S. Jalkanen, J. Immunol. 160 (1998) 5563–5571.
[45] H.J. Moon, J. Finney, L. Xu, D. Moore, D.R. Welch, et al., J. Biol. Chem. 288 (2013) 30000–30008.
[46] S.H. Lee, E. Hao, F. Levine, P. Itkin-Ansari, Islets 3 (2011) 358–366.