An androgen receptor mutation in the MDA-MB-453 cell line model of molecular apocrine breast cancer compromises receptor activity

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Abstract

Recent evidence indicates that the estrogen receptor- α -negative, androgen receptor (AR)-positive molecular apocrine subtype of breast cancer is driven by AR signaling. The MDA-MB-453 cell line is the prototypical model of this breast cancer subtype; its proliferation is stimulated by androgens such as 5 α -dihydrotestosterone (DHT) but inhibited by the progestin medroxyprogesterone acetate (MPA) via AR-mediated mechanisms. We report here that the *AR* gene in MDA-MB-453 cells contains a G-T transversion in exon 7, resulting in a receptor variant with a glutamine to histidine substitution at amino acid 865 (Q865H) in the ligand binding domain. Compared with wild-type AR, the Q865H variant exhibited reduced sensitivity to DHT and MPA in transactivation assays in MDA-MB-453 and PC-3 cells but did not respond to non-androgenic ligands or receptor antagonists. Ligand binding, molecular modeling, mammalian two-hybrid and immunoblot assays revealed effects of the Q865H mutation on ligand dissociation, AR intramolecular interactions, and receptor stability. Microarray expression profiling demonstrated that DHT and MPA regulate distinct transcriptional programs in MDA-MB-453 cells. Gene Set Enrichment Analysis revealed that DHT- but not MPA-regulated genes were associated with estrogen-responsive transcriptomes from MCF-7 cells and the Wnt signaling pathway. These findings suggest that the divergent proliferative responses of MDA-MB-453 cells to DHT and MPA result from the different genetic programs elicited by these two ligands through the AR-Q865H variant. This work highlights the necessity to characterize additional models of molecular apocrine breast cancer to determine the precise role of AR signaling in this breast cancer subtype.

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Introduction

In contrast to estrogen receptor- α -positive (ER(+)) breast cancers, which are amenable to antiestrogenic hormonal therapies, treatment options for ER-negative (ER(–)) tumors are limited. Hence, there is an urgent imperative to develop new therapeutic targets for

ER(–) breast cancer, especially as it has a higher prevalence in young women, tends to be more aggressive than ER(+) disease, and is associated with worse prognosis (Carey *et al.* 2010). Genome profiling studies have identified a ‘molecular apocrine’ subtype of ER(–) breast cancer that is associated with

apocrine differentiation and has a gene profile characterized by active androgen receptor (AR) signaling (Farmer *et al.* 2005, Doane *et al.* 2006).

Gene profiling studies have also classified the MDA-MB-453 breast cancer cell line as molecular apocrine (Doane *et al.* 2006), and therefore, this cell line has been adopted as a model of this breast cancer subtype. We and others have previously shown that MDA-MB-453 cells express very high levels of AR compared with other breast cancer cell lines, with the levels being comparable to those seen in the LNCaP prostate cancer cell line (Hall *et al.* 1994, Birrell *et al.* 1995, Lehmann *et al.* 2011, Robinson *et al.* 2011). Moreover, like prostate cancer cells, but in contrast to most AR(+), ER(+) breast cancer cell lines, proliferation of MDA-MB-453 cells is stimulated by androgens in an AR-dependent manner (Birrell *et al.* 1995, Doane *et al.* 2006, Naderi & Hughes-Davies 2008, Ni *et al.* 2011). Recent studies have shown that AR signaling in MDA-MB-453 cells stimulates a forkhead box A1 (FOXA1)-dependent transcriptional program that is similar to that associated with estrogen signaling in ER(+) luminal breast cancers or cell line models of luminal disease (Doane *et al.* 2006, Robinson *et al.* 2011). Furthermore, AR signaling induces Wnt/ β -catenin signaling in a manner that subsequently augments the Erbb2 pathway to promote proliferation in MDA-MB-453 cells (Ni *et al.* 2011). These findings support the development of therapeutic strategies for ER(–) breast cancer that target the AR, particularly for tumors that possess a steroid receptor profile characteristic of apocrine breast cancer, i.e. positive for AR immunoreactivity in the absence of ER and progesterone receptor (PR). Indeed, clinical trials are currently underway to determine the efficacy of the AR antagonist, bicalutamide, or the androgen biosynthesis inhibitor, abiraterone, as therapeutic options for advanced breast cancers with steroid receptor profiles similar to molecular apocrine disease (clinicaltrials.gov Identifier: NCT00468715 and NCT00755885 respectively).

Whereas proliferation of MDA-MB-453 cells is stimulated by both natural (e.g. 5 α -dihydrotestosterone, DHT) and synthetic (e.g. methyltrienolone, R1881) non-aromatizable androgens, it is inhibited by the synthetic progestin medroxyprogesterone acetate (MPA; Birrell *et al.* 1995, Bentel *et al.* 1999, Doane *et al.* 2006, Naderi & Hughes-Davies 2008, Ni *et al.* 2011). These proliferative responses are attenuated by co-treatment with an AR antagonist, suggesting that they are mediated by the AR (Birrell *et al.* 1995, Bentel *et al.* 1999). Therefore, the goal of the current study was to investigate the activation of the AR by DHT and

MPA in a molecular apocrine context to better understand the mechanisms behind the observed divergent proliferative responses to these ligands. We report here a missense mutation in the AR gene in MDA-MB-453 cells that leads to altered transactivation activity of the resultant AR variant relative to the wild-type AR (wtAR) and differential responses to stimulation by DHT and MPA. Additionally, our findings suggest that alternative *in vitro* models of molecular apocrine disease should be characterized and compared to the MDA-MB-453 cell line.

