Resveratrol activates the histone H2B ubiquitin ligase, RNF20, in MDA-MB-231 breast cancer cells

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\textbf{ABSTRACT}

Resveratrol, a polyphenolic compound found in grapes, has been shown to partially exert its anti-cancer function through the modulation of epigenetic events. However, the epigenetic targets of resveratrol in breast cancer are not yet fully understood. Here, we identified that the gene encoding the histone H2B ubiquitin ligase RNF20 (ring finger protein 20), a chromatin modifying enzyme and putative tumour suppressor, is up-regulated by resveratrol in MDA-MB-231 breast cancer cells. Up-regulation of RNF20 is achieved through an increase of active histone marks around its promoter. The increase in RNF20 in MDA-MB-231 likely contributes to p21 regulation and the anti-growth effects of resveratrol, as RNF20 knockdown diminished these effects. Our findings suggest that RNF20 is a novel epigenetic target of resveratrol, and increase our understanding of how it contributes to the chemopreventive effect of resveratrol in a more aggressive breast cancer cell line, MDA-MB-231.

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1. Introduction

Breast cancer is by far the most common cancer amongst women worldwide, accounting for almost a quarter (23%) of all cases of cancer (Jemal et al., 2011; Youlden et al., 2012). Despite a decline in its mortality rate (Parkin, Bray, Ferlay, & Pisani, 2005; Youlden et al., 2012), breast cancer remains the leading cause of cancer death in women, responsible for 14% of total cancer deaths in 2008 (Jemal et al., 2011; Youlden et al., 2012). The molecular mechanisms of breast cancer development are complex and incompletely understood (Cebrian et al., 2006; Pennock & Wang, 2008; Veeck & Esteller, 2010). Although genetic abnormalities are thought to play a major role in breast carcinogenesis, it has become apparent that epigenetic alterations are also a contributing factor (Cebrian et al., 2006; Jovanovic, Ronneberg, Tost, & Kristensen, 2010; Lo & Sukumar, 2008; Veeck & Esteller, 2010). The hallmarks of epigenetic mechanisms are DNA methylation at cytosine (C) residues in CpG dinucleotides and histone post-translational modifications, both of which regulate gene

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activity by altering chromatin structure and DNA–protein interactions without changing the DNA sequences (Jenuwein & Allis, 2001; Jones & Takai, 2001). The most prevalent aberrant epigenetic events in breast cancer patients are hypermethylation of the promoters of tumour suppressors, such as breast cancer 1 (BRCA1) and cyclin-dependent kinase inhibitor 2A (CDKN2A or p16INK4a) by DNA methyltransferases (DNMTs) (Birgisdottir et al., 2006; Silva et al., 2003). Another common epigenetic mechanism underlying breast cancer is global hyperacetylation of histone H3 and H4; this mechanism represses tumour suppressor genes in cooperation with DNA methyla-
tion (Esteller, 2007; Lo & Sukumar, 2008; Neven et al., 2006).

Recent studies indicate that dysregulation of the enzyme machinery required for histone H2B ubiquitylation (H2Bub) may also contribute to breast cancer development (Espinosa, 2008; Michel & Parsons, 1988).

H2Bub plays a critical role in gene regulation (Kao et al., 2004; Zhang, 2003). Evidence from cell lines and clinical samples suggest that the histone H2B ubiquitin ligase, ring finger protein 20 (RNF20), which is required for H2Bub ubiquitylation (Kim, Hake, & Roeder, 2005; Zhu et al., 2005) acts as a tumour suppressor, perhaps by helping prevent the inappropriate activation of proto-oncogenes (Shema, Kim, Roeder, & Oren, 2011; Shema et al., 2008). Thus, cells depleted for RNF20 exhibit higher oncogenic potential (Shema et al., 2008). The RNF20 promoter has been reported to be hypermethylated in clinical breast tumour tissue as compared with normal tissue (Shema et al., 2008). It has also been reported that levels of H2Bub decrease with the extent of breast cancer progression, and knockdown of a gene encoding a second histone H2B ubiquitin ligase, ring finger protein 40 (RNF40), results in oestrogen-independent cell proliferation (Michel & Parsons, 1988). Thus, aberrant RNF20 expression and the level of H2Bub are connected to breast malignancy through their effects on gene regulation (Michel & Parsons, 1988; Shema et al., 2008).

