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PRECLINICAL STUDY

Identifying molecular features that distinguish fluvastatin-sensitive breast tumor cells

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Abstract Statins, routinely used to treat hypercholesterolemia, selectively induce apoptosis in some tumor cells by inhibiting the mevalonate pathway. Recent clinical studies suggest that a subset of breast tumors is particularly susceptible to lipophilic statins, such as fluvastatin. To quickly advance statins as effective anticancer agents for breast cancer treatment, it is critical to identify the molecular features defining this sensitive subset. We have therefore characterized fluvastatin sensitivity by MTT assay in a panel of 19 breast cell lines that reflect the molecular diversity of breast cancer, and have evaluated the association of sensitivity with several clinicopathological and molecular features. A wide range of fluvastatin sensitivity was observed across breast tumor cell lines, with fluvastatin triggering cell death in a subset of sensitive cell lines. Fluvastatin sensitivity was associated with an estrogen receptor alpha (ERα)-negative, basal-like tumor subtype, features that can be scored with routine and/or strong preclinical diagnostics. To ascertain additional candidate sensitivity-associated molecular features, we mined publicly available gene expression datasets, identifying genes encoding regulators of mevalonate production, non-sterol lipid homeostasis, and global cellular metabolism, including the oncogene *MYC*. Further exploration of this data allowed us to generate a 10-gene mRNA abundance signature predictive of fluvastatin sensitivity, which showed preliminary validation in an independent set of breast tumor cell lines. Here, we have therefore identified several candidate predictors of sensitivity to fluvastatin treatment in breast cancer, which warrant further preclinical and clinical evaluation.

Keywords Statin · Fluvastatin · Breast cancer · Estrogen receptor · Gene expression · Drug sensitivity

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Introduction

Breast cancer encompasses several tumor subtypes that differ in their molecular characteristics, therapeutic response, and prognosis. Clinical evaluation of anticancer agents may therefore benefit from subtype stratification in breast cancer [1, 2], and preclinical identification of markers predictive of therapeutic sensitivity can have major implications for clinical trial design [3, 4]. Statins have been prescribed for decades for the management of

hyperlipidemia [5], but also appear to have pleiotropic anticancer effects in breast cancer [6, 7]. These drugs inhibit the rate-limiting enzyme of the mevalonate pathway, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) [8, 9], which is normally tightly regulated at several levels, including feedback responses that upregulate HMGCR upon mevalonate depletion [9, 10]. The antiproliferative responses of tumor cells to statin treatment have been hypothesized to reflect dysregulation of the mevalonate pathway in cancer [6, 7]. Given that statins are FDA approved, well tolerated, and affordable, they present an opportunity for accelerated repositioning as therapeutics for the treatment of breast cancer.

Prospective clinical evaluations of the use of statins in breast cancer treatment are in their infancy, but show promising signs of therapeutic activity. For example, perioperative statin use may decrease proliferation in a subset of breast tumors [11, 12]. Preclinically, statin sensitivity has been proposed to be associated with activated NFκB levels [13], lack of expression of ERα [13, 14], and mutation of *TP53* [15], yet these studies have examined only two to three breast cell lines. Given the appreciable molecular and clinical heterogeneity of breast cancer [16–20], a comprehensive analysis of molecular features associated with statin sensitivity clearly remains necessary in this tumor type. Here, we have characterized fluvastatin sensitivity in 25 breast cell lines and evaluated whether sensitivity is associated with specific molecular features or known tumor subtypes. Fluvastatin sensitivity was moderately associated with lower HMGCR expression, and more strongly with an ERα-negative status, basal-like subtype, and a 10-gene fluvastatin sensitivity signature. These features may immediately inform selection of preclinical models for the study of statin treatment in breast cancer, and may be further evaluated as predictive markers of statin sensitivity in clinical trials.

Materials and methods

Materials

Cell culture media was prepared at the Ontario Cancer Institute Tissue Culture Media Facility (Toronto, ON, Canada). Fetal bovine serum (FBS) was obtained from Hyclone or Gibco. Horse serum and trypsin–EDTA were obtained from Gibco. Human epidermal growth factor and Matrigel were acquired from R&D Systems and BD Bioscience, respectively. RNAse was purchased from Roche. Fluvastatin was purchased from United States Biological and (*E/Z*)-4-hydroxytamoxifen from Sigma; both were dissolved as 10 mM stocks in ethanol. Mevalonate was obtained from Sigma and dissolved as a 1 M stock in PBS. Unless otherwise specified, all other reagents were obtained from Sigma.

Cell culture

All cell lines represented in the ATCC repository were authenticated by short-tandem repeat (STR) profiling at The Centre for Applied Genomics (Toronto, ON, Canada). SUM149PT and SUM159PT lines were obtained within two passages from original receipt from Asterand. MCF7 cells were provided by Dr. Amadeo Parissenti (Health Sciences North, Sudbury, ON, Canada). T47D cells were from ATCC and cultured in RPMI 1640 (10 % FBS, 0.01 mg/mL insulin). BT20, HS578T, MDAMB436, MDAMB468, SUM149PT, and SUM159PT cells were provided by Dr. Benjamin Neel (Ontario Cancer Institute, Toronto, ON, Canada). BT20 cells were cultured in RPMI 1640 (10 % FBS) and MDAMB436 and MDAMB468 cells were grown in DMEM H21 (10 % FBS). All other cell lines were the kind gift of Dr. Mona Gauthier (Campbell Family Institute for Breast Cancer Research, Toronto, ON, Canada). Unless otherwise specified, cells were cultured as previously described [16].

MTT assays

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed essentially as previously described [21]. 750–15,000 cells/well were seeded in 96-well plates and treated in triplicate with 0–200 or 400 μM fluvastatin and ethanol vehicle control for 72 h. MTT_{50} values, the concentration at which MTT reduction activity in the cell population was decreased by 50 %, were computed using GraphPad Prism (v5.0). In the MDAMB415, HCC202 and CAMA1 cell lines, where 200 μM was consistently unable to suppress MTT reduction by more than 50 %, MTT_{50} values were ceiled at 200 μM for correlative analyses.

