Integrated Systems and Technologies

An RNA Interference Screen Identifies Metabolic Regulators

NR1D1 and PBP as Novel Survival Factors for Breast Cancer Cells with the ERBB2 Signature

Antonis Kourtidis1, Ritu Jain1, Richard D. Carkner2, Cheryl Eifert1, M. Julia Brosnan2, and Douglas S. Conklin1

Abstract

Overexpression of the adverse prognostic marker ERBB2 occurs in 30% of breast cancers; however, therapies targeting this gene have not proved to be as effective as was initially hoped. Transcriptional profiling meta-analyses have shown that there are ~150 genes co-overexpressed with ERBB2, suggesting that these genes may represent alternative targets for influencing ERBB2-positive tumors. Here we describe an RNA interference–based analysis of these genes that identifies transcriptional regulators of fat synthesis and storage as being critical for the survival of these cells. These transcription factors, nuclear receptor subfamily 1, group D, member 1 (NR1D1) and peroxisome proliferator activated receptor γ binding protein (PBP), both reside on ERBB2-containing 17q12-21 amplicons and are part of the ERBB2 expression signature. We show that NR1D1 and PBP act through a common pathway in upregulating several genes in the de novo fatty acid synthesis network, which is highly active in ERBB2-positive breast cancer cells. Malate dehydrogenase 1 and malic enzyme 1, enzymes that link glycolysis and fatty acid synthesis, are also regulated by NR1D1. The resulting high-level fat production from increased expression of these genes likely contributes to an abnormal cellular energy metabolism based on aerobic glycolysis. Together, these results show that the cells of this aggressive form of breast cancer are genetically preprogrammed to depend on NR1D1 and PBP for the energy production necessary for survival. Cancer Res. 70(3): 1783–92. ©2010 AACR.

Introduction

Overexpression of the ERBB2 oncogene is one of the most clinically relevant abnormalities in breast cancer. It occurs in 20% to 30% of breast cancer cases and is an established adverse predictor of relapse time and survival (1). A meta-analysis of the relationship between ERBB2 status and breast cancer prognosis revealed that in 90% of the studies either ERBB2 amplification or protein overexpression was correlated with poor clinical outcome of the patients (2). This association with aggressive disease has made ERBB2 an attractive therapeutic target for breast cancer. Herceptin, a monoclonal antibody targeting the extracellular domain of ERBB2, was widely hailed as the first “next generation” cancer therapy. However, its success has been tempered by response rates of only 11% to 26% when used as a single-agent therapy in patients with ERBB2-positive breast cancer (3), leading other groups to propose that factors beyond ERBB2 and its downstream signaling pathway influence therapy escape of these tumors (4).

Clues to other causes that might contribute to the aggressiveness of ERBB2-positive breast cancer have come from transcriptional profiling meta-analyses that have shown the existence of more than a hundred genes consistently co-overexpressed with ERBB2 in this tumor type (5–7). Overexpression of a number of these genes is the result of amplification of the 17q12-q21 region that contains ERBB2 (8). Most, however, do not map to this region. We have performed a functional RNA interference (RNAi) screen (9–11) to determine whether any of the genes overexpressed with ERBB2 are causal to the aggressiveness of this type of breast cancer. In the study, each overexpressed gene was systematically targeted one at a time in cells of this tumor type so that new therapeutic targets could be identified for ERBB2-positive breast cancer whether or not they are functionally related to the ERBB2 protein itself (12). The screen reveals genes that induce an abnormal cellular physiology as critical for the survival of this type of cells.
**Materials and Methods**

**Cell culture and chemicals.** Breast cancer cell lines were obtained from American Type Culture Collection. HMECs were obtained from Cambrex. HEK 293FT cells were obtained from Invitrogen. MCF10A cells were a gift of the Julio Aguirre-Ghiso laboratory. All cell lines were cultured in DMEM (Hyclone) supplemented with 10% fetal bovine serum (FBS; Hyclone) and 100 units/μL of penicillin-streptomycin (Cellgro), except for MDA-MB-361 cells cultured in RPMI 1640 (Hyclone) supplemented with 20% FBS, HMECs cultured in MEGM medium (Cambrex), and MCF10A cells cultured as indicated in ref. 13. The peroxisome proliferator activated receptor γ (PPARγ) antagonists GW9662 and T0070907 were obtained from Sigma-Aldrich.