As epigenetic events are more easily modified and reversed than genetic changes, epigenetic therapies are potentially useful in combating breast cancer. Extensive studies have shown that a variety of dietary compounds can modulate epigenetic disorders in breast tumours, and act as cancer chemopreventive agents (Neven et al., 2006; Ross et al., 2008). One of the most promising dietary phytochemicals is resveratrol (trans-3,5,4’-trihydroxystilbene), a stilbenoid abundant in red wine and grapes (Harikumar & Aggarwal, 2008; Kundu & Surih, 2004) that has reported anti-atherogenic effects (Chang, Lee, & Sheu, 2012). Resveratrol also has anti-proliferative, pro-apoptotic, anti-metastatic and radio-sensitising effects on various human cancer cells and anti-carcinogenic effects in animal models (Aggarwal et al., 2004; Bishayee, 2009; Liu et al., 2010; Mao et al., 2010; Vanamala, Reddivari, Radhakrishnan, & Turver, 2010) through its effects on gene regulation of multiple intracellular targets.

During the last decade, studies have shown that the gene modulation ability of resveratrol in breast cancer cells is also driven by epigenetic mechanisms (Paluszczak, Krajka-Kuzniak, & Baer-Dubowska, 2010; Papoutsis, Lamore, Wondrak, Selmin, & Romagnolo, 2010; Wang et al., 2008). Resveratrol has been reported to inhibit the activity and expression of DNA methyltransferase 1 (DNMT1) in the human breast cancer MCF-7 cell line (Paluszczak et al., 2010), which prevents epigenetic silencing of the BRCA1 tumour suppressor (Papoutsis et al., 2010). In addition, resveratrol has also been shown to activate a Class III histone deacetylase (HDAC), sir-tuin 1 (SIRT1) (Howitz et al., 2003; Lagouge et al., 2006), through which it facilitates histone hypoacetylation and regulates gene activity. Resveratrol modulates translation and proliferation of oestrogen receptor-positive (ER+) and ER-negative (ER–) breast cancer cells through 5’ adenosine monophosphate-activated protein kinase (AMPK) activation in a SIRT1-dependent manner (Lin et al., 2009). Furthermore, induction of SIRT1 by resveratrol also impairs β-catenin and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB)-p65 signalling cascades in MCF-7 cancer cells (Bourguignon, Xia, & Wong, 2009), and inhibits the growth of BRCA1 mutant tumours through repression of survivin, an anti-apoptotic protein (Wang et al., 2008). Therefore, resveratrol exerts its chemopreventive properties in breast cancer cells through epigenetic mechanisms. As disruption of RNF20 and the level of H2Bub has been connected to breast cancer development (Shema et al., 2008), it is possible that RNF20 may help mediate the chemopreventive function of resveratrol in human breast cancer cells. To investigate this possibility, we examined the effects of resveratrol on RNF20 on two commonly used breast cancer cell lines, MCF-7 (ER+) and MDA-MB-231 (ER–).

2. Materials and methods

2.1. Cell culture and resveratrol treatment

Human breast-cancer cell lines MCF-7 and MDA-MB-231 were purchased from the American Type Culture Collection. MCF-7 cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 2 mM l-glutamine, 10% foetal calf serum and antibiotics (100 units/ml penicillin G, 100 μg/ml streptomycin sulphate and 0.25 μg/ml amphotericin B) at 37 °C in 5% CO2. MDA-MB-231 cells were maintained in Leibovitz’s L-15 supplemented with 2 mM l-glutamine, 10% foetal calf serum and antibiotics (as above) at 37 °C in 5% CO2.

Resveratrol (Sigma–Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulphoxide (DMSO) to form a stock with a concentration of 100 mM. The stock was stored at −20 °C prior to use. To test the dose–effect of resveratrol, human breast cancer cells were incubated with various concentrations (10, 25, 50 μM) of resveratrol or vehicle (DMSO). For efficient uptake, resveratrol stock was added to foetal calf serum for 30 min and then mixed with culture media. The final concentration of DMSO was 0.05% (v/v, equal to 50 μM of resveratrol). After 24 h, cells were washed with phosphate buffered saline (PBS) and collected for mRNA and protein measurement. To investigate the effect of varying treatment duration, breast cancer cells were treated with a single dose of resveratrol (50 μM final) or vehicle (DMSO). After incubation for 24, 48 or 72 h, cells were washed with PBS and collected for mRNA and protein analysis.

