FULL PAPER

BJC

British Journal of Cancer (2013) 109, 2751–2762 | doi: 10.1038/bjc.2013.583

Keywords: fulvestrant resistance; MCF-7 breast cancer cells; GPER; CDK6; hSWI/SNF

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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Received 6 August 2013; revised 29 August 2013; accepted 30 August 2013; published online 29 October 2013

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 ERα 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 (Selever *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 resistance was achieved by treating MCF-7 cells with increasing concentrations (up to 1 μM) of FUL over a period of 6 months and the resistant cell line (FR.MCF-7) was maintained in the above-described medium and in the presence of 500 nM FUL. All cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2. If not mentioned otherwise, all media and supplements were obtained from Invitrogen (Karlsruhe, Germany).

Reagents and antibodies. 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), p21 (C-19; Cat. no. sc-397), cyclin A (H-432; Cat. no. sc-751), cyclin E (M-20; Cat. no. sc-481), CDK 4 (C-22; Cat. no. sc-260), α-tubulin (TU-02; Cat. no. sc-8035) and β-tubulin (H-235; Cat. no. sc-9104) were from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA, USA). Anti-MYC (AB-02, 9E10; Cat. no. MS-139-D1) was purchased from Neomarkers (Fremont, CA, USA), and p53 antibody (Cat. no. 1767) from Immunotech (Marseille, France). Anti-CDK 6 (Cat. no. 3136), retinoblastoma (RB1; Cat. no. 9309), phospho(Ser780)RB1 (Cat. no. 3590), SmarcC2/BAF170 (Cat. no. 8829) and SmarcC1/BAF 155 (Cat. no. 9053) were from Cell Signalling (Danvers, MA, USA). Anti-ARID1A/BAF250A (Cat. no. ab-50878) and SmarcA4/BRG1 (Cat. no. ab-4081) and anti-SNF5/BAF47 (EPR6966; Cat. no. ab-126734) were from Abcam (Cambridge, UK), anti-SmarcE1/BAF57 (Cat. no. NB100-2591) from Novus Biologicals (Cambridge, UK), anti-ACTL6A/BAF53A (Cat. no. A301-391 A) from Bethyl Antibodies (Montgomery, TX, USA) and anti-mouse and anti-rabbit IgGs were from Dako (Glostrup, Denmark).

SDS gel electrophoresis and western blotting. Cells were grown in petri dishes (6 cm diameter) to 80% confluence and treated with 500 nM FUL or 50 nM PD0332991. Then, cells were washed two times with cold PBS and lysed in a buffer containing 150 mM NaCl, 50 mM Tris (pH 8.0), 1% Triton X-100, 1 mM phenylmethylsulphonyl fluoride and 1 mM Protease Inhibitor Cocktail (Sigma-Aldrich). The lysates were centrifuged at 12 000 r.p.m. for 20 min at 4 °C and the supernatants transferred to 1.5 ml tubes and stored at −20 °C until further analysis. Equal amounts of protein lysate were mixed with (2 ×)SDS (sodium dodecyl sulphate) sample buffer, separated by 10% polyacrylamide gel electrophoresis containing SDS (SDS–PAGE) and electrotransferred onto PVDV membranes (Hybond-P; Amersham), 4 °C overnight. Western blotting was performed according to the protocol described by Giessrigl *et al* (2012). In short, staining membranes with Ponceau S controlled equal sample loading, and after washing with Tris-buffered saline (TBS) (pH 7.6), membranes were blocked in 5% non-fat dry milk in TBS containing 0.1% Tween-20 for 1 h. Membranes were incubated with the first antibody (in blocking solution, dilution 1 : 500–1 : 1000) by gently rocking at 4 °C overnight, washed with TBS containing 0.1% Tween-20 and further incubated with the second antibody (peroxidase-conjugated swine anti-rabbit IgG or rabbit anti-mouse IgG, dilution 1 : 2000–1 : 5000 in blocking solution) for 1 h. Chemoluminescence was developed by the ECL plus detection kit (GE Healthcare, Buckinghamshire, UK) and analysed using a Lumi-Imager F1 Workstation (Roche, Basel, Switzerland).

