**Fulvestrant induces resistance by modulating GPER and CDK6 expression: implication of methyltransferases, deacetylases and the hSWI/SNF chromatin remodelling complex**

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**Background:** Breast cancer is the leading cause of cancer death in women living in the western hemisphere. Despite major advances in first-line endocrine therapy of advanced oestrogen receptor (ER)-positive breast cancer, the frequent recurrence of resistant cancer cells represents a serious obstacle to successful treatment. Understanding the mechanisms leading to acquired resistance, therefore, could pave the way to the development of second-line therapeutics. To this end, we generated an ER-positive breast cancer cell line (MCF-7) with resistance to the therapeutic anti-oestrogen fulvestrant (FUL) and studied the molecular changes involved in resistance.

**Methods:** Naive MCF-7 cells were treated with increasing FUL concentrations and the gene expression profile of the resulting FUL-resistant strain (FR.MCF-7) was compared with that of naive cells using GeneChip arrays. After validation by real-time PCR and/or western blotting, selected resistance-associated genes were functionally studied by siRNA-mediated silencing or pharmacological inhibition. Furthermore, general mechanisms causing aberrant gene expression were investigated.

**Results:** Fulvestrant resistance was associated with repression of GPER and the overexpression of CDK6, whereas ERBB2, ABCG2, ER and ER-related genes (GREB1, RERG) or genes expressed in resistant breast cancer (BCAR1, BCAR3) did not contribute to resistance. Aberrant GPER and CDK6 expression was most likely caused by modification of DNA methylation and histone acetylation, respectively. Therefore, part of the resistance mechanism was loss of RB1 control. The hSWI/SNF (human SWItch/Sucrose NonFermentable) chromatin remodelling complex, which is tightly linked to nucleosome acetylation and repositioning, was also affected, because as a stress response to FUL treatment-naive cells altered the expression of five subunits within a few hours (BRG1, BAF250A, BAF170, BAF155, BAF47). The aberrant constitutive expression of BAF250A, BAF170 and BAF155 and a deviant stress response of BRG1, BAF170 and BAF47 in FR.MCF-7 cells to FUL treatment accompanied acquired FUL resistance. The regular and aberrant expression profiles of BAF155 correlated directly with that of CDK6 in naive and in FR.MCF-7 cells corroborating the finding that CDK6 overexpression was due to nucleosome alterations.

**Conclusion:** The study revealed that FUL resistance is associated with the dysregulation of GPER and CDK6. A mechanism leading to aberrant gene expression was most likely unscheduled chromatin remodelling by hSWI/SNF. Hence, three targets should be conceptually addressed in a second-line adjuvant therapy: the catalytic centre of SWI/SNF (BRG1) to delay the development of FUL resistance, GPER to increase sensitivity to FUL and the reconstitution of the RB1 pathway to overcome resistance.

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Oestrogen is the main stimulant for the growth of breast cancer cells. As a consequence, oestrogen receptor alpha (ERα) is the most important target in breast cancer treatment (Jensen and Jordan, 2003). Over the past 30 years, antagonists of steroid hormones are clinically important in the management of receptor-positive breast cancer and the ER antagonist tamoxifen (TAM), which is a non-steroidal selective ER modulator (SERM), is widely used as the gold standard for antihormonal therapy. However, the duration of response of advanced breast cancer is limited (progression-free survival <10 month) because of the development of hormone-independent tumours in virtually all cases (Badia et al., 2000; Miyoshi et al., 2010). Therefore, anti-oestrogen resistance is frequently observed in patients after long-term treatment with TAM, stressing the development of resistance to endocrine therapy as a clinically important problem. Reportedly, resistance to TAM correlated significantly with CpG hypomethylation of ERβ (Chang et al., 2005) and its overexpression (Speirs et al., 1999) and resistance to FUL with ERx downregulation (Fan et al., 2006). Selective ER modulator resistance is associated with the acquisition of oestrogen-independent growth (Badia et al., 2000; Schiff et al., 2004; Sabnis et al., 2005; Miyoshi et al., 2010), which is accomplished in particular by the upregulation of ERBB2 (Shou et al., 2002; Shou et al., 2006; Gutierrez et al., 2005). Also, increased ABCG2 levels cause TAM resistance (Sleever et al., 2011).