**Constructions, RNAi screen, and transfections.** The flag-RevErhα (flag-NR1D1) construct was a gift of M. Lazar. The LSXN-neu* (ERBB2) construct was a gift of L. Petti. To overexpress nuclear receptor subfamily 1, group D, member 1 (NR1D1) in MCF10A cells, the NR1D1 cDNA was obtained from the Dana-Farber/Harvard Cancer Center DNA Resource Core (clone ID: HsCD00005059) and subcloned into the pMAXIV-Puro retroviral expression vector using standard cloning techniques. Short hairpin RNA (shRNA) constructs were expressed from the pSHAG-MAGIC 2 (pSM2) vector and derived from a genome-wide shRNA library (ref. 14; Open Biosystems). We targeted all the genes overexpressed in ERBB2-positive breast cancer with constructs from the initial release of this library available at the time of the screen, irrespective of their level of overexpression and without posing any other criteria. For mature sequences of shRNAs, see Supplementary Table S1. ShRNAs targeting the firefly luciferase gene were used as negative controls. Transfection efficiency was monitored by cotransfection with a MSCV-Puro vector expressing green fluorescent protein (GFP). Transfections were performed using FuGENE 6 and HD (Roche) according to the manufacturer’s protocol in an EpMotion 5070 fluidics station (Eppendorf). For quantification of alamarBlue, we used a BioTek HT Synergy plate reader. The alamarBlue (Biosource) assay was performed 96 h posttransfection, because BT474 cells have a population doubling time of ∼100 h. The 10% of the shRNAs that resulted in the highest decrease in alamarBlue signal (alarBlue < 55%, P < 0.05) were subjected to a second round of transfections to confirm the initial results. The hits that consistently produced a close to or >50% decrease on cell proliferation are shown in Fig. 1.

**Cell viability–apoptosis assays–immunoblotting.** For live cell counts, cells grown on 96-well plates were fixed with 2.5% formaldehyde and stained with Hoechst 33342 (Molecular Probes–Invitrogen). Pictures of cells were acquired using an In Cell Analyzer 1000 (GE Healthcare) high-content imaging system, with a 20× objective. At least 30 fields were imaged per single experiment. Statistics were performed using the In Cell Investigator 3.4 image analysis software (GE Healthcare). Cleaved caspase-3 and activated Bax immunofluorescence was performed at 48 h of treatments, using standard protocols. Cells were imaged using the In Cell Analyzer 1000. At least 500 cells were counted for each experiment. For Western blots, cell extracts were obtained using radioimmunoprecipitation assay buffer supplemented with complete cocktail of proteinase inhibitors (Roche). Protein extracts were separated by SDS-PAGE, transferred to Immobilon-P (Millipore) membranes, blotted according to standard protocols, and imaged using a FluorChem HD (Alpha Innotech) imaging system. Antibodies used were cleaved caspase-3 (Asp175, #9661; Cell Signaling Technology), Bax (6A7; BD Biosciences), anti-flag (M2; Stratagene), PPARγ binding protein (PPB; TRAP220, C-19; Santa Cruz Biotechnology), glyceroldehyde-3-phosphate dehydrogenase (V-18; Santa Cruz), Alexa Fluor 568 goat anti-rabbit IgG (#A-11011; Invitrogen), Alexa Fluor 568 goat anti-mouse IgG (#A-11040; Invitrogen), antirabbit IgG-HRP (sc-2004, Santa Cruz), antigoat IgG-HRP (sc-2768, Santa Cruz), and antiamoegus IgG-HRP (#31430; Pierce Biotechnology).

**Quantitative reverse transcription–PCR.** For all reactions, cells were harvested 48 h posttransfection. Due to low shRNA transfection efficiency of BT474 cells, GFP-positive cells were sorted using a BD FACSAria sorting system. Final population enrichment of transfected cells was 65% to 70%. Total RNA was extracted using TRIzol (Invitrogen), and cDNA was synthesized by a reverse transcription of 2 μg of RNA in a 20-μL reaction using Moloney murine leukemia virus reverse transcriptase (Promega) at 42°C for 1 h. Quantitative reverse transcription–PCR (qRT-PCR) was performed on ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems). The primer pairs used were designed using ABI’s Primer Express software (Supplementary Table S2). Initial amplification was at 95°C for 2.5 min, the amplification protocol consisted of 40 cycles of a 95°C–15 s step and a 60°C–60 s step. Product levels were calculated after normalization with β-actin control.