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:

$$\text{doubling time} = \log 2 \times h \text{ cultivation time}/\log N - \log N0,$$

where h is the cultivation time, N is the cell number after the time of cultivation and $N0$ 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 glyceral-aldehyde 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, HS00981957; 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 (1.0×10^5) were seeded onto 6 cm cell culture plates and cultivated for 24 h. On the day of transefction, 3.6 μg siRNA (corresponding to 360 nM final concentration) was diluted in 500 μl medium. Forty-three microlitres 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 1×10^5 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 data images were processed with GeneChip Operating Software 1.4 (Affymetrix). A quality control report was subsequently made using Bioconductor (open source software for bioinformatics; hosted by Fred Hutchinson Cancer Research Center, Seattle, WA, USA), and normalization and signal extraction was carried out with the RMA (Robust Multichip Average) approach (Bolstadt *et al*, 2003).

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 ERα 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.

Aberrant mRNA expression of GPER and CDK6 is reversed by AZA and TSA, respectively. Expression arrays revealed that ERs and PGR levels were unchanged in FR.MCF-7 cells, yet genes associated with oestrogen signalling (*RERG*, *GREB1* and *GPER*) were repressed. Genes known to contribute to chemoresistance of breast cancer, that is, *BCAR1* (poor overall survival when overexpressed), *BCAR3* (homologous to *BCAR1*), *ABCG2* and *ERBB2* (van Agthoven *et al*, 1998; van der Flier *et al*, 2000; Hu and Mokbel, 2001; Selever *et al*, 2011), were upregulated in FR.MCF-7 cells (Table 1). Notably, CDK6 expression was also increased. However, only the downregulation of GPER and the upregulation of BCAR1, ABCG2 and CDK6 could be confirmed in FR.MCF-7 cells by Q-PCR (Figure 2), but not the aberrant expression levels of GREB1, BCAR3 and ERBB2.

Deprotection of genomic DNA regions can be triggered by modulating the acetylation of histone octamers. In consequence, promoter and enhancer regions, which were previously masked by nucleosomes, become now accessible by methyltransferases. The methylation of CpG islands prevents transcription factor binding and this causes changes in overall protein expression, which may lead to chemoresistance (Chang *et al*, 2005; Fan *et al*, 2006; Fiegl *et al*, 2006). Tamoxifen was shown to epigenetically modulate gene expression that is relevant to acquired resistance (Encarnación *et al*, 1993; Badia *et al*, 2000; Stone *et al*, 2012; Vesuna *et al*, 2012). Hence, gene expression was examined after treatment of FR.MCF-7 cells with TSA (inhibitor of class I and II mammalian histone deacetylases) and AZA (cytidine analogue that inhibits methyltransferases, thereby hypomethylating the DNA) to modulate nucleosome acetylation and to reverse CpG methylation, respectively. Treatment with AZA restored GPER expression in FR.MCF-7 cells to approximately similar levels as in naive MCF-7 cells, but GPER expression remained virtually unaffected upon TSA treatment in the resistant cell line. Conversely, the increased expression of CDK6 in FR.MCF-7 cells was significantly reversed by TSA but became highly induced by AZA. In contrast, aberrant expression levels of BCAR1 and ABCG2 did not revert to normal levels neither by AZA nor by TSA, but were increased even further. The expression of GREB1 was suppressed by AZA and TSA (Figure 2). Hence, GPER inhibition and CDK6 induction in FR.MCF-7 cells were likely due to methylation and deacetylation events, respectively.

GPER contributes to FUL resistance. The siRNA-mediated downregulation of GPER in naive MCF-7 cells decreased the sensitivity to FUL (Figure 3A), suggesting that repression of GPER contributed to FUL resistance in FR.MCF-7 cells. Notably, siRNA suppressed gene expression only by 50% (data not shown), and therefore, the acquisition of a FUL-resistant phenotype by naive MCF-7 cells was weak, yet significant. For control reasons, the expression of GREB1 and RERG was knocked down by specific siRNAs. As with GPER, the suppression of GREB1 reduced the sensitivity to FUL in naive MCF-7 cells, whereas suppression of RERG did not (Figure 3A). Hence, GREB1 can also contribute to FUL resistance (however, the aberrant GREB1 expression detected by GeneChip could not be confirmed by Q-PCR).