It was shown that TAM resistance could be overcome by another SERM, fulvestrant (FUL; synonym: faslodex, ICI 182 780; Gutierrez et al., 2004; Sabnis et al., 2005). Consequently, oestrogen receptor alpha (ERα) is the most important target in breast cancer treatment (Jensen and Jordan, 2003). Over the past 30 years, antagonists of steroid hormones are clinically important in the management of receptor-positive breast cancer and the ER antagonist tamoxifen (TAM), which is a non-steroidal selective ER modulator (SERM), is widely used as the gold standard for antihormonal therapy. However, the duration of response of advanced breast cancer is limited (progression-free survival <10 month) because of the development of hormone-independent tumours in virtually all cases (Badia et al., 2000; Miyoshi et al., 2010). Therefore, anti-oestrogen resistance is frequently observed in patients after long-term treatment with TAM, stressing the development of resistance to endocrine therapy as a clinically important problem. Reportedly, resistance to TAM correlated significantly with CpG hypomethylation of ERβ (Chang et al., 2005) and its overexpression (Speirs et al., 1999) and resistance to FUL with ERx downregulation (Fan et al., 2006). Selective ER modulator resistance is associated with the acquisition of oestrogen-independent growth (Badia et al., 2000; Schiff et al., 2004; Sabnis et al., 2005; Miyoshi et al., 2010), which is accomplished in particular by the upregulation of ERBB2 (Hu and Mokbel, 2001; Chung et al., 2002; Shou et al., 2004; Gutierrez et al., 2005). Also, increased ABCG2 levels cause TAM resistance (Sleever et al., 2011).

It was shown that TAM resistance could be overcome by another SERM, fulvestrant (FUL; synonym: faslodex, ICI 182 780; Shaw et al., 2006), which is a pure anti-oestrogen without agonistic and solely antagonistic features. As FUL follows TAM, we addressed the question regarding the mechanisms that become induced when breast cancer cells develop resistance to FUL.

Materials and Methods

Cell culture. The MCF-7 breast cancer cell line was purchased from ATCC (Rockville, MD, USA) and was cultivated in DMEM/ F-12 1 : 1 medium supplemented with 10% heat-inactivated FCS, 1% l-glutamine and 1% penicillin/streptomycin. Fulvestrant (5-aza-2′-deoxycytidine (AZA), trichostatin A (TSA), fumitremorgin C (FTC)), 17-β oestradiol (E2) and CDK6 inhibitor PD0332991 were purchased from Sigma-Aldrich (Munich, Germany), and trastuzumab (TRA) from Roche (Basel, Switzerland). Amersham ECLplus Western Blotting Detection System was from GE Healthcare (Buckinghamshire, UK).

Mouse monoclonal anti-β-actin (ascites fluid; clone AC-15, Cat. no. A5441) was from Sigma-Aldrich. Anti-cyclin D1 (M-20; Cat. no. sc-718), anti-p21 (C-19; Cat. no. sc-397), cyclin A (H-432; Cat. no. A5441) was from Sigma-Aldrich. Anti-cyclin D1 (M-20; Cat. no. sc-718), anti-p21 (C-19; Cat. no. sc-397), cyclin A (H-432; Cat. no. A5441) was from Sigma-Aldrich. Anti-cyclin D1 (M-20; Cat. no. sc-718), anti-p21 (C-19; Cat. no. sc-397), cyclin A (H-432; Cat. no. A5441) was from Sigma-Aldrich.

Proliferation analysis. MCF-7 cells were seeded in 24-well plates at a concentration of 1 × 10^5 cells per ml allowing logarithmic growth within 96 h. Afterwards, cells were incubated with FUL or PD0332991. The cell number was determined using an electronic cell counter (CASY; Roche Applied Science, Mannheim, Germany). Proliferation rates were calculated as described (Maier et al., 2006; Strasser et al., 2006). Cell duplication was calculated as follows:

\[
\text{doubling time} = \log 2 \times \frac{\text{cultivation time}}{\log N} - \log N_0
\]

where \(h\) is the cultivation time, \(N\) is the cell number after the time of cultivation and \(N_0\) the cell number at the beginning of cultivation.

Quantitative RT-PCR. MCF-7 and FR.MCF-7 cells (1 × 10^5) were seeded in six wells, cultivated for 24 h, harvested and homogenised using Qia-shredder (Qiagen, Hilden, Germany) and further processed according to the instructions of RNeasy Mini Kit (Qiagen). The total RNA concentration was measured using a NanoDrop Fluorospectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Complementary DNA synthesis from 1 μg RNA was performed using Superscript-first-strand synthesis systems for RT–PCR (Invitrogen, Carlsbad, CA, USA). The transcript levels of GREB1, RERG, GPER, BCAR1, BCAR3, CDK6, ERBB2 and ABCG2 were investigated by real-time PCR using Taqman detection system (Applied Biosystems, Carlsbad, CA, USA). The expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control. Assay ID numbers of the Taqman gene expression kits were: GAPDH, HS99999905_m1; GREB1, HS00536409; RERG, HS00922947; GPER, HS01116133; BCAR1, HS01547079; BCAR3, HS00981927; CDK6, HS01026371; and ERBB2, HS01001580. Cycle programme (95°C for 10 min to activate
polymerase followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min) was started on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Real-time PCR was performed in duplicate for each gene investigated. Negative controls, containing water instead of cDNA, confirmed the absence of RNA/DNA in all reagents applied in the assay.

siRNA knockdown. MCF-7 and FR.MCF-7 cells (1.0 × 10⁵) were seeded onto 6 cm cell culture plates and cultivated for 24 h. On the day of transfection, 3.6 µg siRNA (corresponding to 360 nm final concentration) was diluted in 50 µl medium. Forty-three micro-litres of RNAiFect transfection reagent (Qiagen) was added to the diluted siRNA and the mixture incubated for 15 min at room temperature to allow the formation of transfection complexes. Then, the solution was added dropwise onto the cells. After incubation for 16 h at 37 °C, the medium was changed, and after further 24 h, cells were used for experiments. The siRNAs were from Applied Biosystems (Life Technologies Inc., Carlsbad, CA, USA) and the Silencer Select siRNA IDs were: GREB1 (s18650), RERG (s224986), GPER (s6503), BCAR1 (s18371) and BCAR3 (s228334); negative control cat. no. 4390843.