Materials and methods

Cell culture

MDA-MB-453, T-47D, PC-3, and COS-1 cells, obtained from the American Type Culture Collection (Manassas, VA, USA), were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS). MFM-223 cells were obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and maintained in Eagle's Minimum Essential Medium (EMEM) containing 10% FBS, 2 mM L-glutamine, and insulin–transferrin–sodium selenite (ITS) supplement.

AR sequencing

RNA prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany) was reverse transcribed using Superscript II (Invitrogen, Carlsbad, CA, USA). The entire AR cDNA was PCR amplified using primers generating seven overlapping fragments (see [Supplementary Materials and methods](#), see section on [supplementary data](#) given at the end of this article). PCR products were sequenced in both orientations. The mutation was confirmed in genomic DNA extracted using the QIAamp DNA Mini Kit (Qiagen) and PCR amplified using genomic DNA-specific primers ([Supplementary Materials and methods](#)) followed by sequencing.

Plasmid constructs

The pCMV-AR expression construct; pVP16-AR-NTD_{1–540}(wt) and pM-AR-LBD_{644–919}(wt) mammalian two-hybrid constructs; and MMTV-luciferase, ARR3-tk-luciferase probasin, and pGK1 reporters have been described previously (Tilley *et al.* 1989, Buchanan *et al.* 2004, Butler *et al.* 2006). The PSA-luciferase reporter plasmid was constructed from PSA630-CATSAT (Brookes *et al.* 1998) from Dr Peter Molloy (CSIRO Division of Molecular Science, Sydney Laboratory, NSW, Australia).

The pCMV-AR-Q865H variant construct was created from pCMV-AR using site-directed mutagenesis as described previously (Sarkar & Sommer 1990, Buchanan *et al.* 2001). pM-AR-LBD_{644–919}(Q865H) was constructed from pCMV-AR-Q865H by subcloning with AspI and SfuI. Construct integrity was confirmed by sequencing of both DNA strands, and DNA concentrations were measured by spectrophotometry and visualization on agarose gels.

Transient transfection and reporter gene assay

Assays measuring AR activity or AR N/C interactions were performed as described previously (Butler *et al.* 2006, Need *et al.* 2009, Buchanan *et al.* 2011). Briefly, cells were seeded in 96-well plates ($1.5\text{--}2.0 \times 10^4$ cells/well) and transfected with reporter and/or receptor constructs using Lipofectamine 2000 (Invitrogen). After 5 h, cells were treated with DHT (Sigma), MPA (Sigma), or vehicle (0.1% ethanol) and assayed for luciferase activity using the Luciferase Assay System (Promega, Madison, WI, USA) 36–48 h later.

Diethylaminoethyl dextran transfection and ligand binding stability assay

COS-1 cells (1×10^6 cells/10 cm dish) were transiently transfected with 2 μg AR expression constructs using the diethylaminoethyl (DEAE) dextran method as described previously (Buchanan *et al.* 2001). The assay for AR ligand binding stability in lysates from transiently transfected COS-1 cells was performed with ^3H -DHT (PerkinElmer, Waltham, MA, USA) and ^3H -MPA (New England Nuclear, Boston, MA, USA) as described previously (Tilley *et al.* 1989, Marcelli *et al.* 1991, Buchanan *et al.* 2001).

Immunoblot analysis

Cells were lysed in RIPA buffer (10 mM Tris, 1 mM EDTA, 150 mM NaCl, and 1% Triton X-100, pH 7.4, containing $1 \times$ complete protease inhibitor tablets; Roche, Indianapolis, IN, USA) and homogenized by freeze-thawing. Lysates were separated by SDS-PAGE and transferred to membrane. Primary antibodies used were AR N-20 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), GAPDH (Millipore, Billerica, MA, USA), PR (Leica Microsystems, Wetzlar, Germany), glucocorticoid receptor (GR; Abcam, Cambridge, UK), and α -tubulin (Millipore). Immunoreactive bands were detected with enhanced chemiluminescence western blotting detection reagents (GE Healthcare, Buckinghamshire, UK).

Molecular modeling

Molecular models of the AR ligand binding domain (LBD) were generated based on the crystal structure coordinates of the human PR-LBD complexed with progesterone as described previously (Williams & Sigler 1998, Matias *et al.* 2000). DHT and MPA were docked into the AR model by superimposing the AR-LBD onto the crystal structure of the PR-LBD bound to progesterone. Energy-minimized structures for DHT and MPA, and their interaction with the AR-LBD, were modeled as described previously (Buchanan *et al.* 2001). Ribbon diagrams were constructed using the MOLSCRIPT/Raster3D program (Kraulis 1991, Merritt & Murray 1994).

Quantitative reverse transcription PCR

MDA-MB-453 cells (3.5×10^5 cells/well in six-well plates) were seeded in phenol red-free RPMI containing 10% charcoal-stripped FBS. MFM-223 cells (5×10^5 cells/well in 12-well plates) were seeded in phenol red-free EMEM containing 10% charcoal-stripped FBS and ITS. After 48 h, the medium was removed and replaced with a medium containing vehicle (0.1% ethanol) or hormones (DHT, MPA, progesterone (Sigma), dexamethasone (Sigma), hydroxyflutamide (Schering-Plough, North Ryde, NSW, Australia), bicalutamide (AstraZeneca, North Ryde, NSW, Australia), and 17β -estradiol (E_2 , Sigma)) for another 6 h. RNA was extracted using TRIzol reagent (Invitrogen), treated with Turbo DNA-free (Ambion, Austin, TX, USA), and reverse transcribed using iScript reverse transcriptase (Bio-Rad, Hercules, CA, USA). Quantitative PCR was carried out using iQ SYBR Green Supermix (Bio-Rad) and primer pairs (as indicated in the [Supplementary Materials and methods](#), see section on [supplementary data](#) given at the end of this article) on a CFX384 real-time PCR detection system (Bio-Rad). Gene expression was normalized to *GAPDH* mRNA levels.