Table 1. Expression of selected genes during the acquisition of resistance to fulvestrant

	mRNA expresion levels (log_2 of fluorescence signal intensities)					Log_2 changes				Fold changes			
	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/ERα													
205225_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
ERβ													
211117_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
211118_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
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
211120_x_at	2.41931594	2.41931594	2.41931594	2.41997222	2.41931594	0.00	0.00	0.00	0.00	1.00	1.00	1.00	1.00
PGR													
208305_at	2.64711754	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	2.4664246	2.41860147	2.41860147	−6.50	−6.45	−6.50	−6.50	0.01	0.01	0.01	0.01
GPER													
211829_s_at	5.46993059	2.52293077	2.45559364	2.45517669	2.45559364	−2.95	−3.01	−3.01	−3.01	0.13	0.12	0.12	0.12
RERG													
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
244745_at	5.2169564	2.48191844	2.4877962	2.47777047	2.4877962	−2.74	−2.73	−2.74	−2.73	0.15	0.15	0.15	0.15
BCAR1													
223116_at	5.01883611	5.643168	5.36275568	6.3038424	6.60106316	0.62	0.34	1.29	1.58	1.54	1.27	2.44	2.99
BCAR3													
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
HER2/ERBB2													
216836_s_at	5.77551358	7.06484765	6.74520161	6.7298695	6.84912997	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	3.70906532	6.16852278	5.74205374	6.13519766	5.55485673	2.46	2.03	2.43	1.85	5.50	4.09	5.37	3.59
243000_at	3.587304	4.85766838	4.79266751	4.90976999	5.30803138	1.27	1.21	1.32	1.72	2.41	2.31	2.50	3.30
224851_at	3.05994179	6.60058684	5.71180726	6.51980722	5.63511869	3.54	2.65	3.46	2.58	11.64	6.28	11.00	5.96
224847_at	3.99474216	7.05347981	6.6185564	6.85674145	6.41759533	3.06	2.62	2.86	2.42	8.33	6.16	7.27	5.36
CDK4													
202246_s_at	9.33117127	8.73725404	8.59249013	9.15993938	9.02712602	−0.59	−0.74	−0.17	−0.30	0.66	0.60	0.89	0.81

Table 1. (Continued)

	mRNA expresion levels (log_2 of fluorescence signal intensities)					Log_2 changes				Fold changes			
	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
CyclinD1/CCND1													
208711_s_at	8.27783749	7.46386809	7.07873121	7.31080566	7.4054925	−0.81	−1.20	−0.97	−0.87	0.57	0.44	0.51	0.55
208712_at	8.92246862	8.10212307	8.17391194	7.69010283	8.02100152	−0.82	−0.75	−1.23	−0.90	0.57	0.60	0.43	0.54
CyclinA1/CCNA1													
205899_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
CyclinE/ CCNE1													
213523_at	4.34233009	4.37096959	3.71975855	4.33789978	4.0303634	0.03	−0.62	0.00	−0.31	1.02	0.65	1.00	0.81
242105_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
RB1													
211540_s_at	2.86046602	2.87976199	2.90609183	2.90609183	2.91292798	0.02	0.05	0.05	0.05	1.01	1.03	1.03	1.04
203132_at	7.38003719	7.06530219	7.34428805	7.11457959	7.42035597	−0.31	−0..04	−0.27	0.04	0.80	0.98	0.83	1.03
SMARCA4/BRG1													
208793_x_at	5.16202275	4.80539527	5.15704531	4.99218921	5.17509037	−0.36	0.00	−0.17	0.01	0.78	1.00	0.89	1.01
215714_s_at	6.71440874	6.24765247	6.39431904	6.43093232	6.62048264	−0.47	−0.32	−0.28	−0.09	0.72	0.80	0.82	0.94
212520_s_at	6.38741158	6.24566189	6.38386571	6.37892656	6.62667113	−0.14	0.00	−0.01	0.24	0.91	1.00	0.99	1.18
208794_s_at	8.61684233	8.34363613	8.4184534	8.38562077	8.49922772	−0.27	−0.20	−0.23	−0.12	0.83	0.87	0.85	0.92
213720_s_at	6.35984633	6.04999371	6.13949257	6.0162242	6.49780524	−0.31	−0.22	−0.34	0.14	0.81	0.86	0.79	1.10
ARID1A/BAF250A													
210649_s_at	5.68719128	5.47550997	6.21648019	6.05608337	6.15312261	−0.21	0.53	0.37	0.47	0.86	1.44	1.29	1.38
212152_x_at	6.16330059	6.03982007	6.54052607	6.49387141	6.57385219	−0.12	0.38	0.33	0.41	0.92	1.30	1.26	1.33
218917_s_at	6.30735516	6.440982	6.96408687	6.61187399	6.81505207	0.13	0.66	0.30	0.51	1.10	1.58	1.24	1.42
SMARCC2/BAF170													
201321_s_at	7.04707272	6.68098868	6.83795129	6.99340344	7.05593534	−0.37	−0.21	−0.05	0.01	0.78	0.87	0.96	1.01
SMARCC1 BAF155													
201073_s_at	4.97628363	4.95107633	4.99645381	4.91913572	5.04197256	−0.03	0.02	−0.06	0.07	0.98	1.01	0.96	1.05
201074_at	6.00222812	5.81380004	6.00971049	5.77978897	5.94965998	−0.19	0.01	−0.22	−0.05	0.88	1.01	0.86	0.96
201075_s_at	5.06128208	4.94809987	4.99815766	4.8526484	5.03390909	−0.11	−0.06	−0.21	−0.03	0.92	0.96	0.87	0.98
SMARCE1/BAF57													
211989_at	7.17721173	7.0890423	7.03167625	7.18292463	7.31623833	−0.09	−0.15	0.01	0.14	0.94	0.90	1.00	1.10
ACTL6A/BAF53A													
202666_s_at	8.49571128	7.69870853	8.108847	8.0617749	8.36929896	−0.80	−0.39	−0.43	−0.13	0.58	0.76	0.74	0.92
P53													
211300_s_at	6.44222284	6.65705658	6.77750484	6.61797418	6.50794689	0.21	0.34	0.18	0.07	1.16	1.26	1.13	1.05
201746_at	8.14239378	7.91879239	8.04818043	7.90568687	7.68159608	−0.22	−0.09	−0.24	−0.46	0.86	0.94	0.85	0.73