Three-dimensional cell culture

3,000–5,000 cells/well of a 96-well plate were seeded in triplicate on 30 μL of Matrigel, suspended in media containing 2.5 % Matrigel, as per standard protocols [22]. After 4 days, cells were treated with 10 μM fluvastatin or ethanol vehicle control for 72 h. Bright-field images were acquired on a Zeiss AxioObserver microscope (5× objective).

Cell death assays

250,000 cells were seeded in 10 cm plates overnight, then treated with ethanol vehicle control, 10 μM fluvastatin, 200 μM mevalonate and/or 10 μM (*E/Z*)-4-hydroxytamoxifen,

as indicated in figure legends. Fixed propidium iodide (PI) and terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) assays were performed as previously described [23]. Events with sub-diploid ("Pre-G1") DNA content or TUNEL-positive staining were considered dead or apoptotic cells, respectively.

Quantitative real-time RT-PCR

Subconfluent cells were harvested and RNA was extracted using 0.5–1 mL TRIZOL reagent. cDNA was synthesized using SuperScript III (Invitrogen) and real-time quantitative RT-PCR was performed using primers to amplify the full-length splice variant of *HMGCR* ("*HMGCR-FL*"), the splice variant lacking exon 13 ("*HMGCR-D13*"), and *GAPDH* as previously described [24]. *HMGCR* transcript levels were determined relative to *GAPDH*, and fluvastatin-induced expression was determined relative to ethanol vehicle control levels.

Immunoblotting

Lysates were prepared from subconfluent cells. For detection of HMGCR, lysates were prepared and analyzed by SDS-PAGE and immunoblotting, as described [24]. For detection of ERα, cells were lysed directly in boiling SDS lysis buffer (1 % SDS, 11 % glycerol, 10 % β-mercaptoethanol, 0.1 M Tris pH 6.8) and boiled prior to SDS-PAGE. Immunoblots were probed overnight with primary anti-HMGCR A9 antibody (1:500; prepared from CRL-1811 hybridoma, ATCC) or anti-ERα (1:1000; Santa Cruz Biotechnology Inc.), and anti-actin (1:3,000; Sigma). Primary antibodies were detected with IRDye-labeled secondary antibodies (1:20,000; LI-COR Biosciences).

Data mining for fluvastatin sensitivity-associated gene expression

Publicly available microarray data for 51 breast cell lines [16] were preprocessed as described in supplementary

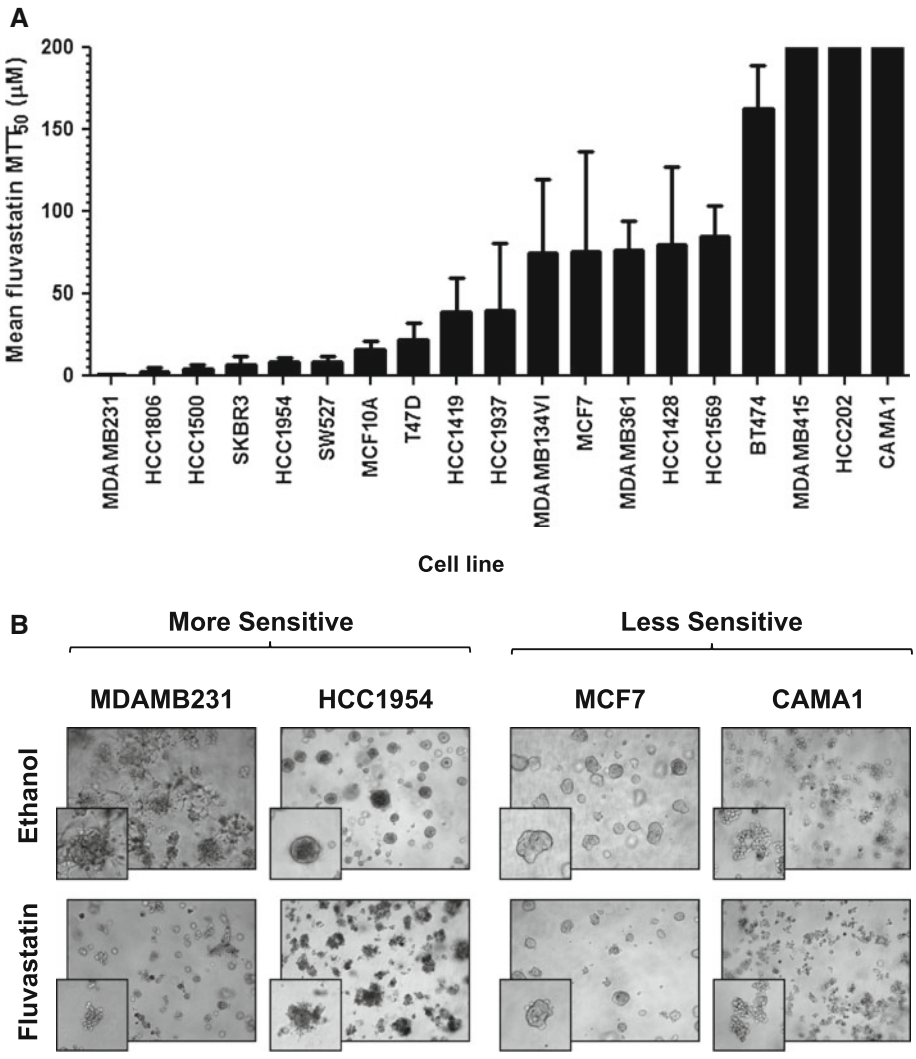


Fig. 1 Breast tumor cell lines display heterogeneous sensitivity to fluvastatin. **a** Fluvastatin sensitivity was assessed in 2-D monolayer culture conditions by MTT assay, indicated by the half-maximal MTT reduction concentration at 72 h (MTT_{50}). *Bars* represent mean MTT_{50} values of three to six independent experiments, with *error bars* indicating standard deviation. The MTT_{50} of non-responsive cell lines was ceiled at 200 μM, as described in the "Materials and Methods" section. Raw MTT_{50} data are found in supplementary Table S2. **b** Representative fluvastatin-sensitive and less sensitive cell lines were grown for four days in 3-D culture conditions on Matrigel, treated with 10 μM fluvastatin or ethanol vehicle control for 72 h, and imaged to assess qualitative changes in acinar morphology. Main *images* are shown at ×5 magnification, with *insets* displaying representative acini at ×10 magnification. *Images* are representative of three independent experiments

Methods. For each gene, we assessed the Pearson correlation between normalized intensity and mean fluvastatin MTT_{50}. A false discovery rate (FDR)-adjusted p value threshold of 0.25 was used to identify genes whose mRNA abundance was significantly associated with fluvastatin sensitivity. Gene Ontology (GO) term enrichment analyses were performed as described in supplementary methods.