Gene expression analysis by Affymetrix. Total RNA was extracted from 1 × 10⁶ cells (grown in six-well plates) by using Qiagen RNeasy Kit (Qiagen). All purified RNA samples were quality controlled by measuring the optical density at 230, 260 and 280nm and by analysing an aliquot of the RNA preparation on an Agilent 2100 Bioanalyzer using RNA 6000 Nano chips (Agilent Technologies, Santa Clara, CA, USA). The Bioanalyzer Expert software (Agilent) was used to calculate RNA integrity numbers, which were above 9.0 in all samples. For Genechip analysis, we followed the standard protocol provided by Affymetrix (Santa Clara, CA, USA) for converting the polyA + fraction of ~ 1.5 µg total RNA into double-stranded cDNA, which was purified with a GeneChip sample cleanup module column and then used as template for in vitro transcription into biotin-labelled complementary RNA (cRNA). We used reagents and materials contained in the GeneChip Expression 3’ Amplification One-Cycle Target Labelling Kit (Affymetrix). Both cRNA preparations and fragmented cRNA samples were quality controlled by analysing on an Agilent 2100 Bioanalyzer. Hybridization was performed to HG-U133 Plus 2.0 GeneChips (Affymetrix) for 16 h at 45 °C with constant rotation at 60 r.p.m. Washing, staining and scanning of the chips was performed using the Fluidics 450 Station and the GeneChip 3000 7G Scanner following the manufacturer’s protocols.

Scanned raw images were processed with GeneChip Operating Software 1.4 (Affymetrix). A quality control report was subsequently generated using the chips was performed using the Fluidics 450 Station and the GeneChip 3000 7G Scanner following the manufacturer’s protocols.

Statistical analyses. For statistical analyses, Prism 5 software package (GraphPad, San Diego, CA, USA) was used. The values were expressed as mean ± s.e.m. and the Student’s t-test was used to compare differences between controls and individual samples, whereas analyses of variance (one-way ANOVA together with Dunnett’s post-test) was used to analyse treatment groups. Statistical significance level was set to P < 0.05.

RESULTS

Resistance to FUL occurs in the presence of functional ER. Naive MCF-7 cells were exposed to increasing concentrations of FUL (application schedule described in ‘Materials and Methods’) to generate FUL-resistant cells (FR.MCF-7; Figure 1A). After 1, 2, 4 and 6 months of FUL treatment, the gene expression profiles were analysed by GeneChip (Affymetrix) (Table 1). Gene expression profiling showed that ERα, ERβ and its target progesterone receptor (PGR) were not downregulated after long-term treatment, but expressed at similar levels in FR.MCF-7 and naive MCF-7 cells, which was confirmed by western blotting (data not shown). FR.MCF-7 cells still responded to 100 nM E2 with increased proliferation (Figure 1B) and to TAM with retarded cell growth (Figure 1C). Thus, neither did long-term exposure to FUL downregulate ERs constitutively (Fan et al, 2006) nor did ERz lose its functionality. However, FR.MCF-7 cells were less sensitive to TAM than naive MCF-7 cells, and therefore, FUL resistance partly compromised a common response mechanism.

GPER and CDK6 are regulated by siRNA knockdown. GPER and CDK6 are overexpressed in FR.MCF-7 cells (Figure 1A). Accordingly, GREB1 expression was suppressed by AZA and TSA. Hence, gene expression was examined after treatment of FR.MCF-7 cells with AZA, with and without TSA (Figure 2). For GPER, the expression was downregulated only by AZA, whereas the expression was upregulated by both AZA and TSA. For CDK6, the expression was downregulated by AZA, whereas the expression was upregulated by TSA. However, the expression was not regulated by AZA and TSA in naive MCF-7 cells. Hence, GPER and CDK6 are regulated by siRNA knockdown.