Table 1. (Continued)

	mRNA expresion levels (log_2 of fluorescence signal intensities)					Log_2 changes				Fold changes			
	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
MYC													
202431_s_at	6.88605453	6.19927673	5.93264485	6.12741337	5.29210142	−0.69	−0.95	−0.76	−1.59	0.62	0.52	0.59	0.33
β-Tubulin													
209026_x_at	10.6524403	10.2076006	10.3941924	10.505446	10.7789747	−0.44	−0.26	−0.15	0.13	0.73	0.84	0.90	1.09
211714_x_at	11.4517527	10.9892812	11.1830457	11.378038	11.5406703	−0.46	−0.27	−0.07	0.09	0.73	0.83	0.95	1.06
212320_at	10.4028034	10.1465532	10.1741946	10.5249237	10.3546692	−0.26	−0.23	0.12	−0.05	0.84	0.85	1.09	0.97
α-Actin													
224594_x_at	13.3896002	13.4093731	13.4914716	13.4348962	13.311052	0.02	0.10	0.05	−0.08	1.01	1.07	1.03	0.95
200801_x_at	13.4138194	13.4553181	13.5406988	13.4912622	13.3463601	0.04	0.13	0.08	−0.07	1.03	1.09	1.06	0.95
213867_x_at	13.4232841	13.3985462	13.493612	13.4381171	13.3237501	−0.02	0.07	0.01	−0.10	0.98	1.05	1.01	0.93

Naive MCF-7 cells were treated with fulvestrant (FUL) and mRNA was isolated after 1, 2, 4, and 6 months (1m, 2m, 4m, 6m) of treatment, subjected to GeneChip analysis and compared with the gene expression of naive MCF-7 cells. Enlisted are only the genes which were analysed in this study.