Microarray analysis

MDA-MB-453 cells were seeded and treated (with DHT (1 nM), MPA (100 nM), or vehicle) as described earlier. RNA was extracted using TRIzol, purified using the RNeasy Mini Kit, and integrity was confirmed using the Experion Automated Electrophoresis System (Bio-Rad). Total RNA (300 ng) was linearly amplified and converted to cDNA using the Ambion WT Expression Kit, then fragmented, and labeled using the GeneChip WT Terminal Labeling and Controls Kit (Affymetrix, Santa Clara, CA, USA). Samples were hybridized to GeneChip Human

Gene 1.0 ST Arrays (Affymetrix) for 17 h at 45 °C before washing, staining, and scanning. Hybridizations were performed for biological quadruplicate samples for each treatment group. Data were analyzed using Partek Genomics Suite (Partek Incorporated, St Louis, MO, USA). Briefly, .cel files were imported using RMA background correction, Partek's own GC content correction, and mean probe summarization. Normalized intensity values from each array are supplied in [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article. Differential gene expression was assessed by ANOVA with the *P* value adjusted using step-up (Benjamini & Hochberg 1995) multiple test correction to control the false discovery rate (FDR). Adjusted step-up *P* values <0.05 were considered significant.

Pathway analysis

GeneCodis 2.0 (Carmona-Saez et al. 2007, Nogales-Cadenas et al. 2009) was used to assess for enrichment of biological pathways in genes uniquely regulated by DHT and MPA in MDA-MB-453 cells (i.e. genes with an adjusted step-up *P* <0.05 and fold change ≥1.2), with the reference consisting of all human annotated genes. This analysis was also performed with IPA 9.0 (Ingenuity Systems, Redwood City, CA, USA), using genes on the Affymetrix Human Gene 1.0 ST Array as a reference set. Pathways with a FDR-corrected hypergeometric *P* <0.05 were considered significantly enriched.

Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (GSEA; Subramanian et al. 2005) was implemented using the Broad Institute's public GenePattern server (<http://genepattern.broadinstitute.org/gp/pages/index.jsf>). Microarray-generated expression profiles from DHT-, MPA-, and vehicle-treated MDA-MB-453 cells were compared to publicly available gene sets. The Cicatiello set comprised genes regulated in MCF-7 breast cancer cells treated with 10 nM E₂ for 6 h (Cicatiello et al. 2010). The Gene Expression Metasignature (GEMS) set comprised genes regulated in MCF-7 cells treated with E₂ for 3–4 h across five independent published studies, with a combined FDR-corrected *P* <0.01 (Ochsner et al. 2009). The KEGG Wnt Signaling Pathway dataset was obtained from the Molecular Signatures Database (Subramanian et al. 2005). Pathway analysis was also performed with GSEA using the KEGG gene set collection from the Molecular Signatures Database.

Statistical analysis

Differences between multiple groups were analyzed using a two-way ANOVA and differences within a single group were analyzed using a one-way ANOVA (with Tukey's *post hoc* test). Tests were performed using PASW 17.0 software (IBM Corporation, Armonk, NY, USA) and statistical significance was accepted at *P* <0.05.

Figure 1 Sequencing and functional analysis of the AR in MDA-MB-453 cells. (A) Partial nucleotide sequence of *AR* exon 7 cDNA in the MDA-MB-453 (left panel) and T-47D (right panel) breast cancer cell lines. The arrowhead indicates nucleotide position 3719, where the G-T nucleotide substitution is located in MDA-MB-453 cells, resulting in the substitution of a glutamine residue for histidine at amino acid 865. (B) Partial nucleotide sequence of *AR* exon 7 genomic DNA in MDA-MB-453 cells. (C and D) MDA-MB-453 cells were transiently transfected with wtAR (blue curves) or AR-Q865H (red curves) expression constructs and the ARR3-tk-luc reporter construct followed by incubation with increasing concentrations (0.01–100 nM) of (C) DHT or (D) MPA or vehicle (0.1% ethanol, control) as indicated. Cell lysates were assayed for luciferase reporter gene activity. (E and F) PC-3 cells were transiently transfected and assayed for luciferase reporter gene activity in response to (E) DHT and (F) MPA as described for panels C and D. Points represent the average ± S.E.M. of quadruplicate wells. **P* <0.05, two-way ANOVA, AR-Q865H vs wtAR. (G and H) AR protein levels in transiently transfected PC-3 cell lysates from the experiments shown in panels E and F were detected by western blot analysis. GAPDH was used as a loading control.

Results

AR sequence and transactivation analysis in MDA-MB-453 cells

As a first step to characterize the AR in MDA-MB-453 cells, we sequenced the entire coding region of the AR gene. A single nucleotide G-T transversion at position 3719 (NM_000044) was identified in exon 7 of the AR in MDA-MB-453 cells but not in the T-47D breast cancer cells, the latter having a wild-type nucleotide sequence at this position (Fig. 1A). This nucleotide substitution in MDA-MB-453 cells was confirmed by sequencing in the antisense orientation (data not shown) and by analysis of genomic DNA (Fig. 1B), and is in agreement with the MDA-MB-453

AR sequence in the National Center for Biotechnology Information nucleotide sequence database (accession number AF162704). This G-T transversion results in the substitution of a glutamine residue for histidine at amino acid 865 in the AR LBD; we have termed this protein the AR-Q865H variant. No other sequence alterations were observed in the coding region of the AR gene in the MDA-MB-453 cell line.