Aberrant CDK6 expression contributes to resistance. Aberrant CDK6 expression contributes to resistance against FUL. CDK6 expression is upregulated in FR.MCF-7 cells (Figure 1A). Hence, CDK6 expression was further investigated. CDK6 expression was upregulated in FR.MCF-7 cells, but not in naive MCF-7 cells. Hence, CDK6 expression contributes to FUL resistance.
## Table 1. Expression of selected genes during the acquisition of resistance to fulvestrant

### mRNA expression levels (log2 of fluorescence signal intensities)

| Genes | Naive MCF-7 | FUL-1m | FUL-2m | FUL-4m | FUL-6m | FUL-1m | FUL-2m | FUL-4m | FUL-6m | FUL-1m | FUL-2m | FUL-4m | FUL-6m |
|-------|-------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| ER1/ER2 |            |        |        |        |        |        |        |        |        |        |        |        |        |
| .205235_at | 7.92940446 | 7.40746887 | 7.36516238 | 7.43586785 | 7.82339317 | -0.52 | -0.56 | -0.49 | -0.11 | 0.70 | 0.68 | 0.71 | 0.93 |
| ER3 |            |        |        |        |        |        |        |        |        |        |        |        |        |
| .21117_x_at | 2.41860147 | 2.41860147 | 2.41860147 | 2.41860147 | 2.41860147 | 0.00 | 0.00 | 0.00 | 0.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| .21118_x_at | 2.41860147 | 2.41860147 | 2.41860147 | 2.41860147 | 2.41860147 | 0.00 | 0.00 | 0.00 | 0.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| .21119_x_at | 2.41860147 | 2.41860147 | 2.41860147 | 2.41860147 | 2.41860147 | 0.00 | 0.00 | 0.00 | 0.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| .21120_x_at | 2.41860147 | 2.41860147 | 2.41860147 | 2.41860147 | 2.41860147 | 0.00 | 0.00 | 0.00 | 0.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| PGR |            |        |        |        |        |        |        |        |        |        |        |        |        |
| .208305_at | 2.46711754 | 2.41860147 | 2.41860147 | 2.41860147 | 2.41860147 | -0.23 | -0.23 | -0.23 | -0.23 | 0.85 | 0.85 | 0.85 | 0.85 |
| GREB1 |            |        |        |        |        |        |        |        |        |        |        |        |        |
| .205862_at | 8.91745287 | 2.41860147 | 2.41860147 | 2.41860147 | 2.41860147 | 0.00 | 0.00 | 0.00 | 0.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| .211119_at | 2.41860147 | 2.41860147 | 2.41860147 | 2.41860147 | 2.41860147 | 0.00 | 0.00 | 0.00 | 0.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| GPER |            |        |        |        |        |        |        |        |        |        |        |        |        |
| .227758_at | 6.60593075 | 2.97879521 | 3.13454835 | 2.70040478 | 3.00687154 | -3.63 | -3.47 | -3.91 | -3.60 | 0.08 | 0.09 | 0.07 | 0.08 |
| RERG |            |        |        |        |        |        |        |        |        |        |        |        |        |
| .223116_at | 5.01883611 | 5.643168 | 5.36275668 | 6.3038424 | 6.6016316 | 0.62 | 0.34 | 1.29 | 1.58 | 1.54 | 1.27 | 2.44 | 2.99 |
| BCAR1 |            |        |        |        |        |        |        |        |        |        |        |        |        |
| .204032_at | 4.51669978 | 5.81232156 | 5.38142902 | 5.80243424 | 6.04847713 | 1.30 | 0.86 | 1.29 | 1.53 | 2.45 | 1.82 | 2.44 | 2.89 |
| BCAR3 |            |        |        |        |        |        |        |        |        |        |        |        |        |
| HER2/ERBB2 |            |        |        |        |        |        |        |        |        |        |        |        |        |
| .216836_s_at | 5.7751358 | 7.06484765 | 6.74520161 | 6.7298695 | 6.8492997 | 1.29 | 0.97 | 0.95 | 1.07 | 2.44 | 1.96 | 1.94 | 2.10 |
| BCRP/ABCG2 |            |        |        |        |        |        |        |        |        |        |        |        |        |
| .209735_at | 4.91355091 | 7.5321778 | 7.25873884 | 8.11165114 | 8.39001347 | 2.62 | 2.35 | 3.20 | 3.48 | 6.14 | 5.08 | 9.18 | 11.13 |
| CDK6 |            |        |        |        |        |        |        |        |        |        |        |        |        |
| .224848_at | 3.7090632 | 5.46852278 | 5.74205374 | 6.13019766 | 5.55485763 | 2.46 | 2.03 | 2.43 | 1.85 | 5.50 | 4.09 | 5.37 | 3.59 |
| CDK4 |            |        |        |        |        |        |        |        |        |        |        |        |        |
| .202246_s_at | 9.33117127 | 8.73725404 | 8.359249013 | 9.15993938 | 9.02712602 | -0.59 | -0.74 | -0.17 | -0.30 | 0.66 | 0.60 | 0.89 | 0.81 |
| mRNA expression levels (log2 of fluorescence signal intensities) | Log2 changes | Fold changes |
|---------------------------------------------------------------|-------------|--------------|
| CyclinD1/CCND1                                                 |             |              |
| Naive MCF-7 FUL-1m FUL-2m FUL-4m FUL-6m FUL-1m FUL-2m FUL-4m FUL-6m FUL-1m FUL-2m FUL-4m FUL-6m |             |              |
| CyclinA1/CCNA1                                                 |             |              |
| Naive MCF-7 FUL-1m FUL-2m FUL-4m FUL-6m FUL-1m FUL-2m FUL-4m FUL-6m FUL-1m FUL-2m FUL-4m FUL-6m |             |              |
| Cyclin/ CCNE1                                                  |             |              |
| Naive MCF-7 FUL-1m FUL-2m FUL-4m FUL-6m FUL-1m FUL-2m FUL-4m FUL-6m FUL-1m FUL-2m FUL-4m FUL-6m |             |              |
| RB1                                                           |             |              |
| Naive MCF-7 FUL-1m FUL-2m FUL-4m FUL-6m FUL-1m FUL-2m FUL-4m FUL-6m FUL-1m FUL-2m FUL-4m FUL-6m |             |              |
| SMARCA4/BRG1                                                  |             |              |
| Naive MCF-7 FUL-1m FUL-2m FUL-4m FUL-6m FUL-1m FUL-2m FUL-4m FUL-6m FUL-1m FUL-2m FUL-4m FUL-6m |             |              |
| ARID1A/BAF250A                                                 |             |              |
| Naive MCF-7 FUL-1m FUL-2m FUL-4m FUL-6m FUL-1m FUL-2m FUL-4m FUL-6m FUL-1m FUL-2m FUL-4m FUL-6m |             |              |
| SMARCC2/BAF170                                                 |             |              |
| Naive MCF-7 FUL-1m FUL-2m FUL-4m FUL-6m FUL-1m FUL-2m FUL-4m FUL-6m FUL-1m FUL-2m FUL-4m FUL-6m |             |              |
| SMARCC1 BAF155                                                 |             |              |
| Naive MCF-7 FUL-1m FUL-2m FUL-4m FUL-6m FUL-1m FUL-2m FUL-4m FUL-6m FUL-1m FUL-2m FUL-4m FUL-6m |             |              |
| SMARCE1/BAF57                                                  |             |              |
| Naive MCF-7 FUL-1m FUL-2m FUL-4m FUL-6m FUL-1m FUL-2m FUL-4m FUL-6m FUL-1m FUL-2m FUL-4m FUL-6m |             |              |
| ACTL6A/BAF533                                                  |             |              |
| Naive MCF-7 FUL-1m FUL-2m FUL-4m FUL-6m FUL-1m FUL-2m FUL-4m FUL-6m FUL-1m FUL-2m FUL-4m FUL-6m |             |              |
| P53                                                           |             |              |