Generation and validation of a candidate fluvastatin sensitivity signature

In the set of 16 cell lines with publicly available gene expression data described above, cell lines were defined as fluvastatin-sensitive if their mean $MTT_{50} \leq 20$ μM, and insensitive otherwise. To decrease possible confounding effects of cell lines whose sensitivity is close to this threshold, we excluded cell lines where 10 μM ≤ mean $MTT_{50} \leq 30$ μM. A k nearest neighbors (KNN) model, as implemented in the class package (v7.3-3) of R (v2.14.2), was used to generate a predictive gene signature. We used leave-one-out cross-validation (LOOCV) to determine the optimal gene signature size by nesting a validation loop within a loop that considered the top n genes for several candidate values of n (i.e., $n = 3$, 5, 10, 20, 30, 40, 50, 75, or 100). Genes were selected according to the statistical significance of their correlation with fluvastatin MTT_{50}. This process led to the selection of an optimal 10-gene signature size; all 14 cell lines were then used to train the 10-gene signature.

Statistical analyses

Differences between two treatment groups were assessed using two-tailed unpaired heteroscedastic t tests. Fluvastatin-induced HMGCR expression was assessed using one-sample t tests. Associations of fluvastatin MTT_{50} values with continuous or dichotomous variables were evaluated by either Pearson or point-biserial correlations as appropriate.

Cell proliferation assays, measurement of cholesterol and lipids, and verification of mRNA abundance correlations with NanoString technology are described in supplementary methods.

Results

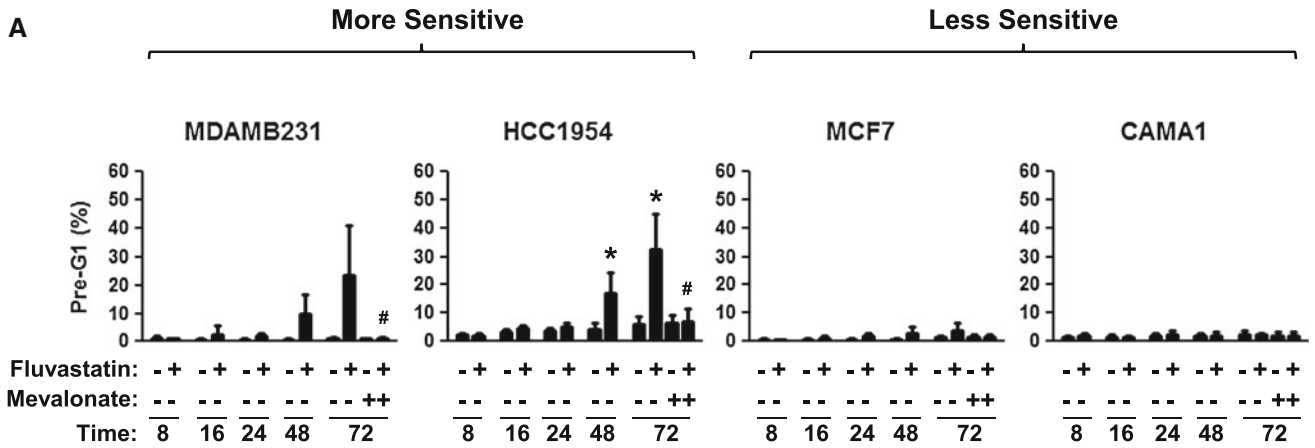
We characterized the anticancer effects of fluvastatin in 19 immortalized breast cell lines, most of which have been extensively characterized [16, 25–27] and display molecular features consistent with a variety of histological and molecular subtypes of breast cancer. Fluvastatin was chosen for study due to its promise in both preclinical and clinical settings [11, 13] and its favorable pharmacokinetic properties for anticancer use [23, 28].

Fig. 2 Fluvastatin induces cell death in sensitive cell lines through inhibition of the mevalonate pathway. **a** Fixed propidium iodide (PI) assays detected a time-dependent induction of cell death in relatively sensitive cell lines upon treatment with 10 μM fluvastatin, assessed as events with Pre-G1 DNA content detected by flow cytometry. At the 72 h time-point, cells were co-treated with 200 μM exogenous mevalonate. *Bars* represent means of three to four independent experiments, with *error bars* indicating standard deviation. *Asterisks* indicate a statistically significant difference ($p < 0.05$) between ethanol vehicle- and fluvastatin-treated samples at each time-point using two-sample unpaired heteroscedastic t tests. *Hatch marks* indicate no significant difference ($p > 0.05$) between fluvastatin and mevalonate co-treatments and vehicle treatment at 72 h, by t test as described above. **b**, **c** The proportion of cells undergoing apoptosis following treatment with 10 μM fluvastatin or ethanol vehicle control for 72 h was determined by TUNEL staining and flow cytometric detection. Representative TUNEL versus PI *dotplots* are shown for a subset of relatively more and less sensitive cell lines in **b**, with representative *dotplots* from additional cell lines included in supplementary Fig. S2. *Numbers highlighted* in the upper right corners of each *dotplot* represent the number of events detected in the TUNEL-positive gate shown. Experiments were repeated three times. Results are compiled in **c**, where *bars* represent means of three independent experiments, with *error bars* indicating standard deviation. *Asterisks* indicate significant differences ($p < 0.05$) detected by two-sample unpaired heteroscedastic t tests comparing fluvastatin to ethanol vehicle treatments

Breast tumor cell lines display heterogeneous sensitivity to fluvastatin

We defined relative fluvastatin sensitivity under two-dimensional (2-D) culture conditions by the MTT assay, using the MTT_{50} at 72 h as an indicator of chemosensitivity. A wide-ranging continuum of sensitivity was observed (Fig. 1a, supplementary Table S1; raw MTT_{50} data in supplementary Table S2). To consider whether the heterogeneity of fluvastatin sensitivity in these cell lines reflected generalized chemoresistance, fluvastatin MTT_{50} values were correlated with published half-maximal growth inhibitory concentrations (GI_{50}) of 74 anticancer drugs [27]. No drugs were identified for which the panel of cell lines exhibited statistically similar sensitivity (data not shown), nor was sensitivity solely associated with proliferative rate (supplementary Fig. S1).