We recreated the AR-Q865H variant in a mammalian expression construct to assess differences in receptor dynamics compared to the wtAR. To investigate the functionality of the AR-Q865H variant, we assessed the activity of ectopically expressed wtAR and the AR-Q865H variant in MDA-MB-453 cells on an AR-dependent probasin reporter gene (Kasper *et al.* 1999). MDA-MB-453 cells were used in order to maintain a molecular apocrine cell context and as there is an emerging appreciation of the requirement for co-transfection of essential transcription factors to elicit the full repertoire of steroid receptor actions in cells that do not normally express them (Kong *et al.* 2011). This approach was considered feasible because the transactivation activity of the endogenous AR was approximately two to three orders of magnitude lower than the transfected exogenous AR in MDA-MB-453 cells (Supplementary Figure 1A, see section on supplementary data given at the end of this article). Therefore, while it is still active in response to ligand (Supplementary Figure 1B), the endogenous

Figure 2 Effect of the Q865H substitution on ligand and intramolecular interactions and AR stability. (A) Stability of ligand-AR complexes. COS-1 cell lysates transiently transfected with wild-type AR (blue curves) or the AR-Q865H variant (red curves) were incubated with DHT (left panel) or MPA (right panel) at 37 °C for the indicated times. Points represent the average $\pm$ S.E.M. of at least three independent experiments. (B) Homology modeling of the holo wtAR (blue) and AR-Q865H (yellow) AF2 region. Diagrams showing the α -helical and β -sheet backbone of the AR-LBD were generated using the MOLSCRIPT/Raster3D program. Important amino acid side chains are depicted in stick form and predicted hydrogen bonds are shown as green dotted lines. Wild-type (Q) and variant (H) residues at position 865 are indicated. (C) Induction of the AR N/C interaction by DHT and MPA was measured using a mammalian two-hybrid assay. COS-1 cells were transiently transfected with the pVP16-AR-NTD(wt) and the pM-AR-LBD(wt) or pM-AR-LBD(Q865H) expression constructs and the pGK1 reporter. Cells were treated with increasing concentrations of DHT (0.1–100 nM, black bars), MPA (1–1000 nM, gray bars), or vehicle (0.1% ethanol, control) as indicated for 48 h and cell lysates were assayed for luciferase reporter gene activity. Bars represent the average $\pm$ S.E.M. of quadruplicate wells. * P < 0.05, two-way ANOVA, DHT vs MPA, # P < 0.05 AR-Q865H vs wtAR. (D) Ligand-induced stabilization of the endogenous AR-Q865H variant in MDA-MB-453 cells. Cells were treated with vehicle control (veh, 0.1% ethanol), DHT (1 nM, 10 nM), or MPA (1 nM, 10 nM) for 24 h and then AR and GAPDH protein levels were measured by western blotting.

AR-Q865H variant makes little contribution to transfected exogenous wtAR or AR-Q865H AR activity measured in these assays. Transactivation activity of the exogenous AR-Q865H variant on the probasin reporter gene was significantly reduced compared with the exogenous wtAR in response to both DHT (Fig. 1C) and MPA (Fig. 1D). At ligand concentrations >0.01 nM, both the AR-Q865H and the wtAR were more sensitive to activation by DHT than to equal concentrations of MPA. The above differences between DHT- and MPA-induced wtAR and AR-Q865H variant activity cannot be explained by MPA acting via the PR as MDA-MB-453 cells do not express PR (Supplementary Figure 2A, see section on supplementary data given at the end of this article and Subik *et al.* (2010)). Western blot analysis also indicated that GR levels were very low but AR is maintained in MDA-MB-453 cells treated under the same steroid-depleted conditions used for the transactivation studies (Supplementary Figure 2B). Collectively, these findings suggest that MPA action is predominantly mediated by the AR in MDA-MB-453 cells. The activity of the AR-Q865H variant compared with the wtAR in response to DHT (Fig. 1E) and MPA (Fig. 1F) was also significantly reduced in AR(–) PC-3 prostate cancer cells. Furthermore, immunoblot analysis demonstrated that this was associated with reduced stability of the AR-Q865H variant compared with the wtAR (Fig. 1G and H).

Effect of the Q865H substitution on ligand-induced AR stability

To investigate the reason for the different activity and stability of wtAR and the AR-Q865H variant, we first examined the stability of DHT and MPA binding to each receptor. Specifically, we assessed the time to 50% loss of specific ligand binding (t_{diss50}) in cytosol fractions of transfected COS-1 cells at 37 °C. As shown in Fig. 2A (left panel), DHT binding to the AR-Q865H variant was less stable (t_{diss50} =50 min) compared with wtAR (t_{diss50} =90 min). Compared to DHT, MPA exhibited lower but comparable binding stability to both receptors (t_{diss50} =35 min for wtAR, 20 min for AR-Q865H, Fig. 2A, right panel). Molecular modeling of wtAR and the AR-Q865H variant predicted changes in the AR intramolecular structure arising from the Q865H substitution (Fig. 2B). In particular, Q865 forms a hydrogen bond with helix 8 in the wtAR, whereas H865 is predicted to form a hydrogen bond with β -strand 4, which comprises the C-terminal 10 amino acids of the receptor. Acquisition of this hydrogen bond is likely to influence folding of the