**Metabolic assays.** For detection of neutral fat stores, cells were either stained with 60% Oil Red O (Fisher Chemicals) or with 10 μg/mL 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY 493/503; Molecular Probes). For Oil Red O staining, cells were counterstained for nuclei with Mayer’s Hematoxylin (Sigma-Aldrich) and visualized using the 60× objective of an Arcturus Veritas (Molecular Devices Corporation) microdissection system. For BODIPY assays, cells were grown on 96-well plates, fixed with 2.5% formaldehyde, stained with 10 μg/mL BODIPY 493/503 and counterstained for nuclei with Hoechst 33342. Transfected cells were monitored by cotransfecting with the pDsRed-Monomer-N1 vector (Clontech). Cells were imaged and analyzed using the In Cell Analyzer 1000–In Cell Investigator 3.4 system. For total fatty acid detection and quantification, total cellular lipids were extracted according to a standard procedure previously described (15).

**Statistics.** In all experiments, SDs from three individual experiments were calculated and presented as error bars. The Student’s two-tailed t test was used for P value calculations. Comparisons in each case refer to the respected controls, unless otherwise indicated.
Results

A function-based screen of genes overexpressed in ERBB2-positive breast cancer. A total of 309 available shRNAs (9) were used to target 141 of the 154 genes identified in a total of 22 studies (refs. 5–7; Supplementary References) as being upregulated in ERBB2-positive breast cancer cell lines and tumors (Supplementary Table S3). These constructs were individually used to silence each gene in the well-characterized, ERBB2-positive BT474 breast cancer cell line. The effects on cells were monitored using alamarBlue, a fluorimetric indicator of cell proliferation and viability (11). Whereas most of the shRNAs corresponding to the overexpressed genes were without significant effect, shRNAs targeting 10 genes result in more than a 50% decrease in proliferation (Fig. 1A; Table 1). This group of shRNAs includes an ERBB2 shRNA, as would be predicted. Other shRNAs with significant effects on proliferation silence genes with previously reported roles in cancer, such as KI67, TPDS2, and CA9 (refs. 16–18; Table 1). Knockdown of ERBB3, another member of the epidermal growth factor receptor family that heterodimerizes with ERBB2 (19), has a modest effect on cell viability (Table 1). shRNAs targeting GRB7 and STARD3, two genes that are closely linked to ERBB2 on the 17q12 amplicon (20), have a moderate effect on cell proliferation in our screen (Table 1), which agrees with previous studies (20).

Surprisingly, three of the shRNAs that have the most dramatic effect on cell proliferation target genes are related to adipogenesis, including NR1D1 (21), PBP (22), and the mitogen-activated protein (MAP) kinase MAP2K6 (ref. 23; Table 1). Three others, fatty acid desaturase 2 (FADS2), STAR-related lipid transfer domain containing 3 (STARD3), and fatty acid synthase (FASN), which, when silenced, have a moderate effect on cell proliferation, are also involved in lipid metabolism (Table 1).

Figure 1. A, the RNAi screen targeting genes overexpressed in ERBB2-positive breast cancer. Three transfection mixes were produced for each shRNA, and each was transfected into triplicate wells of BT474 cells. AlamarBlue was used to monitor cell proliferation and viability. The averages of the nine parallel cultures were calculated for each shRNA, normalized to transfection efficiency, expressed as percentage of the control shRNA (luciferase), and sorted on the basis of effect. B, NR1D1 and PBP are specifically necessary for viability of ERBB2-positive cells. Several breast cancer (BC) cell lines, as well as HMEC and HEK 293 cells, were transfected and assayed as in A. Effects of each shRNA on each cell line were subjected to hierarchical cluster analysis using Cluster 3.0 (M. Eisen) and displayed using Treeview. The ERBB2 and ER status of each cell line is indicated at the bottom.
**NR1D1 and PBP are required specifically for ERBB2-positive cell survival.** To assess the specificity of effect of each shRNA, those causing the greatest decrease in BT474 cell proliferation were transfected in ERBB2 overexpressing and nonoverexpressing breast cancer cell lines (24), in normal human mammary epithelial cells and a non-breast cell line (Fig. 1B). Proliferation rates of shRNA-transfected cells were measured for each cell line (Supplementary Table S4), and the results were subjected to cluster analysis and displayed as a heat map and dendrogram (Fig. 1B). The NR1D1 and PBP shRNAs cluster with ERBB2, because each specifically decreases the viability of ERBB2-positive cells, yet is without effect on other cell lines or HMECs (Fig. 1B). Notably, their effect on cell survival is independent of the estrogen receptor (ER) status of the cell lines used (Fig. 1B).