Aberrant CDK6 expression contributes to resistance.

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The cause for the aberrant expression of BCAR and ABCG2 remained unclear (Figure 2). Neither the specific inhibition of ABCG2 with FTC (Figure 3B) nor the downregulation of BCAR1 by specific siRNA (data not shown) re-established sensitivity to FUL in FR.MCF-7 cells.

Overexpression and activation of ERBB2 indicates the acquisition of an ER-independent growth mechanism in TAM-insensitive breast cancer (Ghayad et al., 2010), and therefore, it was tested whether ERBB2 (although an increased expression could not be confirmed by Q-PCR) contributed to insensitivity to FUL. For this, FR.MCF7 cells were treated with 1 \( \text{mg} \cdot \text{ml}^{-1} \) TRA, which is a specific inhibitor of ERBB2. Trastuzumab had no effect on the proliferation of FR.MCF-7 cells (Figure 3C), and therefore, basal ERBB2 expression did not desensitise breast cancer cells to FUL.

CDK6 contributes to FUL resistance. The overexpression of CDK6 was confirmed by western blotting (Figure 4A). The expression of CDK4 was unchanged. The study of cell cycle protagonists such as CDK6 by siRNA approaches is difficult,
because resulting transfectants cannot be expanded for subsequent analyses. Therefore, we chose a different approach to confirm or disregard whether CDK6 has a role in FUL resistance by analysing the phosphorylation status of serine 780 of retinoblastoma protein (RB1), which is the direct target of CDK6. Indeed, in FR.MCF-7 cells Ser780RB1 was constitutively phosphorylated (thereby inactivating RB1). As a consequence, the indirect downstream effector of inactivated RB1, CCNA1, was also overexpressed (Henglein et al., 1994). Fulvestrant treatment induced CDK6 in naive cells after 24 and 48 h, which remained constitutively high in FR.MCF-7 cells (Figure 4B). Interestingly, the expression of CCNE1 was reduced (Figure 4A) and CCND1 was also slightly downregulated in FR.MCF-7 cells (Table 1, Figure 4B). Thus, FR.MCF7 cells became independent of CCND1 and CCNE1 expression, which was surprising since CCND1 was shown to have a significant role in the development of breast cancer (Dean et al., 2010). Furthermore, FUL treatment induced p21, particularly in FR.MCF-7 cells, and this suggested that the resistant cells managed to bypass effectively the p21 cycle arrest signal (Figure 4B). The loss of CCND1 dependence was supported by the fact that the proliferation of FR.MCF-7 cells was significantly faster than that of naive MCF-7 cells (Figure 4C). Hence, the constitutive down-regulation of CCND1 should not be considered as a cause, but rather as a consequence of resistance.