2.2. RNA extraction and reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was isolated using TRI reagent (Ambion, Monza, Italy). Two micrograms of total RNA were reverse-transcribed using SuperScript III (Invitrogen, Carlsbad, CA, USA) and oliga
dT primer. The cDNA was diluted 1:5-1:100 with ddH2O, and 4 µl aliquots were used for qPCR using 160 nM primers and FastStart SYBR Green Master mix (Roche, Mannheim, Germany) (LightCycler® 480: 95 °C 10 s, 60 °C 10 s, 72 °C 10 s, 45 cycles; Roche, Penzberg, Germany). Primer sequences are listed in Table S1. mRNA levels were normalised to β-actin. Relative mRNA expression was calculated using the formula 2^(-ΔΔCq), where △△Cq = △Cq(treatment) − △Cq(control), △Cq is Cq(gene) − Cq(β-actin), and Cq is the quantification cycle, also known as the crossing point (C0) (Bustin et al., 2009).

2.3. Western blot analysis

Total protein was extracted using lysis buffer (20 mM Tris–HCl, pH 7.5, 1 mM ethylenediamine tetracetic acid disodium salt (Na2EDTA), 150 mM sodium chloride (NaCl), 1 mM ethylene glycol tetracetic acid (EGTA), 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate (Na3VO4), 15 µg/ml leupeptin, 1 mM phenylmethylsulphonyl fluoride (PMSF)) at 4 °C for 30 min. Subsequently, 35–50 µg total cellular protein were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon P, Millipore, Bedford, MA, USA), and then probed with primary antibody followed by secondary antibody. The following antibodies were used: anti-RNF20 (Sigma–Aldrich, St. Louis, MO, USA), anti-α-tubulin (Serotec), anti-H2B (Millipore, Temecula, CA, USA), anti-H2Bub (Millipore, Temecula, CA, USA), anti-DNMT1 (Santa Cruz, Heidelberg, Germany), anti-HDAC1 (Santa Cruz, Heidelberg, Germany) and anti-p53 (Santa Cruz, Heidelberg, Germany). The immunocomplexes were visualised with enhanced chemiluminescence kits (Millipore, Temecula, CA, USA).

2.4. Chromatin immunoprecipitation (ChIP)

ChIP was carried out as described in the Acetyl-Histone H3 Immunoprecipitation Assay Kit (Millipore, Temecula, CA, USA). Briefly, cells were treated with resveratrol (50 µM final) or DMSO. After incubation for 24 h, cells were lysed and chromatin samples were sonicated to generate fragments shorter than 500 bp, and were then immunoprecipitated with antibodies against H3K4me3 (Abcam, Cambridge, UK), H3K9/K14Ac (Millipore, Temecula, CA, USA), H4K16Ac (Millipore, Temecula, CA, USA), H4tetraAc (Millipore, Temecula, CA, USA) or Iga dynabeads (GE Healthcare Biosciences AB, Uppsala, Sweden). After protein digestion, eluted DNA was diluted 1:5 with ddH2O, and 4 µl aliquots were used for qPCR. Primer sequences are shown in Table S1. DNA was quantified using a calibration curve generated from MDA-MB-231 genomic DNA with a linear interval (Cq < 40). Percent enrichment in each pull-down was calculated relative to input DNA.

2.5. siRNA transfection

siRNA oligonucleotides against RNF20 (ON-TARGETplus SMARTpool, L-007027-00-0005) and non-targeting siRNA (siCon) were purchased from Thermo Scientific (Dharmacon RNAi Technologies, Lafayette, CO). Sequences targeting RNF20 are listed in Table S2. Cells were seeded onto 6- or 12-well plates in maintenance media, at a cell density of 50%. Transfections with siRNA (25 nM, final) and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) were carried out the day after plating. At 24 h post-transfection, cells were treated with either maintenance media, or media containing vehicle (DMSO) or resveratrol (50 µM final) for a further 24–48 h.

2.6. Cell viability assay and FACS analysis

Approximately 5,000 cells were seeded onto 96-well microtiter plates (4 replicates), and treated with vehicle (DMSO) or resveratrol (10, 25, or 50 µM) for 72 h. The proportion of viable cells was determined using the MTT (Sigma–Aldrich, St. Louis, MO, USA) reduction assay at 12 h intervals. MTT stock (5 mg/ml in PBS) was added to each well at the indicated times to a final concentration of 0.1 mg MTT/ml. After 2 h of incubation at 37 °C, MTT solution was removed, and the formazan crystals were dissolved in 200 µl DMSO by incubating at 37 °C with constant shaking for 10 min. The absorbance at a wavelength of 570 nm was recorded, and the results calculated as percentage of cell viability, with the vehicle sample acting as a reference at each time point.