We next examined whether relative fluvastatin sensitivity defined under 2-D growth conditions was preserved under three-dimensional (3-D) culture conditions. A representative subset of two fluvastatin-sensitive and two less sensitive cell lines were grown on Matrigel and treated with 10 μM fluvastatin, a dose predicted to be clinically achievable in plasma, extrapolating from levels reported following a high but well-tolerated dose of lovastatin [29] (Fig. 1b). Changes in acinar morphology were observed in the fluvastatin-sensitive MDAMB231 and HCC1954 cell


A
More Sensitive
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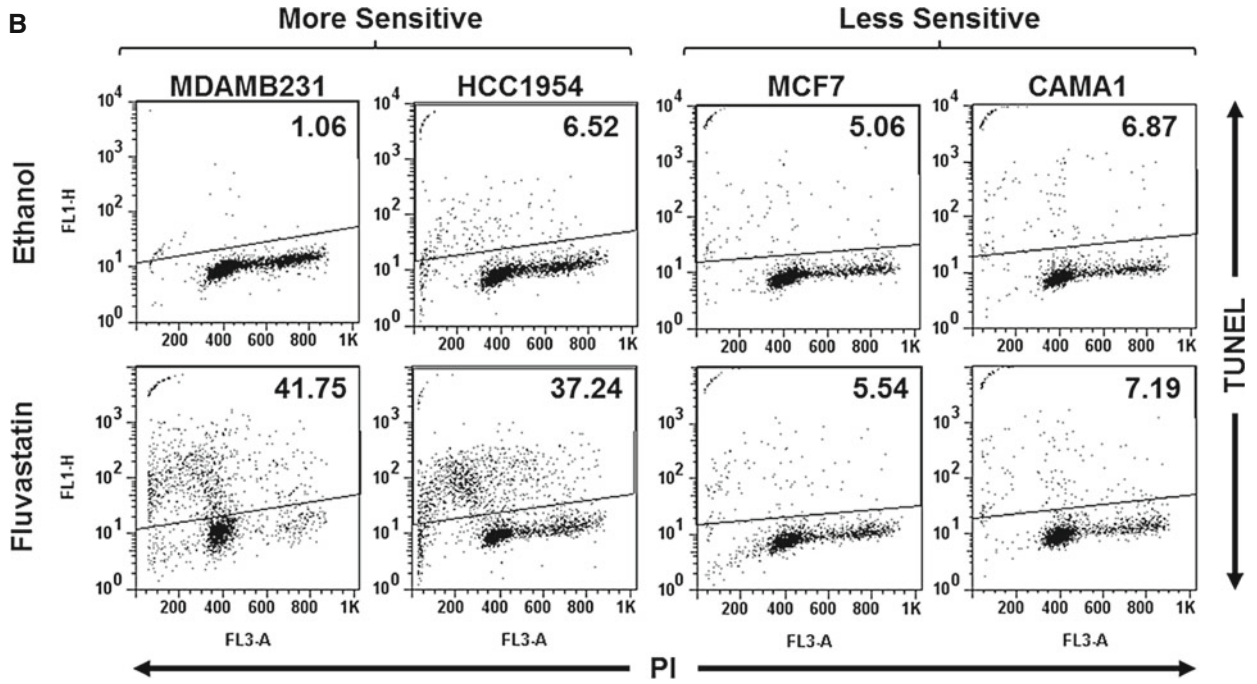
B
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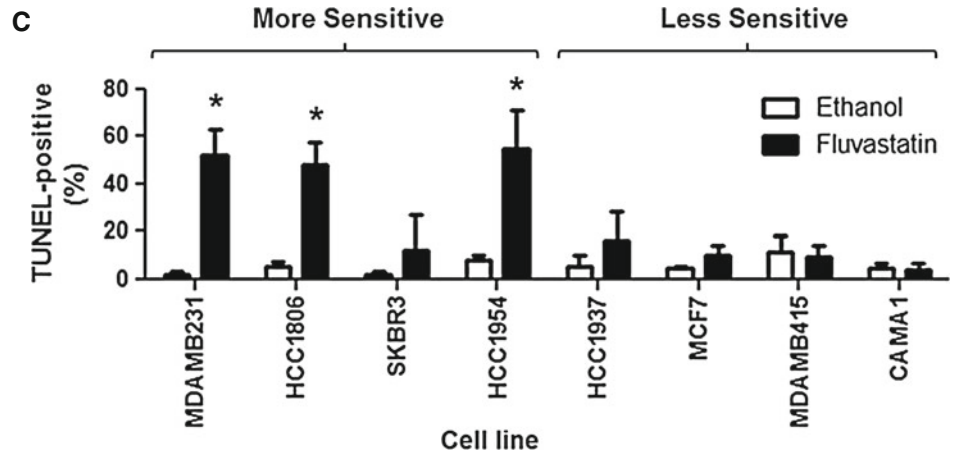
C
More Sensitive
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Ethanol
Fluvastatin
MDAMB231
HCC1806
SKBR3
HCC1954
HCC1937
MCF7
MDAMB415
CAMA1
Cell line

lines upon fluvastatin exposure, but not in the less sensitive MCF7 and CAMA1 cell lines. Thus, the relative fluvastatin sensitivity of these cell lines appears consistent between 2-D and 3-D conditions.

Fluvastatin induces cell death in sensitive cell lines

To assess fluvastatin-induced cell death, we performed fixed PI assays (Fig. 2a). Fluvastatin induced time-dependent cell death in the sensitive MDAMB231 and HCC1954 cell lines, which was inhibited by co-treatment with exogenous mevalonate. Fluvastatin did not induce cell death in the less sensitive MCF7 and CAMA1 cell lines. These results were confirmed using TUNEL as an independent cell death assay (Fig. 2b) and in additional cell lines (supplementary Fig. S2). The lack of TUNEL positivity observed in the SKBR3 cell line suggests that other non-apoptotic antiproliferative mechanisms may contribute to its relatively low MTT_{50}.