The role of high CDK6 expression was tested by treating FR.MCF-7 cells and naive MCF-7 cells with the specific inhibitor PD0332991. The proliferation of FR.MCF-7 cells was significantly more attenuated by 50 nM PD0332991 than that of naive cells (Figure 4D). Rendering FR.MCF-7 cells more susceptible to PD0332991 strongly indicated that the rapid growth of these cells relied on the high expression of CDK6. The functionality of CDK6 in FR.MCF-7 cells was confirmed by the inhibited phosphorylation of Ser780RB1 upon PD0332991 treatment (Figure 4E). Therefore, CDK6 activity contributed to unrestricted cell growth, which was acquired during long-term treatment with FUL. The upregulation of CDK6 during PD0332991 treatment might have been a compensatory feedback mechanism to keep up CDK6 signalling.

Treatment with FUL modulates the expression of subunits of the hSWI/SNF chromatin remodelling complex. The catalytic subunit of hSWI/SNF (human SWItch/Sucrose NonFermentable),

![Figure 2. Gene expression upon treatment with AZA and TSA.](https://example.com/figure2)

Fulvestrant-resistant MCF-7 cells were pretreated for 24 h with 100 ng ml⁻¹ TSA and for 72 h with 2.5 μM AZA. Then, cells were lysed, RNA was extracted, mRNA reverse transcribed to cDNA and Q-PCR performed. The expression levels of GREB1, BCAR1, BCAR3, GPER, CDK6, ABCG2 and ERBB2 were standardised to GAPDH mRNA expression in FR.MCF-7 cells and naive MCF-7 cells. Experiments were carried out in duplicate and error bars indicate s.e.m.

![Figure 3. Testing of specific gene products required for the cellular response to FUL.](https://example.com/figure3)

(A) MCF-7 cells were transfected with the indicated small interfering RNAs (siRNAs) or with control RNA in which no complementary cellular RNA exists, were treated with 500 nM FUL or solvent (Co) for 48 h and then cells were counted. (B, C) Fulvestrant-resistant MCF-7 cells were exposed to 500 nM FUL alone or (B) in combination of 2.5 μM FTC or (C) in combination with 1 μg ml⁻¹ TRA, and the cell number was measured after 48 h. Experiments were carried out in triplicate, error bars indicate s.e.m. and asterisks denote significance (t-test).
the ATPase BRG1, binds directly to RB1 (Strobeck et al, 2000; Zhang et al, 2000), and furthermore, BRG1 is strictly required to maintain ER function (Ichinose et al, 1997; Belandia et al, 2002; Inoue et al, 2002; Reisman et al, 2009). Hence, hSWI/SNF links the RB1 pathway to ER function and may have a role in the acquisition of FUL resistance, which was shown to involve RB1 signalling (Thangavel et al, 2011).

Acetylases, deacetylases and the hSWI/SNF chromatin remodeling complex are tightly associated (Zhang et al, 2000; Naidu et al, 2009) with each other, and in dependence of the hSWI/SNF subunit composition, the gene expression pattern changes (Nagl et al, 2007; Jones et al, 2010). Therefore, the expression of constant (BAF47, BAF53A, BAF57, BAF155, BAF170) and of variable (BRG1, BAF250A) subunits of the hSWI/SNF chromatin remodeling complex was analysed. Upon FUL treatment, BAF250A, BAF155 and BAF47 became transiently upregulated and BGR1 and BAF170 downregulated in naive MCF-7 cells (Figure 5). This stress response to FUL was also observed for BRG1, BAF250A and BAF47 in FR.MCF-7 cells, although the response times were attenuated and weaker for BAF47 and accelerated for BRG1.

In FR.MCF-7 BAF155 did not respond any longer to FUL and BAF170 became even induced, which was contrary to the response observed in naive MCF-7 cells (Figure 5). The expression of BAF53A and BAF57 remained unchanged in both cell lines. The constitutive upregulation of BAF250A protein in FR.MCF-7 cells was also reflected by the slight increase of the transcript, whereas...
constitutive upregulation of BAF155 and downregulation of BAF170 was likely due to post-transcriptional/post-translational events, because from the GeneChip data both genes were similarly expressed in naive and resistant cells (Table 1). The constitutive changes of gene expression as well as the aberrant stress response are attributes of the acquired resistance phenotype.

The expression of MYC and p53 was shown to be under the control of the hSWI/SNF complex (Albert et al., 2001; Lee et al., 2003, 2005; Chung and Levens, 2005; Chen et al., 2006, 2007; Sims et al., 2007; Naidu et al., 2009). Fulvestrant treatment downregulated the expression of MYC and p53 in naive MCF-7 cells, which correlated inversely with the expression of BAF47 (BAF47 is bona fide tumour suppressor).