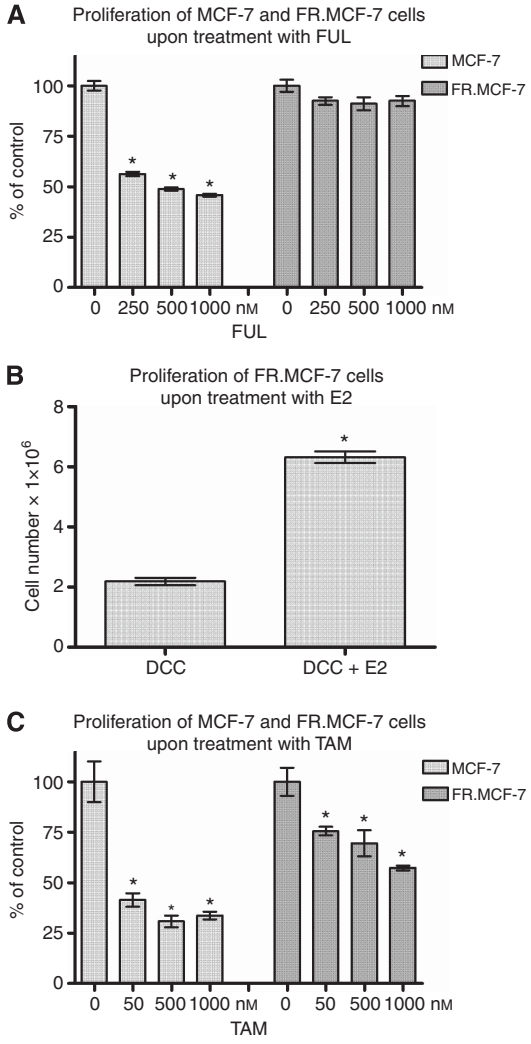

Figure 1. Sensitivity of MCF-7 and FR.MCF-7 cells to FUL, TAM and E2. (**A**) MCF-7 and FUL-resistant (FR) MCF-7 cells were exposed to increasing concentrations of FUL or (**C**) TAM and cell proliferation was measured after 48 h. (**B**) Fulvestrant-resistant.MCF-7 cells were grown in hormone-deprived (DCC) medium ± E2 for 96 h when cells were counted. Experiments were carried out in triplicate and error bars indicate s.e.m. and asterisks denote significance. (**A**, **C**) One-way ANOVA and Dunnett's post-test and (**B**) *t*-test.

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.MC-7 cells were treated with 1 $\mu g\,ml^{-1}$ 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,

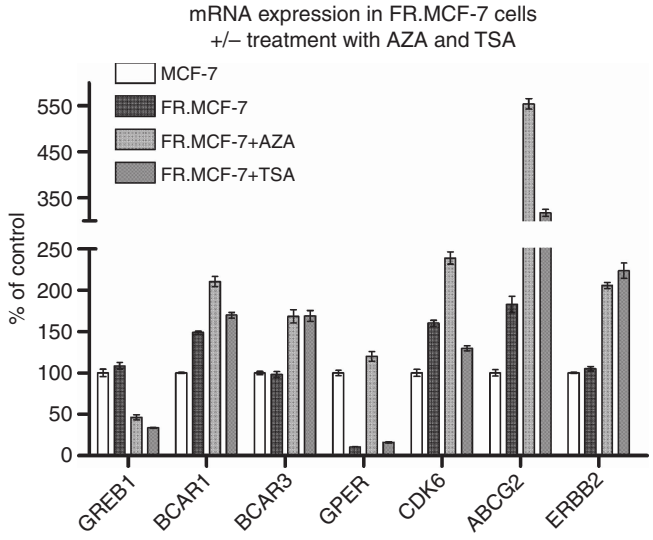

Figure 2. Gene expression upon treatment with AZA and TSA. Fulvestrant-resistant.MCF-7 cells were pretreated for 24 h with 100 ng ml^{-1} TSA and for 72 h with 2.5 μM AZA. Then, cells were lysed, RNA was extracted, mRNA reverse transcribed to cDNA and Q-PCR performed. The expression levels of GREB1, 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.

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.