Figure 3 Response of endogenous AR in MDA-MB-453 breast cancer cells to androgenic and non-androgenic ligands and antagonists. (A) Partial sequence alignment of steroid receptor family members. The gray shading surrounds residues homologous to AR amino acid 865. (B) Cells were transiently transfected with the MMTV-luciferase reporter plasmid followed by incubation with vehicle (veh, 0.1% ethanol, control) or 10 nM of DHT, MPA, progesterone (Prog), dexamethasone (Dex), hydroxyflutamide (OHF), bicalutamide (Bic), or 17 β -estradiol (E₂). Cell lysates were then assayed for luciferase reporter gene activity. (C, D and E) Cells were treated with the above ligands and then the mRNA levels for (C) *FKBP5*, (D) *UGT2B28*, and (E) *C10RF116* were measured by quantitative RT-PCR. Bars represent the average $\pm$ S.E.M. of triplicate samples. * P <0.05, one-way ANOVA, hormone vs vehicle control.

adjacent helix 12 over the ligand binding pocket in response to ligand binding, and as such the formation of the essential activation function-2 (AF-2). AF-2 is a three-dimensional ligand-induced LBD structure considered necessary for AR agonist activity; it acts as an interaction surface for specific LXXLL-like sequences in receptor coregulators and the structurally similar FQNLF peptide of the AR amino-terminal transactivation domain (NTD) in the AR N/C interaction (Ikonen *et al.* 1997, He *et al.* 2000, 2002, Callewaert *et al.* 2003). Indeed, mammalian two-hybrid assays were able to demonstrate that the DHT-induced N/C interaction for AR-Q865H was weaker than that observed for the wtAR, with a significant reduction observed at 10 and 100 nM DHT (Fig. 2C). The DHT-induced N/C interaction for both the wtAR (as previously reported Kempainen *et al.* (1999) and Birrell *et al.* (2007)) and the AR-Q865H variant was significantly stronger than that induced by equal concentrations of MPA. Consistent with this observation, DHT induced a dose-dependent stabilization of AR protein in MDA-MB-453 cells; however, MPA did not stabilize AR to the same extent as DHT (Fig. 2D). This is further supported by the stabilization of wtAR and, to a lesser extent, the AR-Q865H variant by DHT (Fig. 1G) but not by MPA (Fig. 1H) in PC-3 cells. Collectively, these findings potentially explain the reduced activity of the AR-Q865H variant compared with wtAR and the differential responses to DHT and MPA in MDA-MB-453 breast cancer cells.

AR-Q865H variant activity in response to androgenic and non-androgenic ligands

Given that the T875A substitution in the AR in LNCaP prostate cancer cells results in promiscuous activation by nonclassical ligands (Veldscholte *et al.* 1990), that the histidine substitution at amino acid 865 restores AR homology with the PR, GR, and mineralocorticoid receptor (MR; Fig. 3A) and the close proximity of the T875A and Q865H substitutions within the AR-LBD, we tested whether the endogenous AR-Q865H variant responds in a similar manner as the LNCaP AR to nonclassical ligands. In MDA-MB-453 cells transiently transfected with the MMTV-luc reporter gene, which can be activated by AR, PR, and GR (Yen *et al.* 1997), DHT and MPA significantly increased luciferase reporter activity compared with control, whereas progesterone, the synthetic glucocorticoid dexamethasone, E₂, and the AR antagonists hydroxyflutamide and bicalutamide did not (Fig. 3B). Quantitative RT-PCR was used to demonstrate that steady-state mRNA levels for *FKBP5*, a well-characterized androgen-, progestin-,

and glucocorticoid-regulated gene (Hubler *et al.* 2003, Vermeer *et al.* 2003, Magee *et al.* 2006), were significantly increased by DHT, MPA, and dexamethasone but not by any other ligands tested (Fig. 3C). *UGT2B28*, a member of the androgen-regulated

Figure 4 Transactivation activity of the endogenous AR in MDA-MB-453 breast cancer cells in response to DHT and MPA. Cells were transiently transfected with the (A) MMTV-luciferase, (B) PSA-luciferase, or (C) ARR3-tk-luciferase reporter plasmids followed by incubation with vehicle (0.1% ethanol, control), DHT (0.01–100 nM, black curves), or MPA (0.01–100 nM, gray curves). Cell lysates were then assayed for luciferase reporter gene activity. Points represent the average $\pm$ S.E.M. of quadruplicate wells. * $P < 0.05$, two-way ANOVA, DHT vs MPA.

Figure 5 Microarray analysis of DHT- and MPA-regulated genes in MDA-MB-453 cells. (A) Volcano plots showing distribution of gene expression for DHT (left panel) or MPA (right panel) compared to vehicle control. Dashed lines represent the $P < 0.05$ statistical cutoff, dotted lines represent the 1.2-fold change (FC, inner lines), and 1.5 FC (outer lines) cutoffs. Black points represent genes with $\text{FC} > 1.5$ ($P < 0.05$), dark gray points represent genes with $\text{FC} = 1.2\text{--}1.5$ ($P < 0.05$), and light gray points represent genes with $\text{FC} < 1.2$ ($P < 0.05$) and/or $P \geq 0.05$. (B) Euler diagrams showing the number of genes uniquely or commonly upregulated or downregulated > 1.2 -fold by DHT (1 nM) and MPA (100 nM) compared with vehicle ($P < 0.05$).