NR1D1 and PBP are tightly linked to ERBB2 and frequently reside on the 17q12-21 amplicons found in ERBB2-positive tumors (4, 8). Several studies have shown that irrespective of the amplicon size they are consistently co-overexpressed with ERBB2 (25, 26) and are among the six genes that comprise the "ERBB2 gene expression signature" seen in breast cancers (7). These genes are also functionally linked. PBP is a coactivator of PPARγ (22), which upregulates 30 genes related to adipogenesis (27) including NR1D1 (28). NR1D1 is a member of the steroid receptor supergene family that promotes adipocyte differentiation and has been also identified as a component of the circadian clock (29). However, its downstream effectors are poorly characterized (21).

**Table 1. The shRNAs that consistently decreased BT474 cell proliferation in the RNAi screen**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Name</th>
<th>Function</th>
<th>Percentage of proliferation of control (%)*</th>
<th>Z score</th>
</tr>
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<tbody>
<tr>
<td>ERBB2</td>
<td>V-erb-b2 erythroblastic leukemia viral oncogene homologue 2</td>
<td>Receptor tyrosine kinase</td>
<td>27.04</td>
<td>−2.86</td>
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<tr>
<td>MKI67</td>
<td>Antigen identified by monoclonal antibody Ki-67</td>
<td>Proliferation</td>
<td>32.99</td>
<td>−2.59</td>
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<td>NR1D1</td>
<td>Nuclear receptor subfamily 1, group D, member 1</td>
<td>Adipogenesis–fat metabolism</td>
<td>35.83</td>
<td>−2.46</td>
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<tr>
<td>PBP</td>
<td>PPAR binding protein PPARBP</td>
<td>Adipogenesis–fat metabolism</td>
<td>37.08</td>
<td>−2.40</td>
</tr>
<tr>
<td>MAP2K6</td>
<td>Mitogen-activated protein kinase 6</td>
<td>Adipogenesis–signal transduction</td>
<td>37.23</td>
<td>−2.39</td>
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<tr>
<td>SLPI</td>
<td>Secretory leukocyte protease inhibitor</td>
<td>Proteolysis</td>
<td>38.65</td>
<td>−2.33</td>
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<tr>
<td>PFN2</td>
<td>Prolin 2</td>
<td>Actin polymerization</td>
<td>41.06</td>
<td>−2.22</td>
</tr>
<tr>
<td>SPINT1</td>
<td>Serine protease inhibitor, Kunitz type 1</td>
<td>Proteolysis</td>
<td>42.87</td>
<td>−2.14</td>
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<tr>
<td>LASP1</td>
<td>LIM and SH3 protein 1</td>
<td>Actin cytoskeleton</td>
<td>43.27</td>
<td>−2.12</td>
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<tr>
<td>TPD52</td>
<td>Tumor protein D52</td>
<td>Secretion</td>
<td>46.50</td>
<td>−1.97</td>
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<td>H2AFY</td>
<td>H2A histone family, member Y</td>
<td>Nucleosome assembly</td>
<td>50.46</td>
<td>−1.79</td>
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<tr>
<td>SIAT4C</td>
<td>Sialyltransferase 4C</td>
<td>Transferase activity</td>
<td>53.30</td>
<td>−1.66</td>
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<td>SFRS7</td>
<td>Splicing factor, arginine/serine-rich 7, 35kDa</td>
<td>Splicing</td>
<td>53.87</td>
<td>−1.64</td>
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<td>BNIPL3</td>
<td>BCL2/adenovirus E1B 19 kDa interacting protein 3-like</td>
<td>Apoptosis</td>
<td>54.62</td>
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<tr>
<td>CA9</td>
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<td>Secretion</td>
<td>54.01</td>
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<td>RPL19</td>
<td>Ribosomal protein 19</td>
<td>Ribosome structure</td>
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<td>−1.60</td>
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<td>FADS2</td>
<td>Fatty acid desaturase 2</td>
<td>Fat metabolism</td>
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<td>STARD3</td>
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<td>Fat metabolism</td>
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<td>−1.00</td>
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<tr>
<td>ERBB3</td>
<td>V-erb-b2 erythroblastic leukemia viral oncogene homologue 3</td>
<td>Receptor tyrosine kinase</td>
<td>69.33</td>
<td>−0.94</td>
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<td>GRB7</td>
<td>Growth factor receptor bound protein 7</td>
<td>Signal transduction</td>
<td>71.41</td>
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<td>FASN</td>
<td>Fatty acid synthase</td>
<td>Fat metabolism</td>
<td>75.40</td>
<td>−0.66</td>
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</table>