The stress response of MYC and p53 to FUL was suspended in FR.MCF-7 cells and also BAF47 induction was weak and delayed. This indicated that hSWI/SNF regulated MYC and p53 expression in MCF-7 cells when treated with FUL, and that abrogation of MYC and p53 regulation was integral to acquired FUL resistance. Interestingly, the MYC transcript in FR.MCF-7 cells as one-third of that in naive MCF-7 cells, yet the protein was almost similarly expressed in both cell lines (Figure 5).

In addition to the direct binding to target genes transient activation of hSWI/SNF was reported to permanently reposition nucleosomes (Schnittler et al., 2001; Ulyanova and Schnitzler, 2005), leading to long-lasting changes in gene expression patterns. This was corroborated by the fact that FUL-induced CDK6 in naive MCF-7 cells and that high CDK6 expression was maintained in FR.MCF-7 cells (Figure 4B). CDK4 expression was not induced by FUL and was similar in naive and FR.MCF-7 cells. Fulvestrant did not further induce CDK6 expression in FR.MCF-7 cells (Figure 4B) as it was already high and CDK6 levels correlated directly with the expression of BAF155 in both cell lines (Figure 5). Thus, affecting nucleosome control by FUL may have been the mechanism responsible for the endurance of CDK6 upregulation, even after withdrawal of FUL, and for acquired resistance.

DISCUSSION

The aim of this investigation was to elucidate the cellular mechanisms that become involved during the acquisition of FUL resistance. Well-known protagonists mediating drug resistance in breast cancer cells, such as activation of ERBB2 (Hu and Mokbel, 2001; Chung et al., 2002; Shou et al., 2004; Gutiérrez et al., 2005) or the overexpression of the breast cancer resistance protein BCRP/ABCG2 (Selever et al., 2011), did not seem to contribute to resistance in this model system nor was loss or inactivation of ERs causal for FUL resistance. However, repression of GPER, an endoplasmic reticulum-bound receptor for oestrogen derivatives (Revankar et al., 2005), caused a significant decrease in the sensitivity to FUL. Hence, GPER partly mediates growth inhibition triggered by FUL. Also GREB1, an oestrogen-regulated gene, exhibited a similar property, although GREB1 did not contribute to resistance in the generated FR.MCF-7 cells.

Recently, it was reported that inactivated RB1 (through phosphorylation of Ser780) caused insensitivity to FUL in LCC9 breast cancer cells and that lack of signature RB1 target regulation is a hallmark of breast cancer cells with spontaneous and acquired resistance to SERM treatment (Lange and Yee, 2011; Thangavel et al., 2011). Disruption of RB1 control was due to maintenance of CCND1 expression, thereby keeping CDK activity high (Thangavel et al., 2011). However, the present study demonstrates that FR.MCF-7 cells became independent of CCND1, because they proliferated significantly faster than naive MCF-7 cells despite reduced CCND1 levels. Instead, CDK6, which is associated with CCND1, was overexpressed, thereby contributing to FUL resistance and CDK6-dependent cell growth made FR.MCF-7 cells more susceptible to the specific inhibitor PD0332991. Thus, resistance of hormone-sensitive breast cancer cells impinging on the RB1 pathway, yet the causal upstream players may vary (Thangavel et al., 2011). Notably, p21 induction by FUL could not arrest FR.MCF-7 cell proliferation underscoring how powerful CDK6-mediated resistance was. This recommends the reconstitution of the RB1 pathway as a subject to target tailored second-line adjuvant therapy. Twenty-one clinical trials testing PD0332991 against different cancer entities are currently recruiting, are active, or have been completed (following trials focus on breast cancer: ‘PD0332991/Paclitaxel in Advanced Breast Cancer’ – ClinicalTrials.gov Identifier: NCT01320592; ‘A Study Of PD-0332991 (Cyclin Dependent Kinase 4/6 Inhibitor) In Japanese Patients With Advanced Solid Tumors’ – ClinicalTrials.gov Identifier: NCT01684215 – both phase I studies, still recruiting; ‘PD 03 32991 and Anastrozole for Stage 2 or 3 Estrogen Receptor Positive and HER2 Negative Breast Cancer’ – ClinicalTrials.gov Identifier: NCT01723774; ‘Letrozole and CDK 4/6 Inhibitor for ER Positive, HER2 Negative Breast Cancer in Postmenopausal Women’ – ClinicalTrials.gov Identifier: NCT01709370 – both phase II studies and still recruiting; ‘Study Of Letrozole With Or Without PD 03 32991 For The First-Line Treatment Of Hormone-Receptor Positive Advanced Breast Cancer’ – ClinicalTrials.gov Identifier: NCT00721409, a phase I/II study and still active; ‘A Study of PD-0332991 + Letrozole vs Letrozole For 1st Line Treatment Of Postmenopausal Women With ER+/HER2– Advanced Breast Cancer’ – ClinicalTrials.gov Identifier: NCT01740427, a phase III study and still recruiting).