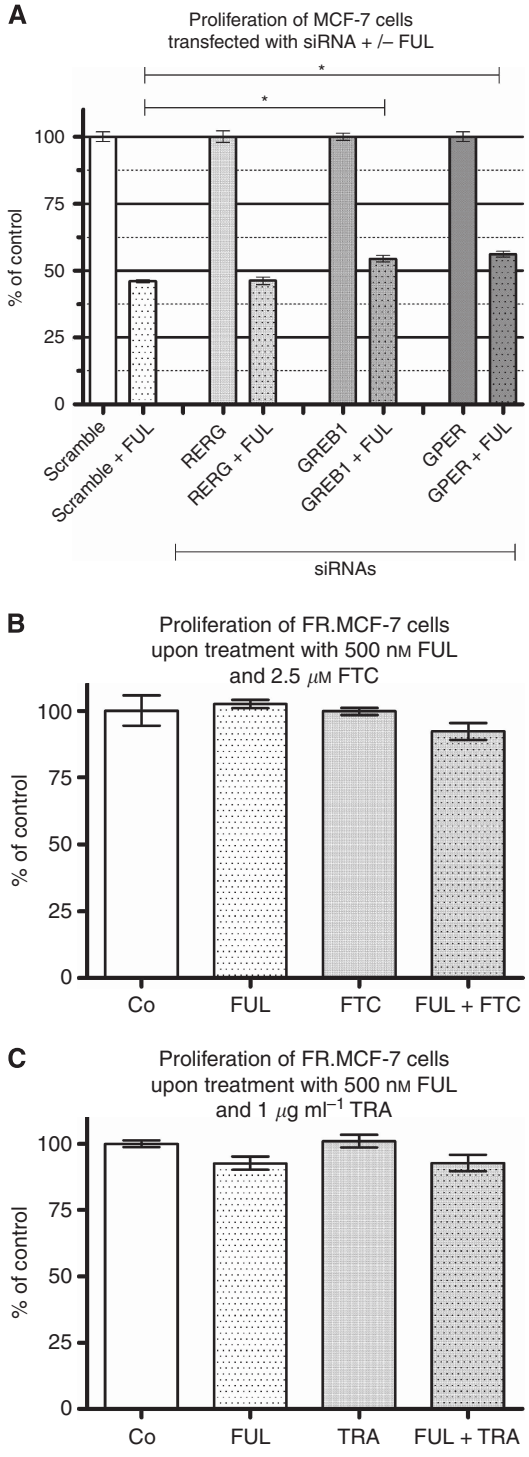

Figure 3. Testing of specific gene products required for the cellular response to FUL. (**A**) MCF-7 cells were transfected with the indicated small interfering RNAs (siRNAs) or with control RNA in which no complementary cellular RNA exists, were treated with 500 nM FUL or solvent (Co) for 48 h and then 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).