*Results shown are statistically significant (P < 0.05).*
indicating that cell death is the cause of the decrease in alamarBlue staining. Increased cleaved caspase-3 (Fig. 2B) and activated Bax signals (Supplementary Fig. S1B) indicate that the cell death is apoptotic. A 2.5- to 3.5-fold increase in apoptosis, which is initially observed within 48 hours of shRNA transfection, results in extensive cell death after 72 hours (Fig. 2A). Three other shRNAs targeting NR1D1 and two targeting PBP also result in apoptosis specifically of BT474 cells confirming the result (Supplementary Fig. S2A and B). These results indicate that NR1D1 and PBP are survival factors for these cells.

Transfection with NR1D1 or PBP shRNAs results in >60% decreased mRNA levels of their targets in BT474 cells (Fig. 2C), as well as in decreased protein levels (Supplementary Fig. S2C). Interestingly, the PBP shRNA also causes a significant decrease in the message levels of NR1D1, whereas the NR1D1 shRNA does not have a significant effect on PBP mRNA levels (Fig. 2C). Because PBP is a coactivator of PPARγ, which is known to regulate NR1D1 expression (28), it is likely that PBP indirectly regulates expression of NR1D1. Simultaneous transfection of the NR1D1 and PBP shRNAs does not have an additive effect on either the cell loss from the population or the apoptosis (Fig. 2D). This indicates that the two genes promote survival of these cells via the same pathway. Because PPARγ is also part of this pathway in other cell types, we examined its potential role in breast cell survival. Inhibition of PPARγ activity using an antagonist (GW9662; ref. 30) dramatically decreases NR1D1 message levels (Supplementary Fig. S3A). Similar to the effects seen with NR1D1 and PBP shRNAs, ERBB2-positive cells are specifically sensitive to GW9662 treatment (ref. 30; Supplementary Fig. S3B). A second PPARγ antagonist produces the same effects (Supplementary Fig. S3C). For either drug, PPARγ inhibition results in apoptosis of BT474 cells (Supplementary Fig. S3D).

Genetic interaction experiments indicate that ERBB2 is not a downstream mediator of the survival advantage that NR1D1 and PBP confer in BT474 cells. Although PBP knockdown decreases ERBB2 message levels, NR1D1 knockdown does not have any effect on ERBB2 expression (Supplementary Fig. S4A). Because the effect of PBP and NR1D1 seems to occur
through a common pathway (Fig. 2C and D), it is unlikely that the effect on cell survival is caused by ERBB2. The different phenotypic effects of NR1D1 and PBP inhibition compared with ERBB2 inhibition support this notion, because ERBB2 inhibition does not result in a significant increase in apoptosis ($P = 0.08$; Supplementary Fig. S4B and C) but only in attenuation of proliferation (Table 1). Furthermore, the effect on growth rate observed in the proliferation of the ERBB2-negative MCF-7 cells, when ERBB2 and NR1D1 are simultaneously overexpressed, is additive (Supplementary Fig. S4D). In the same context, simultaneous knockdown of ERBB2 and NR1D1 had an additive effect on BT474 cell viability (Supplementary Fig. S4E), further supporting the notion that the two genes regulate different pathways. It is also unlikely that NR1D1 and PBP are effectors of ERBB2, because an ERBB2 shRNA does not alter message levels of PBP or NR1D1 in BT474 or HEK 293 cells (Supplementary Fig. S4F) and because overexpression of either wild-type or constitutively active forms of ERBB2 in MCF10A cells does not increase levels of NR1D1 and PBP (data not shown). However, we still cannot formally exclude the possibility that the different phenotypes observed are due to bifurcation of a single pathway.