The overexpression of CDK6 was reversed by TSA (but not AZA), indicating a functional involvement of nucleosome acetylation in the aberrant expression of CDK6 and in the acquisition of FUL resistance.
resistance. Acquired TAM resistance was also shown to involve chromatin remodelling through nucleosome acetylation (Badia et al., 2000).

Another mechanism that contributed to FUL resistance was DNA methylation, as the downregulation of GPER was reversed by AZA (but not TSA). Nucleosome (histone) acetylation and DNA (CpG island) methylation requires the accessibility of acetyl transferases and methylases, respectively, to previously protected areas. The positioning of nucleosomes on the DNA (regulated by chromatin remodelling complexes) and the higher order structure of the histone octamer core (controlled by acetyl transferases and deacetylases) facilitate stochastic access for transcription factors to bind promoter regions, or methylases to switch off genes epigenetically. Acetyl transferases, histone deacetylases and chromatin remodelling complexes such as hSWI/SNF were shown to cooperate in this process (Zhang et al., 2000; Naidu et al., 2009). Human SWItch/Sucrose NonFermentable is involved in embryonic development, differentiation and cancer (Reisman et al., 2009) and alters nucleosome positioning (Flaus and Owen-Hughes, 2001; Sims et al., 2007, 2008). Human SWItch/Sucrose NonFermentable consists of ‘core’ and ‘variable’ subunits and changing their composition triggers the movement of nucleosomes from high-affinity (default) positions to different DNA regions (Nagl et al., 2007; Jones et al., 2010). Redistribution of histone octamers within the chromatin by hSWI/SNF causes deprotection or occlusion of DNA regions facilitating or preventing the access to DNA and giving rise to aberrant gene expression. Human SWItch/Sucrose NonFermentable was shown to regulate the expression of p21 and CCNA1 (Murphy et al., 1999) and the subunits BAF250A and BAF47, both described as bona fide tumour suppressors, down-regulate MYC (Nagl et al., 2006, 2007) and CCND1 (Rao et al., 2008), as it was observed in our investigation. BAF250A-containing hSWI/SNF complexes repress E2F (Van Rechem et al., 2009) and E2F is required for the expression of MYC (Oswald et al., 1994). Here we show that FUL induced BAF47 and this correlated with MYC downregulation in naive MCF-7 cells, whereas this axis was disturbed in FR.MCF-7 cells.

The catalytic centre of hSWI/SNF, BRG1, is a transcriptional coactivator of p53 and of nuclear hormone receptors (Chen et al., 2006; Simons, 2006). Taken together with BAF170 and BAF155, BRG1 integrates signals of anti-oestrogens directly at the ER promoter (Zhang et al., 2000). This is in agreement with the reduced responsiveness of FR.MCF-7 cells to TAM, because in FR.MCF-7 cells the expression patterns of these subunits was tilted: BRG1 expression was less robust in FR.MCF-7 cells than in naive cells, and BAF170 was repressed and BAF155 overexpressed. Decreased BRG1 levels predispose mice to cancer formation (Klochendler-Yeivin et al., 2002; Simons, 2006), whereas another investigation demonstrates the capacity of BRG1 to induce a tumour-initiating cell phenotype (Okamoto et al., 2011). Therefore, the expression of BRG1 acts in different directions depending on the molecular context.

The composition of the hSWI/SNF subunits is subject to alterations and represents an own level of control to access DNA. Changes in the hSWI/SNF subunit constitution ATP dependently redistributes nucleosomes from default DNA positions to other nucleotide sequences within the chromatin (Schnitzler et al., 2001; Ulyanova and Schnitzler, 2005; Teif and Rippe, 2009) with consequences for chromatin structure and gene expression. Resumption to normal subunit composition may shuttle nucleosomes back to their default positions. This would explain the transient nature of MYC and p53 repression. However, based on the array data, it is more likely that FUL suppressed both genes at a post-transcriptional level. Even transient alterations in hSWI/SNF subunit composition were shown to provoke a steady-state redistribution of nucleosomes (Schnitzler et al., 2001; Ulyanova and Schnitzler, 2005) causing enduring changes in gene expression, that is, when hSWI/SNF subunits become directly affected.

Notably, we observed stable overexpression of BAF250A and BAF155 and constitutive suppression of BAF170 in FR.MCF-7 cells and FUL treatment swiftly caused this induction/inhibition of BAF250A/BAF155/BAF170 (respectively) already in naive cells. Hence, the increase of constitutive CDK6 expression (at the transcriptional and translational level) can be explained as an immediate consequence of aberrant hSWI/SNF subunit expression and chromatin remodelling upon FUL treatment, which ultimately resulted in FUL resistance. It is further of note that the expression of BAF155 and CDK6 correlated in both cell strains. BRG1, containing the catalytic centre and being responsible for the general activity of hSWI/SNF, was only transiently downregulated by FUL treatment and remained expressed in FR.MCF-7 cells. Therefore, specifically and temporally inhibiting BRG1 activity throughout adjuvant therapy might prevent the redistribution of nucleosomes through inactivation of hSWI/SNF and possibly the development of a resistance phenotype.

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