Baseline expression of HMGCR is moderately associated with fluvastatin sensitivity

We hypothesize that dysregulated expression of mevalonate pathway genes may contribute to tumorigenesis and/or statin sensitivity [6, 15, 24, 30]. Two alternatively spliced isoforms of HMGCR have been detected in cancer cells [24, 30]—HMGCR-FL and HMGCR-D13. We observed a moderate correlation between fluvastatin MTT_{50} and baseline (i.e., untreated) mRNA levels of *HMGCR-FL* ($r = 0.43$, $p = 0.07$) and *HMGCR-D13* ($r = 0.45$, $p = 0.05$; Fig. 3a). While total levels of HMGCR protein detected by immunoblot were variable across the panel of cell lines, most of the cell lines expressing the highest levels of HMGCR protein were among the least fluvastatin-sensitive (Fig. 3b). We found no evidence that either statin-induced upregulation of HMGCR or baseline cholesterol/lipid pools differed with fluvastatin sensitivity (supplementary Fig. S3).

Data mining identifies additional candidate fluvastatin sensitivity-associated genes

Sixteen of the cell lines profiled for fluvastatin sensitivity also have available baseline mRNA abundance profiles generated by Neve et al. [16], allowing us to identify additional genes whose mRNA abundances correlated with fluvastatin sensitivity. We identified 1,167 genes significantly correlated with fluvastatin sensitivity ($Q \leq 0.25$; supplementary Table S3), of which 62.2 % (725 genes) was positively correlated with MTT_{50}. These included several genes encoding proteins associated with the mevalonate or sterol pathways (supplementary Table S4). These genes were significantly enriched for those annotated with 260 GO terms ($p < 0.05$; supplementary Table S5), including cellular metabolism, ribosome biogenesis, cell division, and mitosis. Notably, the v-myc avian myelocytomatosis viral oncogene homolog (MYC) oncoprotein can contribute to several of these metabolic processes [31, 32] and is commonly deregulated in breast cancer [33]. Expression of *MYC* was strongly associated with fluvastatin MTT_{50} ($r = -0.724$, $q = 0.16$), expressed at higher levels in more sensitive cell lines. Conversely, the opposite relationship was observed for two genes encoding negative regulators of the myc-associated factor X (MAX)-dependent functions of MYC [32] (MAX dimerization protein 3, *MXD3*: $r = 0.756$, $q = 0.14$; MAX dimerization protein 4, *MXD4*: $r = 0.560$, $q = 0.24$).

To provide confidence in the publicly available microarray data, we compared it to independent measurements of mRNA abundance in the cell lines we evaluated for fluvastatin sensitivity. We selected 25 genes with a range of predicted associations to fluvastatin sensitivity and profiled their mRNA abundance in our breast cell lines using NanoString technology [34]. Hierarchical clustering demonstrated that all but one set of biological duplicate samples clustered closely together (supplementary Fig. S4a). Moderate or strong correlations between mRNA levels assessed by the two platforms support the generalizability of the public expression data to those observed in our cell line panel (supplementary Table S6). Furthermore, the relationship of mRNA abundance with fluvastatin MTT_{50} remained similar whether mRNA levels were assessed by microarray or by NanoString (supplementary Fig. S4b; supplementary Table S6).

ERα-negative and basal-like breast tumor subtypes are associated with fluvastatin sensitivity

To identify fluvastatin sensitivity-associated features with routine and/or strong preclinical diagnostics, we correlated fluvastatin MTT_{50} with known clinicopathological and molecular features of our cell lines [16, 27] (Fig. 4a; supplementary Table S7). Published ERα status demonstrated a trend toward correlation with fluvastatin sensitivity, with cell lines reported to be ERα-negative having lower fluvastatin MTT_{50} values ($r_{pb} = 0.464$, $p = 0.07$). Assessment of ERα status in all 19 cell lines in our panel by immunoblot identified one line—HCC1500—for which discordance existed between the published report [16] and our immunoblots (Fig. 4a), but the overall trend was maintained ($r_{pb} = 0.377$, $p = 0.11$).

Both primary breast tumors and cell lines can be classified into intrinsic molecular subtypes [16, 18–20, 27]. Most of the cell lines in our panel represent either luminal or basal-like subtypes [16, 27] and fluvastatin sensitivity