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),

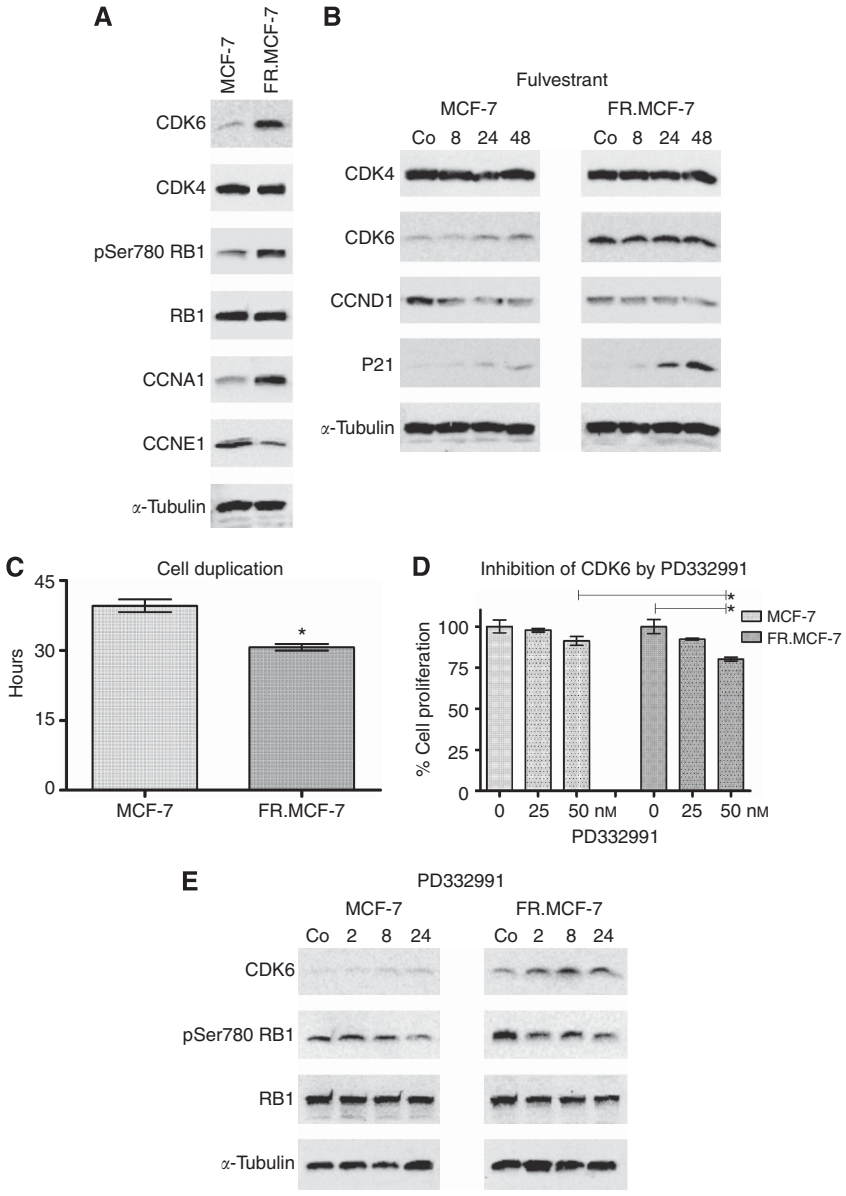

Figure 4. Effects of FUL and PD0332991 on cell cycle regulators. (**A**) MCF-7 and FR.MCF-7 cells were grown to 70% confluence, or (**B**) treated with 500 nM FUL or solvent (Co) for the indicated times when they were lysed and similar amounts of protein subjected to electrophoretic separation and western blot analysis with the indicated antibodies. Equal sample loading was confirmed by Ponceau S staining and α-tubulin analysis. (**C**) MCF-7 and FR.MCF-7 cells were grown for 24 and 48 h when cells were counted and the duplication time calculated. Experiments were carried out in triplicate, error bars indicate s.e.m. and asterisk denotes significance (*t*-test). (**D**) MCF-7 and FR.MCF-7 cells were exposed to 25 and 50 nM PD0332991 and cell proliferation was measured after 48 h. Experiments were carried out in triplicate, error bars indicate s.e.m. and asterisks denote significance (*t*-test). (**E**) MCF-7 and FR.MCF-7 cells were grown to 70% confluence and treated with 50 nM PD0332911 or solvent (Co) for the indicated times when they were lysed and similar amounts of protein subjected to electrophoretic separation and western blot analysis with the indicated antibodies. Equal sample loading was confirmed by Ponceau S staining and α-tubulin analysis.

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 remodelling 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 remodelling 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

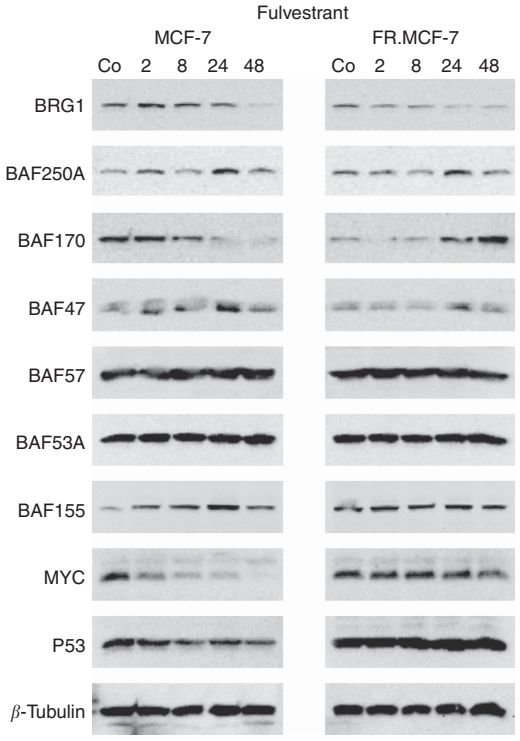

Figure 5. Effects of fulvestrant on SWI/SNF subunits and targets. MCF-7 and FR.MCF-7 cells were incubated with 500 nM FUL and harvested after 2, 8, 24 and 48 h of treatment. Cells were lysed, protein samples subjected to electrophoretic separation and to western blot analysis with the indicated antibodies. Equal sample loading was confirmed by Ponceau S staining and β-tubulin analysis.

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; Nagl *et al*, 2006, 2007; Simone, 2006; 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-7cells, yet the protein was almost similarly expressed in both cell lines.

In addition to the direct binding to target genes transient activation of hSWI/SNF was reported to permanently reposition nucleosomes (Schnitzler *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; Gutierrez *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 endoplasmatic 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 impinges 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 0332991 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 0332991 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. 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; Simone, 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; Simone, 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.

ACKNOWLEDGEMENTS

We thank Toni Jäger for preparing the figures. The work was supported by the Herzfelder Family Foundation to GK.

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