**NR1D1 and PBP induce excessive fat accumulation in ERBB2-positive cells.** The emergence of genes related to lipid metabolism in the RNAi screen suggests that fat production is critically important to ERBB2-positive cell survival. Stains of neutral fat reveal that ERBB2-positive cells exhibit ~20-fold higher level of accumulated fat in lipid stores when compared with HMEC cells and a 10-fold increase when compared with other breast cancer cell lines (Fig. 3A and B; Supplementary Fig. S5A). When quantified by mass spectrometry, differences are also observed in total cellular fats, because BT474 cells are found to contain more than twice the amount of fatty acids of MCF-7 cells (Supplementary Fig. S5B). Targeting NR1D1 and PBP with shRNAs causes a modest although significant decrease in fat stores in BT474 cells of 47% and 30%, respectively, in the first 48 hours after transfection (Fig. 4A and B; Supplementary Fig. S5C). The decrease in cellular fats begins at 24 hours (Fig. 4A) and maximizes at 48 hours after NR1D1 and PBP shRNA transfection, whereas apoptosis under the same conditions is not significant at 24 hours but maximizes after 48 hours (Supplementary Fig. S1B). This indicates that apoptosis caused by NR1D1 and PBP knockdown is a result of the observed changes in the metabolic state of the cells. Knockdown of ERBB2 results only in 1% and 17% decreases in fat stores of BT474 cells after 24 and 48 hours, respectively (Supplementary Fig. S5D). In addition, when NR1D1 is overexpressed in the immortalized, non-tumorigenic MCF10A breast cells (Fig. 4C), a 4-fold increase in fat accumulation is observed in these cells compared with the control cells (Fig. 4D). In contrast, overexpression of a constitutively active ERBB2 causes only a 1.6× increase in fats in MCF10A cells. Wild-type ERBB2 is without effect (Supplementary Fig. S5E).
The fat synthetic process is required for survival of ERBB2-positive breast cancer cells. The importance of fatty acid synthesis to the survival of ERBB2-positive cancer cells is not due to the level of stored fats but rather to the synthetic process itself. Targeting NR1D1 and PBP with shRNAs significantly decreases viability (Fig. 2A and B) and fat stores in BT474 cells (Fig. 4A and B). However, similar decreases in fat stores of BT474 cells grown in media containing the alternative fuel sources galactose or fructose (Supplementary Fig. S6A–C) do not lead to cell death (Supplementary Fig. S6D). These results indicate that the survival function provided by NR1D1 and PBP is due to increased activity of the fatty acid synthesis pathway and not to the increased levels of the end products of this pathway.

To identify potential downstream targets of NR1D1 and PBP that are related to fat metabolism and pertinent to breast cancer cell survival, we looked at mRNA levels of the three major enzymes of de novo fatty acid synthesis: ATP citrate lyase (ACLY), acetyl-CoA carboxylase α (ACACA), and FASN. The transcript level of each enzyme is significantly reduced after transfection with NR1D1 and PBP shRNAs (Fig. 5A) or PPARγ antagonist treatment (Supplementary Fig. S7A). Transcript levels of FADS2, a fat metabolism-related gene that was identified in the screen (Table 1), are also downregulated under the same conditions (Fig. 5A; Supplementary Fig. S7A). ShRNAs targeting ACLY, ACACA, and FASN significantly decrease BT474 cell viability (Fig. 5A; Supplementary Fig. S7B), showing that these genes are mediators of the survival advantage that NR1D1 and PBP confer to ERBB2-positive cells. It has already been established that FASN (31, 32) and ACACA (4) are upregulated in ERBB2-positive breast tumors. A search in the Oncomine database (Edition 4) also revealed strong correlations of PBP and NR1D1 to ACLY and ACACA expression in ERBB2-positive breast tumors (see Supplementary Table S5).