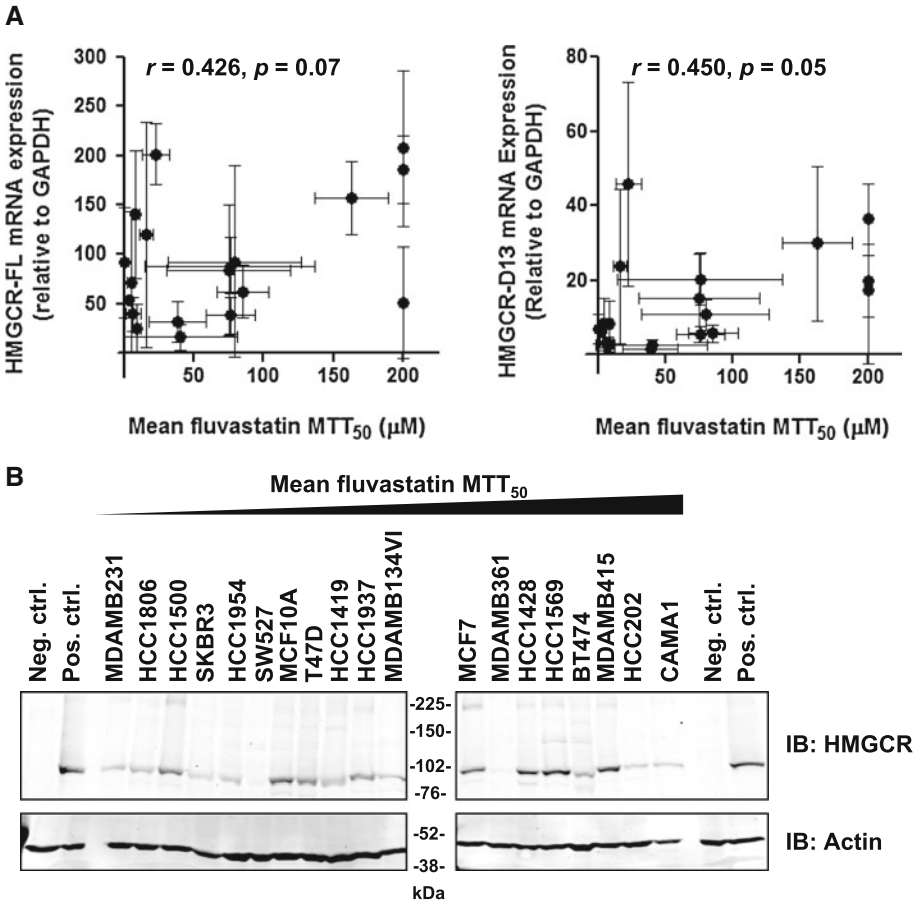

Fig. 3 Expression of HMGCR shows a trend toward moderate correlation with fluvastatin sensitivity. **a** mRNA expression of either *HMGCR-FL* or *HMGCR-D13* splice variants was assessed in the panel of 19 cell lines by RT-PCR, relative to *GAPDH* levels. Mean expression levels from three to five independent experiments were plotted against mean fluvastatin MTT_{50}, and a Pearson correlation coefficient, *r*, was computed. *Error bars* indicate standard deviation. **b** Protein levels of HMGCR were assessed in the panel of 19 cell lines by SDS-PAGE and immunoblotting with the A9 monoclonal antibody, with actin as a loading control. Identical negative and positive HMGCR control lysates were loaded on each gel. The negative control ("Neg. ctrl") lysate was from the HMGCR-deficient UT2 cell line ectopically expressing a control vector, and the positive control lysate was from the UT2 cell line ectopically expressing HMGCR-FL ("Pos. ctrl."; Goard and Penn unpublished data). *Immunoblots* shown are representative of three independent experiments

was significantly correlated with these published subtypes, with basal-like cell lines demonstrating significantly lower fluvastatin MTT_{50} values than cell lines of the luminal subtype ($r_{pb} = 0.563$, $p = 0.02$) (Fig. 4a; supplementary Table S7). ERα-negativity and basal-like molecular subtype are highly correlated [16, 18–20, 27]. There may remain, however, a subset of ERα-positive tumors that are also fluvastatin-sensitive. Since these tumors would likely be treated with endocrine therapy, we sought to examine the effect of concomitantly antagonizing ERα with the selective estrogen receptor modulator (SERM) 4-hydroxytamoxifen and treating with fluvastatin (Fig. 4b). More cell death was induced by fluvastatin in two ERα-negative cell lines than in two ERα-positive cell lines, and the reverse was true upon 4-hydroxytamoxifen treatment. In ERα-negative cell lines, 4-hydroxytamoxifen did not potentiate fluvastatin-induced cell death; in ERα-positive cell lines, co-treatment with fluvastatin and 4-hydroxytamoxifen led to higher levels of cell death than with treatments of either drug alone.

A novel candidate gene expression signature predicts fluvastatin sensitivity

While an ERα-negative status or basal subtype may allow selection of breast tumor cell lines enriched for those that are responsive to fluvastatin, it is clear from Fig. 4a that using these features as predictors would not lead to completely accurate classification. We therefore evaluated whether existing mRNA abundance data could be used to generate a signature to identify fluvastatin-sensitive breast tumor cells. We dichotomized fluvastatin sensitivity of breast cell lines into two classes—fluvastatin-sensitive if their mean $MTT_{50} \leq 20$ μM, and fluvastatin-insensitive