UGT2B family (Chouinard et al. 2006), was significantly induced only by DHT (Fig. 3D). *CIORF116*, which is specifically regulated by AR but not by GR (Steketee et al. 2004), was significantly induced by DHT and MPA (Fig. 3E). These results suggest that the variant AR in MDA-MB-453 cells is not strongly activated by non-androgenic ligands or antagonists.

Effect of DHT and MPA on endogenous AR-Q865H variant transactivation activity in MDA-MB-453 cells

To further characterize AR-Q865H variant activity, we investigated DHT- and MPA-induced transactivation activity of the endogenous AR-Q865H in MDA-MB-453 cells transiently transfected with three different androgen-responsive reporter genes. Both DHT and MPA stimulated endogenous AR-Q865H variant activity on the *MMTV* (Fig. 4A) and *PSA* (Fig. 4B) promoters, although sensitivity to DHT was at least two orders of magnitude greater than that for MPA. In contrast, only DHT significantly stimulated ARR3-tk-luc probasin reporter activity (Fig. 4C), albeit to a lesser degree than either the *MMTV* or *PSA* reporters. These findings suggest that the Q865H substitution may differentially affect DHT- and MPA-induced recruitment of the AR to target genes in MDA-MB-453 cells.

DHT- and MPA-induced gene expression profiles in MDA-MB-453 cells

Given the reduced potency of MPA compared with DHT in inducing the transactivation activity of the endogenous AR-Q865H variant on different reporter genes and endogenous genes in MDA-MB-453 cells, we performed genome-wide profiling to determine whether differences in DHT- and MPA-induced regulation of AR target genes could explain the divergent proliferative effects of these two ligands on

MDA-MB-453 cells. We have used 1 nM DHT and 100 nM MPA as these concentrations have previously been shown to be required for proliferative effects of the respective ligands on MDA-MB-453 cells (Birrell et al. 1995, Bentel et al. 1999). Furthermore, reporter gene assays (Fig. 4) and microarray analyses performed in this study (data not shown) indicate that 1 nM MPA is not sufficient to induce robust activation of AR in MDA-MB-453 cells. DHT and MPA significantly regulated 557 and 1807 genes > 1.2 -fold, respectively (adjusted $P < 0.05$), and 49 and 134 genes > 1.5 -fold, respectively, compared with vehicle-treated cells (Fig. 5A). There was considerable overlap between the DHT- and MPA-responsive gene profiles (Fig. 5B); however, genes upregulated by DHT showed significantly less overlap with MPA upregulated genes compared with DHT and MPA downregulated genes (contingency table analysis χ^2 test, $P < 0.0001$).

Genes uniquely regulated by DHT (but not regulated by MPA) or regulated by MPA (but not DHT), potentially contribute to the divergent proliferative effects of these ligands in MDA-MB-453 cells. GeneCodis was used to identify significantly enriched KEGG and GO pathways in uniquely DHT- and MPA-regulated gene sets (Supplementary Table 2, see section on supplementary data given at the end of this article). Whereas pathways involved in metabolism, transport, and signal transduction were enriched in the DHT-regulated gene list, MPA-regulated pathways included those associated with transcription (including p53 signaling), apoptosis, and cell cycle. Similar results (Supplementary Table 2) were observed with Ingenuity Pathway Analysis and GSEA. Collectively, these analyses highlight the potential of DHT and MPA to regulate distinct cellular pathways in MDA-MB-453 cells via the AR-Q865H variant, which may be responsible for the divergent proliferative effects of these two ligands.

Figure 6 Comparison of DHT- and MPA-regulated genes in MDA-MB-453 (black bars) and MFM-223 (gray bars) cells. Cells were treated with vehicle control (veh, 0.1% ethanol), DHT (1 nM), or MPA (100 nM) for 6 h. mRNA levels for target genes were measured by quantitative RT-PCR. (A) *C10RF116*, (B) *SLC15A2*, (C) *FKBP5*, (D) *RANBP3L*, (E) *SEC14L2*, (F) *DUSP10*, (G) *PCDH20*, and (H) *TMPRSS2*. Points represent the average $\pm$ S.E.M. of triplicate samples. * $P < 0.05$, one-way ANOVA, hormone vs vehicle control.

wtAR and the AR-Q865H variant differentially regulate endogenous gene expression

In order to further ascertain whether regulation of endogenous target genes by the AR-Q865H variant in MDA-MB-453 cells was compromised compared to wtAR, we compared the regulation of select candidate genes identified in MDA-MB-453 to that in another cell line, MFM-223, which expresses a wtAR (Magklara *et al.* 2002). MFM-223 cells are potentially another useful model of molecular apocrine breast cancer as they are AR(+) but do not express ER or PR and they cluster with MDA-MB-453 in transcriptome profiling analyses (Hackenberg *et al.* 1991, Lehmann

et al. 2011). Quantitative reverse transcription PCR analysis demonstrated that candidate genes, such as *C10RF116* (Fig. 6A) and *SLC15A2* (Fig. 6B), show similar regulation in MDA-MB-453 and MFM-223 cell lines, with both genes being more strongly regulated by DHT than MPA. Other genes showed qualitative and quantitative differences in regulation by DHT and MPA in the two cell lines. *FKBP5* (Fig. 6C) and *RANBP3L* (Fig. 6D) were more strongly induced by MPA than DHT in MDA-MB-453 cells but were induced to a comparable level by both ligands in MFM-223 cells. *SEC14L2* (Fig. 6E) was more strongly induced by DHT than MPA in MDA-MB-453 cells but

more equally induced by both ligands in MFM-223 cells. *DUSP10* (Fig. 6F) is specifically induced by DHT in MDA-MB-453 cells but is repressed by MPA in MFM-223 cells. *PCDH20* (Fig. 6G) was repressed only by MPA in MDA-MB-453 cells but induced by DHT in MFM-223 cells. *TMPRSS2* (Fig. 6H) is not regulated by DHT or MPA in MDA-MB-453 cells but is induced by both ligands in MFM-223 cells; similarly, *KLK3* (data not shown) is not expressed in MDA-MB-453 cells under any treatment conditions but is induced by both ligands in MFM-223 cells. Taken together, these results indicate that the AR-Q865H variant in MDA-MB-453 cells potentially regulates a different set of endogenous genes or differentially regulates certain AR target genes when compared with the wtAR in MFM-223 cells.