Because both synthesis and elongation of fatty acids use NADPH as a cofactor, it is necessary that these reactions be continuously supplied with NADPH. This also predicts that enzymes that generate cytoplasmic pools of NADPH using NADH might be required for fat synthesis and consequently for ERBB2-positive breast cancer cell survival. Two genes involved in these processes are malate dehydrogenase 1 (MDH1), the key enzyme in the production of cytoplasmic malate from oxaloacetate using NADH (33), and malic
enzyme 1 (ME1), which converts malate to pyruvate and is the primary source of NADPH required by FASN for palmitate synthesis (34). Message levels of the MDH1 and ME1 genes after NR1D1 shRNA treatment are significantly decreased (Fig. 5B). Although PBP acts upstream of NR1D1, we did not observe the same effect on MDH1 and ME1 message levels, after PBP knockdown. These results may suggest that MDH1 and ME1 are more acutely affected by loss of NR1D1 than PBP. Such a situation might be caused by the kinetics of the pathway. Importantly, targeting MDH1 and ME1 with shRNAs results in decreased viability of BT474 cells, but not MCF-7 cells (Fig. 5B; Supplementary Fig. S7C and data not shown). Simultaneous knockdown of MDH1 or ME1 with either NR1D1 or PBP did not result in an additive effect on cell viability (Supplementary Fig. S7D), consistent with the notion that these two genes are downstream targets of NR1D1. Together, these results suggest that NR1D1 and PBP maximize fatty acid synthesis in favor of an altered physiology that provides a survival advantage to the ERBB2-positive breast cancer cells (Fig. 5C).

Discussion

Our functional genomics approach revealed that a small fraction of the genes that are overexpressed in ERBB2-positive breast cancer plays a role in the aggressiveness of this type of cancer. Downregulation of 10 of the 141 genes targeted in our RNAi screen resulted in 50% or more decrease in proliferation of the ERBB2-positive BT474 breast cancer cells. Most interestingly, the screen revealed a number of genes associated to fatty acid metabolism as being necessary for the survival of this type of cells. Three of them, NR1D1, PBP, and MAP2K6, were among the most significant hits of the screen. Our interrogation of the system identified not just individual genes that are the most important for ERBB2-positive breast cancer but also a whole pathway and a particular metabolic state that is required for the survival of the cells of this type of cancer, showing the power of this approach.

Knockdown of NR1D1 and PBP induces apoptosis specifically in ERBB2-positive cells, indicating that these genes are required for the survival of these cancer cells.

Figure 5. NR1D1 and PBP upregulate the de novo fatty acid synthesis pathway. A, qRT-PCRs of BT474 cells treated with luciferase (control), PBP, or NR1D1 shRNAs for detection of ACLY, ACACA, FASN, and FADS2 mRNAs (left; *, P < 0.005). BT474 cells were also transfected with ACLY, ACACA, and FASN shRNAs, cotransfected with GFP, and counted as in Fig. 2B (right; **, P < 0.01). B, MDH1 and ME1 qRT-PCRs of BT474 cells treated with luciferase (control), PBP, or NR1D1 shRNAs (left; *, P < 0.002). BT474 cells were also transfected with luciferase (control), MDH1, and ME1 shRNAs and assayed as in Fig. 2B (right; *, P = 0.0003). C, the model of NR1D1- and PBP-mediated regulation of fatty acid metabolism and energy balance in ERBB2-positive cells.
and fundamentally contribute to their aggressiveness. Although the effects on viability of inhibiting \(NR1D1\) and \(PBP\) are seen only in \(ERBB2\)-positive cells, they are not mediated by \(ERBB2\) itself. Our results indicate that \(NR1D1\) and \(PBP\) are factors that contribute to the aggressiveness of what is clinically designated as “\(ERBB2\)-positive breast cancer.” \(NR1D1\) and \(PBP\) are part of the \(ERBB2\)-positive gene expression signature \((7)\), which has been established to better describe \(ERBB2\)-positive tumors. One reason of \(NR1D1\) and \(PBP\) being highly active in this type of cancer cells is their tight genetic linkage to \(ERBB2\) in the 17q12 amplicon. This tight genetic linkage between \(NR1D1\), \(PBP\), and \(ERBB2\) causes co-overexpression of these gene products such that, as indicated by the present study, \(ERBB2\)-positive breast cancer cells are genetically preprogrammed to depend on \(NR1D1\) and \(PBP\) for survival. It is unlikely, however, that these genes drive cells to become tumorigenic by themselves. For example, overexpression of \(NR1D1\) in MCF10A cells does not confer the ability to form colonies in soft agar (data not shown).