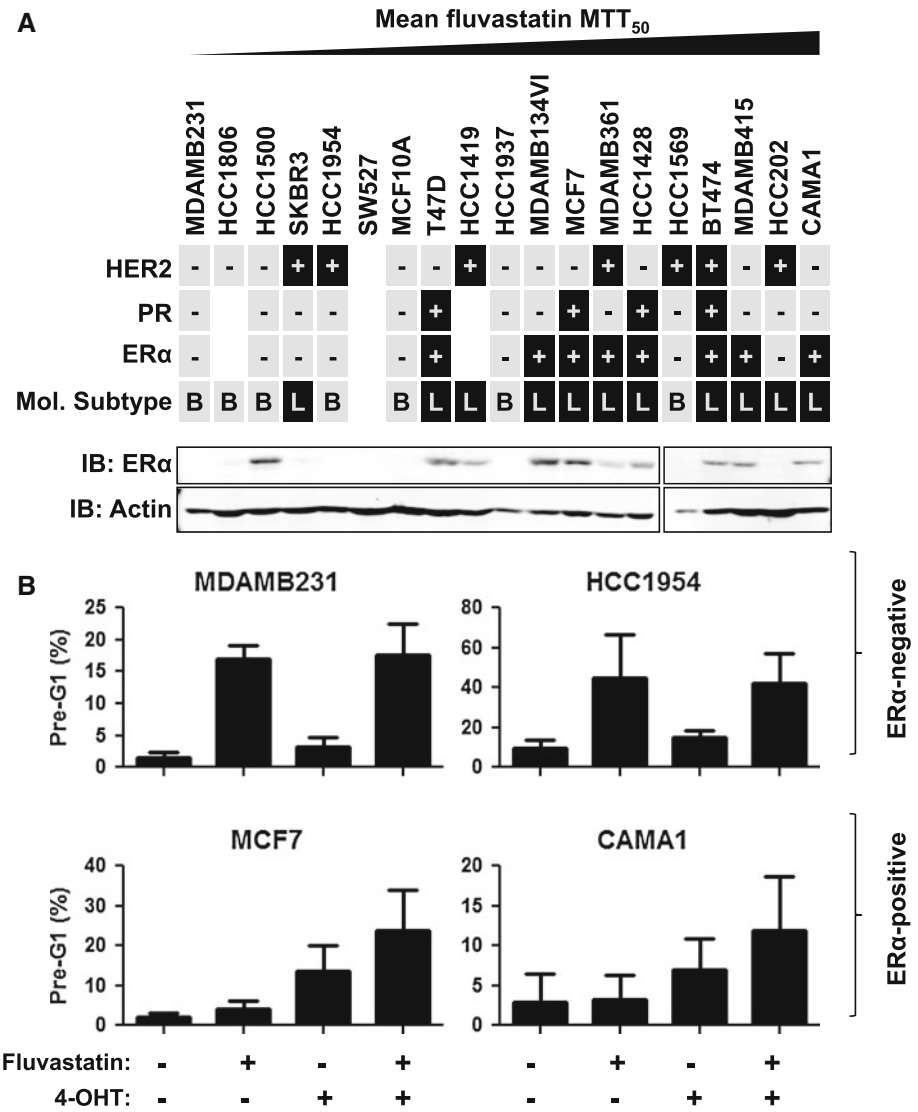

Fig. 4 Fluvastatin sensitivity is associated with an ERα-negative, basal-like subtype in breast tumor cell lines. **a** Published molecular features of 19 cell lines with differing fluvastatin sensitivity are depicted (*upper panel*), including HER2, PR, and ERα status (negative, "-", or positive, "+") and molecular subtype ("Mol. Subtype"; basal-like, "B", or luminal, "L") [16, 27]. ERα expression status was confirmed by SDS-PAGE and immunoblotting, using actin as a loading control (*lower panel*). *Immunoblots* are representative of three independent experiments. **b** Fluvastatin does not interfere with ERα antagonism in cell culture. ERα-negative and ERα-positive cell lines were treated for 72 h with ethanol vehicle control, 10 μM fluvastatin, 10 μM 4-hydroxytamoxifen ("4-OHT"), or both combined. Cell death was assessed by fixed propidium iodide (PI) staining and detection of the fraction of cells with Pre-G1 DNA content by flow cytometry. *Bars* represent means of five to seven independent experiments, with *error bars* indicating standard deviation. No significant difference from additivity was observed upon combination treatment, determined by general linear modeling (data not shown)

otherwise. By this definition, seven cell lines were deemed fluvastatin-sensitive, and 12 cell lines were fluvastatin-insensitive (supplementary Table S1). To reduce noise in the training data set, we focused on cell lines with unambiguous classifications (i.e., 10 μM $\leq$ MTT_{50} $\leq$ 30 μM). Fourteen cell lines remained that had available mRNA abundance data, which were used to discover a predictive signature (Fig. 5a; Table 1). Leave-one-out cross-validation estimated a predictive accuracy of 93 % for this 10-gene signature. To evaluate the predictive accuracy of the candidate fluvastatin-sensitivity signature, we determined the fluvastatin MTT_{50} in six additional cell lines with relatively low HMGCR expression (Fig. 5b), ERα-negative status (Fig. 5b, c), and reported basal-like molecular subtype (Fig. 5c), all hallmarks of fluvastatin-sensitive breast tumor cells. We also considered the MCF10A and T47D cell lines that were not included in the 10-gene signature training. Our 10-gene signature correctly