Association of DHT- and MPA-regulated genes in MDA-MB-453 cells with established growth regulatory pathways

The synthetic androgen methyltrienolone (R1881) stimulates MDA-MB-453 cell proliferation and induces a gene expression signature resembling that of estrogen-responsive ER(+) breast cancer cell lines, such as MCF-7 (Doane et al. 2006). This raised the possibility that androgen signaling stimulates MDA-MB-453 cell proliferation by activating genes associated with E₂-induced proliferation. To test this, we used GSEA to compare the gene profiles regulated by DHT and MPA in MDA-MB-453 cells with those regulated by E₂ in MCF-7 cells. Analysis of two independent E₂-regulated gene sets, namely Cicatiello (Fig. 7A) and GEMS (Fig. 7B) (Ochsner et al. 2009, Cicatiello et al. 2010), demonstrated that genes upregulated by DHT in MDA-MB-453 cells are significantly associated with those upregulated by E₂ in MCF-7 breast cancer cells (Table 1). In contrast, genes regulated by MPA in MDA-MB-453 cells were not associated with genes activated by E₂ (Table 1). In light of a recent study demonstrating that androgens can also modulate the Wnt signaling pathway to induce MDA-MB-453 cell proliferation (Ni et al. 2011), we used GSEA to also investigate the association between DHT- and MPA-regulated genes from our microarray study with the Wnt signaling pathway. Genes in the Wnt signaling pathway were significantly enriched in the DHT-regulated transcriptome but not in the MPA-regulated transcriptome (Fig. 7C and Table 1). These results suggest that the ability of the AR-Q865H variant to regulate pathways associated with proliferation in molecular apocrine breast cancer is dependent on the nature of the bound ligand.

Figure 7 Association of DHT- and MPA-regulated genes in MDA-MB-453 with E₂-regulated genes in MCF-7 breast cancer cells and genes in the Wnt signaling pathway as assessed by GSEA. The change in expression of each gene in MDA-MB-453 in response to DHT treatment is shown as a heatmap (top panel). Probe sets in the datasets were collapsed to gene level, assigned a score based on a signal-to-noise ratio algorithm, and rank-ordered by this score. E₂ upregulated genes from (A) the Cicatiello dataset or (B) the GEMS dataset, in the ordered MDA-MB-453 dataset are shown as black lines (middle panel), and the running enrichment score (ES) is plotted (bottom panel). The maximum ES and *P* value are indicated. (C) GSEA results comparing DHT-regulated genes in MDA-MB-453 with genes in the KEGG Wnt signaling pathway.

Discussion

Given that ER(−), PR(−) breast cancers are not treated with standard hormonal therapies, and there are limited alternative treatment options, there is an urgent requirement to characterize the mechanisms regulating their growth and metastasis. Of particular interest is to identify alternative hormone-mediated mechanisms regulating the growth of ER(−), PR(−) breast tumors

Table 1 GSEA results, enrichment score (ES), and *P* values for DHT- and MPA-regulated genes in MDA-MB-453 cells with genes in the Cicatiello, GEMS, and KEGG Wnt signaling pathway datasets

Dataset	Cicatiello MCF-7 E ₂ (UP)		GEMS MCF-7 E ₂ (UP)		KEGG Wnt signaling	
	ES	<i>P</i> value	ES	<i>P</i> value	ES	<i>P</i> value
DHT	0.482	<0.001	0.520	<0.001	0.249	<0.001
MPA	0.394	0.121	0.435	0.124	0.215	0.514

as this may afford options for immediate therapeutic intervention. Recent studies using the MDA-MB-453 breast cancer cell line have suggested that AR signaling may, at least in part, drive the growth of ER(–) breast cancers with a molecular apocrine phenotype. Thus, the AR has become an attractive therapeutic target for this breast cancer subtype. In the current study, we examined AR structure and function in MDA-MB-453 cells and identified a mutation in the *AR* gene. The resulting AR-Q865H variant has compromised activity in response to DHT and MPA but does not confer responsiveness to non-androgenic ligands or AR antagonists. These findings should be taken into account when using the MDA-MB-453 cell line as a model of molecular apocrine breast cancer.

Several other breast cancer cell lines, including HCC202, SUM185PE, and CAL-148, have now been shown to have a steroid receptor profile resembling that of MDA-MB-453 cells and their proliferation is stimulated by androgen and/or inhibited by the AR antagonist bicalutamide (Lehmann *et al.* 2011, Naderi *et al.* 2011, Ni *et al.* 2011). Conversely, proliferation of the MFM-223 breast cancer cell line, which clusters with MDA-MB-453 in transcriptome profiling analyses (Lehmann *et al.* 2011) and expresses wtAR, is inhibited by DHT and MPA (Hackenberg *et al.* 1991, 1993, Magklara *et al.* 2002). Two additional breast cancer cell lines, HCC1937 and HCC1954, are also ER(–), PR(–), and AR(+); although the proliferative response of these cell lines to DHT is yet to be reported, they are inhibited by the adrenal androgen dehydroepiandrosterone sulfate (Gazdar *et al.* 1998, Tomlinson *et al.* 1998, Hardin *et al.* 2007, Naderi & Liu 2010). Collectively, these studies indicate that not all ER(–), PR(–), AR(+) cell types are growth stimulated by AR signaling, which calls into a question about the suitability of therapies that inhibit AR activity in molecular apocrine breast cancer.