We have shown that overexpression of \(NR1D1\) and \(PBP\) are responsible for the upregulation of all three major enzymes of the de novo fatty acid synthesis pathway. The phenotypic outcome of \(NR1D1\) and \(PBP\) overexpression is that \(ERBB2\)-positive breast cancer cells possess extraordinarily high levels of fats. Downregulation of \(NR1D1\) or \(PBP\) directly results in rapid decrease of fatty acid stores within 48 hours. These results support the notion that the major effect of inhibition of \(NR1D1\) and \(PBP\) is an acute inhibition of the de novo fatty acid synthesis pathway that is coordinated by these transcription factors. This has a more profound effect on \(ERBB2\)-positive breast cancer cells causing apoptosis, which likely reflects the overall higher metabolic rate of the fatty acid synthesis pathway in these cells and their dependence upon this pathway for survival. Although the mechanism of \(NR1D1\) and \(PBP\) regulating fat metabolism is at present unclear, our results are in accord with a recent study, which finds that \(NR1D1\) overexpression in the livers of transgenic mice causes the upregulation of several genes related to fat synthesis through a poorly understood effect on the sterol response element binding protein SREBP1c \((35)\).

Our approach also identified an extended metabolic network required for the high level fat production that is found in \(ERBB2\)-positive breast cancer cells. The role of fatty acid synthesis in the overall metabolic balance of these cells provides a rationale for its essential function. Our finding that it is not the decrease in the amount of stored fats followed by \(NR1D1\)-\(PBP\) inhibition that is responsible for the apoptosis observed in the \(ERBB2\)-positive breast cancer cells but the process of synthesizing these fats supports this notion. Recently published results showing that exogenous supplementation of fatty acids is toxic to \(ERBB2\)-positive cells is also indicative of this physiology \((30)\). A rationale for fat production as a survival mechanism has been suggested by studies showing that increased fatty acid synthesis in some cancer cells is a feature of aerobic glycolysis \((36)\), the altered tumor cell energy metabolism first proposed by Warburg \((37)\). Oxygen does not serve as the terminal electron acceptor in cells with this physiology. To avoid low NAD\(^+\)/NADH ratios that would eventually feed back to inhibit glycolysis, electrons are incorporated into other molecules such as lactate with the concomitant regeneration of NAD\(^+\). Because both synthesis and elongation of fatty acids use NADPH as a cofactor, it is thought to play a role in indirectly regenerating NAD\(^+\) under these conditions. This implies that enzymes that might couple the transfer of electrons from glycolysis to fat synthesis would also be required for \(ERBB2\)-positive breast cancer cell survival. MDH1 and ME1 are two such enzymes that mediate interconversion of NAD-NADH used in glycolysis to NADP-NADPH used in fatty acid synthesis. Our results show that both these genes are regulated by \(NR1D1\) and are critical for the survival specifically of the \(ERBB2\)-positive breast cancer cells. Overall, the transcriptional regulation by \(NR1D1\) and \(PBP\) of enzymes involved in fatty acid synthesis and storage and whose concerted action is required for clearing the electrons that are produced during glycolysis provides an explanation for the essential nature of these genes. These results suggest that there is a greater metabolic requirement served by maximized fatty acid synthesis in the \(ERBB2\)-positive breast cancer cells and that this requirement is satisfied by \(NR1D1\) and \(PBP\) overexpression.

The central role that the \(NR1D1\)-\(PBP\) pathway plays in the physiology of these cells may have several implications for this type of cancer. The action of \(NR1D1\) and \(PBP\) establishes a physiologic program that causes products of glycolysis to be stored as triglycerides rather than to be exported as lactate. This may bestow \(ERBB2\)-positive breast cancer cells with increased metabolic autonomy in vivo, which could directly improve clonal dissemination and survival during metastasis and tumor relapse. Supporting this notion, recent expression-profiling studies identified \(PBP\) in the group of genes that are differentially expressed in invasive ductal carcinoma \((38)\) and in brain metastases of breast cancer \((39)\). This altered physiologic program may also implicate \(NR1D1\), a proposed link between the circadian clock and cellular metabolism \((29)\), as a potential mediator of the tumorigenic influences of diet and the “night shift” that have been recently established for breast cancer \((40)\). Further study of \(NR1D1\) and \(PBP\) promises the elucidation of key functions associated to breast cancer progression.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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Antonis Kourtidis, Ritu Jain, Richard D. Carkner, et al.

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