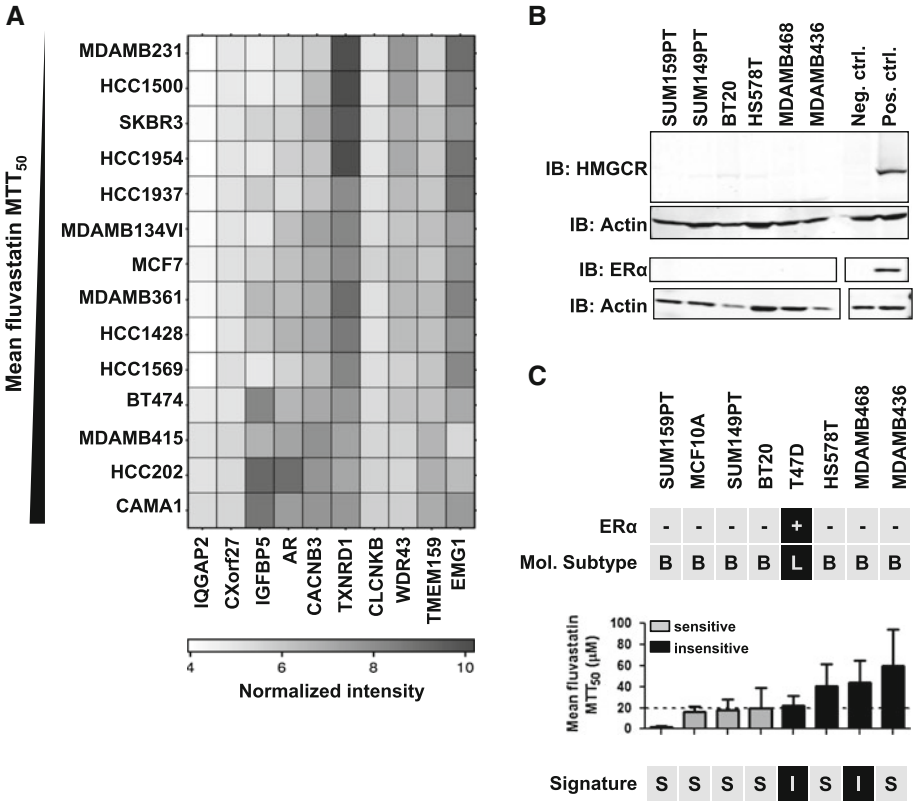

Fig. 5 A 10-gene candidate signature predicts fluvastatin sensitivity in an external validation set of breast cell lines. **a** Mining available baseline mRNA abundance data from 14 cell lines led to the generation of a 10-gene signature predictive of fluvastatin sensitivity in all cell lines in the training set. The *heatmap* shown displays normalized mRNA abundance intensity values for each of the ten genes (highlighted in supplementary Table S3) across the training set of 14 cell lines. **b** A set of ERα-negative breast tumor cell lines with low HMGCR expression was selected for external signature validation. HMGCR levels were assessed by immunoblot using the A9 monoclonal antibody, with actin as a loading control. Identical negative and positive HMGCR control lysates were loaded on each gel. The negative control ("Neg. ctrl") lysate was from the HMGCR-deficient UT2 cell line ectopically expressing a control vector, and the positive control lysate was from the UT2 cell line ectopically expressing HMGCR-FL ("Pos. ctrl."; Goard and Penn unpublished data). ERα status was determined by immunoblot, with actin as a loading control. An ERα-negative control ("Neg. ctrl."; MDAMB231) and ERα-positive control ("Pos. ctrl."; MCF7) were included on each gel. All *immunoblots* shown are representative of three independent experiments. **c**. The 10-gene candidate signature performs at least as well as subtype features in predicting fluvastatin sensitivity in external validation set cell lines. ERα status (negative, "-", or positive, "+") and published molecular subtype ("Mol. Subtype"; basal-like, "B", or luminal, "L") [16, 27] for external validation set cell lines are depicted (*upper panel*). Fluvastatin sensitivity was confirmed in these cell lines by MTT assay, where cell lines were considered sensitive ("S") if their mean $MTT_{50} \leq 20$ μM and insensitive ("I") otherwise (*middle panel*). *Bars* represent means of three to six experiments, with *error bars* indicating standard deviation. The *dashed line highlights* the sensitivity threshold of 20 μM. Fluvastatin sensitivity predictions by the 10-gene candidate signature are highlighted below (*lower panel*)

identified all four sensitive cell lines, while making two false-positive calls (Fig. 5c).

Discussion

While the pleiotropic anticancer effects of statins are well known, heterogeneity in statin sensitivity within a single tumor type remains poorly understood [13, 30, 35, 36], and most studies rely on few cell lines. Recent high-throughput studies correlating transcriptomic and genomic characteristics with sensitivity to a wide range of anticancer drugs have not yet considered statins [25–27]. Effective translation of fluvastatin to breast cancer treatment will likely require identification of intrinsically sensitive tumors.

We observed a trend toward greater fluvastatin sensitivity in cell lines derived from ERα-negative breast tumors. Our confidence in this trend achieves statistical significance when the additional six cell lines used for external validation are included ($r_{pb} = 0.437$, $p = 0.03$). Xenografts of ERα-negative tumor cells have also responded to treatment with lipophilic statins [13, 15]. We further identified an even stronger association of fluvastatin sensitivity with a basal-like molecular breast cancer subtype, which overlaps considerably with the ERα-negative

Table 1 A 10-gene candidate fluvastatin-sensitivity signature

Gene symbol	Gene name
IQGAP2	IQ motif-containing GTPase-activating protein 2
CXorf27	Chromosome X open-reading frame 27
IGFBP5	Insulin-like growth factor-binding protein 5
AR	Androgen receptor
CACNB3	Calcium channel, voltage-dependent, beta 3 subunit
TXNRD1	Thioredoxin reductase 1
CLCNKB	Chloride channel Kb
WDR43	WD repeat domain 43
TMEM159	Transmembrane protein 159
EMG1	EMG1 nucleolar protein homolog (*S. cerevisiae*)

r, Pearson correlation coefficient between published gene expression levels [16] and experimentally determined mean fluvastatin MTT_{50}, with FDR, *q*

subtype [18–20]. These tumor subtypes include aggressive "triple negative" tumors (ERα-, PR-, and HER2-negative) that are difficult to treat upon recurrence and have few effective targeted therapeutic options [37]. Thus far, two prospective window-of-opportunity clinical trials have evaluated the impact of short-term perioperative treatment with lipophilic statins (fluvastatin and atorvastatin) in breast cancer patients [11, 12]. In both cases, the primary trial endpoint was a decrease in Ki67 staining, as a proxy for decreased tumor cell proliferation. While the numbers of ERα-negative tumors examined in these small studies were limited, no statistically significant associations of ERα status with antiproliferative response following statin treatment were detected [11, 12]. Indeed, some ERα-positive tumors have also displayed clinical sensitivity to fluvastatin [11], and we have shown that the combination of fluvastatin and a SERM can have at least additive effects on cell death in ERα-positive cell lines. Of note, our definition of fluvastatin sensitivity in breast cell lines using the MTT assay reflects the combined effects of cell growth, proliferation, and apoptosis. An examination of both antiproliferative and proapoptotic responses to fluvastatin in larger panels of primary breast tumors may therefore clarify the clinical relationship between fluvastatin sensitivity and ERα status, suggesting that further subtype-stratified clinical evaluations of fluvastatin in breast cancer are warranted.

We also observed a moderate trend toward greater fluvastatin sensitivity in breast cell lines displaying lower abundances of HMGCR protein and mRNA. This is in contrast to the window-of-opportunity clinical study of atorvastatin, where breast tumors expressing HMGCR protein detected by immunohistochemistry had a greater reduction in Ki67 staining than those with undetectable basal HMGCR expression [12]. While the reasons underlying this discordance are not yet clear, it is probable that differences in the growth of breast tumor cells in culture and in vivo impact HMGCR expression, and in turn, the potential of this protein as a biomarker for statin sensitivity.

While our data suggest that ERα-negative, basal-like breast tumors may be enriched for those that would respond to fluvastatin treatment, a multiparametric molecular predictor may provide additional value. Our hypothesis-generating data mining approach identified several cellular processes and pathways to probe for functional and mechanistic relevance to fluvastatin sensitivity and predictive value in future work. For example, our data demonstrate that fluvastatin-sensitive breast cell lines have a higher abundance of *MYC* mRNA. This is of interest, as statins have been shown to interfere with MYC-driven tumourigenesis in mouse models of lymphoma and hepatocellular carcinoma [38, 39]. Moreover, the mRNA abundance of several genes encoding regulators of the mevalonate pathway was also correlated with sensitivity. Thus, our identification of fluvastatin sensitivity-associated genes provides a resource to guide further research into the functional relationship of these genes with each other, and their contribution to statin sensitivity.

By considering the genes most strongly associated with fluvastatin sensitivity, we generated a 10-gene signature that performed at least as well as ERα status or molecular subtype as a predictor of fluvastatin sensitivity in an external validation panel of breast tumor cell lines. This "first-generation" multi-gene mRNA abundance-based fluvastatin sensitivity signature provides proof of principle that this approach merits further exploration, with the ultimate goal of evaluating primary breast tumor tissue from prospective clinical trials.

Taken together, this work provides a foundation of molecular features associated with the anticancer effects of fluvastatin in breast tumor cells that may ultimately guide us in the design of appropriately targeted clinical trials.

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Conflict of interest The authors declare no potential competing financial interests.

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