Identification of an *AR* gene variant giving rise to a functionally distinct AR in MDA-MB-453 cells raises the question of whether this or other *AR* mutations are important in the pathology of molecular apocrine breast cancers. In contrast to the stimulatory effects on ER(–), PR(–), AR(+) MDA-MB-453 cells,

androgen signaling has a predominantly growth inhibitory effect on ER(+), PR(+), AR(+) breast cancer cell lines (Poulin *et al.* 1988, Birrell *et al.* 1995, Ando *et al.* 2002, Ortmann *et al.* 2002, Cops *et al.* 2008). Those findings are consistent with the clinical utility of androgenic agents as breast cancer therapies before the advent of tamoxifen (Fels 1944, The Cooperative Breast Cancer Group 1961), the favorable prognosis associated with *AR* expression in ER(+) breast cancers (Gonzalez-Angulo *et al.* 2009, Peters *et al.* 2009, Castellano *et al.* 2010), and the ability of AR to inhibit ER activity (Peters *et al.* 2009). This disparity in the function of AR in different subtypes of breast cancer cells is considered to arise due to the presence or absence of *ER* expression, and the possibility of AR assuming a stimulatory, ER-like role in ER(–) cells (Robinson *et al.* 2011). However, our data indicate that, in addition to ER status, AR structure and function may determine the response to androgen in breast cancer cells. Moreover, different AR-mediated proliferative responses can be elicited in the same cell line depending on the ligand and the structural integrity of the receptor. Additional studies are warranted to determine whether AR variants are present in molecular apocrine breast cancer and should be considered in developing new therapeutic strategies targeting the AR.

Our current studies demonstrated that DHT and MPA regulate distinct but overlapping sets of target genes in MDA-MB-453 breast cancer cells and that endogenous genes regulated via the AR-Q865H variant are distinct from those regulated by the wtAR. This may, at least in part, be explained by the differential ability of DHT and MPA to induce an N/C interaction in the AR-Q865H variant and subsequent structural differences that likely affect ligand-dependent DNA response element binding, interaction with coregulator molecules, and possibly engagement with pioneering factors such as FOXA1 and/or the transcriptional machinery. An alternative explanation for the differences in DHT- and MPA-regulated gene expression profiles in MDA-MB-453 cells is that MPA may be binding to and activating GR at the concentration used in the current experiments (Moore

et al. 2012). However, western blot analysis of GR and quantitative PCR of GR-regulated genes (data not shown) indicated that GR is unlikely to be mediating the transcriptional responses observed in this study. Although there are a number of genes commonly regulated by DHT and MPA in MDA-MB-453 cells, the distinct gene expression profiles and pathways induced by each ligand likely contribute to their dichotomous proliferative effects. For example, the DHT-regulated gene expression profile in ER(–) MDA-MB-453 cells resembled that of estrogen signaling in the E₂-stimulated and ER(+) MCF-7 breast cancer cell line, highlighting the potential of AR to act as a surrogate ER (Robinson *et al.* 2011). We also showed that DHT modulates the Wnt signaling pathway, which has recently been shown to contribute to the proliferative effects of androgen in MDA-MB-453 cells (Ni *et al.* 2011). While these analyses have provided insight into the mechanisms by which androgens and the AR promote proliferation of MDA-MB-453 cells, AR activity appears to be dependent on the nature of the ligand bound, as MPA (unlike DHT) did not induce an ER-like gene signature or genes associated with Wnt signaling. Rather, in the presence of MPA, AR activity is redirected to regulate alternative target genes and pathways that lead to antiproliferative effects in MDA-MB-453 cells.

In summary, we have identified a mutation in the AR gene in MDA-MB-453 cells that elicits altered receptor activity relative to the wtAR. While the AR variant present in MDA-MB-453 cells retains transcriptional responsiveness to DHT and MPA, the transcriptomes regulated by these ligands are distinct, which likely contributes to their divergent proliferative effects in this cell line. As the AR is considered a critical driver of molecular apocrine breast cancer, our finding of an AR variant with altered activity compared with wtAR in the widely used MDA-MB-453 cell line model makes it important to determine in future studies whether i) molecular apocrine cancers express AR variants and ii) the MDA-MB-453 cell line is a suitable model for this subtype of breast cancer. Additionally, characterization of alternative models of molecular apocrine breast cancer, including those with a wtAR, may lead to a better understanding of AR signaling in this disease. Such studies will facilitate the rational development of effective treatment strategies for molecular apocrine breast cancer.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-12-0065>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

N L Moore and W D Tilley designed the study. N L Moore, G Buchanan, J M Harris, L A Selth, and A R Hanson performed and contributed to analysis of the experiments. N L Moore prepared the manuscript with contributions from L M Butler, T E Hickey, and W D Tilley while G Buchanan, J M Harris, L A Selth, T Bianco-Miotto, S N Birrell, L M Butler, T E Hickey, and W D Tilley contributed to experimental design, manuscript editing, and review.

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