Cell cycle analysis was performed using propidium iodide (PI) staining. MCF-7 (300,000 cells/well) and MDA-MB-231 (250,000 cells/well) were cultured in 6-well plates, and treated with vehicle (DMSO), resveratrol, non-targeting siRNA (siCon) or siRNF20, as described above. Cells for FACS analysis were trypsinised, washed twice with ice-cold PBS and fixed in ice-cold 70% ethanol overnight at −20 °C. After centrifugation, the fixed cells were washed with ice-cold PBS, and stained with PI buffer (PI 0.02 mg/ml, RNase A 0.2 mg/ml, 0.1% Triton X-100 in PBS) for 15 min at 37 °C. DNA content was analysed by BD FACSCanto II flow cytometry (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA). Data from 3,000–10,000 events/sample were collected and analysed using FlowJo Software version 7.6.1 (Tree Star Software, Ashland, OR).

2.7. Statistical analysis

At least two replicates per sample were tested for each experiment, and two or three independent experiments were performed. All values are expressed as mean ± SEM. Student’s t test was used for statistical comparison between control and treatment groups (DMSO vs 50 µM resveratrol; siCon vs siRNF20). One-way analysis of variance (ANOVA) was used to analyse the effects of resveratrol dosage or treatment length, and the effects of varying the duration of RNF20 knockdown. A p-value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Resveratrol up-regulates the H2B ubiquitin ligase gene, RNF20, in MDA-MB-231

To examine the effects of resveratrol on RNF20 expression in MCF-7 and MDA-MB-231 breast cancer cell lines, we applied different doses of resveratrol (10–50 µM) to each cell line. After 24 h, a dose-dependent increase of RNF20 mRNA and protein was observed in MDA-MB-231, but not in MCF-7 cancer cells.
In mammalian cells, RNF20 forms a heteromeric complex with RNF40 (Kim et al., 2005; Zhu et al., 2005) to regulate H2Bub. However, we found that RNF40 expression was unaffected in both cell lines, even at the highest dose of resveratrol (50 μM) used (Fig. S1). Thus, resveratrol specifically up-regulates RNF20 in MDA-MB-231 breast cancer cells. Since RNF40 is a H2B ubiquitin E3 ligase, we asked whether the increase in RNF20 directly impacts on the level of H2Bub. We measured the latter’s abundance in cells cultured with resveratrol (10–50 μM) for 24 h, and found it increased (albeit not significantly) with dose in MDA-MB-231 but not in MCF-7, as normalised to total H2B (Fig. 1C). The change in H2Bub level upon resveratrol treatment is consistent with the pattern of RNF20 expression in both cell lines (Fig. 1B and C).

3.2. Resveratrol primes the RNF20 gene promoter for chromatin remodelling

Next, we were curious as to how resveratrol regulates RNF20 expression in the MDA-MB-231 cancer cell line. As RNF20 was previously reported to be silenced in breast cancer tissues through epigenetic mechanisms (Shema et al., 2008), and resveratrol potentially regulates epigenetic events in these cells (Paluszczak et al., 2010; Papoutsis et al., 2010), we hypothesised that the observed increase in RNF20 transcription may arise through an epigenetic process. Dysregulation of epigenetic enzymes such as DNMT1 and HDACs has been linked to breast tumorigenesis (Hoshino & Matsubara, 2010; Lo & Sukumar, 2008; Veeck & Esteller, 2010). Previous studies have also demonstrated that both DNMT1 and HDACs are potential targets of resveratrol in modulating aberrant gene activity in cancer cells (Howitz et al., 2003; Lagouge et al., 2006; Paluszczak et al., 2010; Papoutsis et al., 2010). Thus, as these two enzymes are often associated with the repression of key genes in cancer cells (Esteller, 2007; Jones & Baylin, 2007), we examined the level of DNMT1 and HDAC1 in our two cell lines. It was found that levels of DNMT1 (Fig. S2A) and HDAC1 (Fig. S3A) in MDA-MB-231 were not changed as compared to untreated cells, even at the highest dose used (50 μM). Similarly, HDAC1 expression was unaffected in MCF-7 (Fig. S3B). However, we observed a non-monotonic effect of resveratrol (10–50 μM) on the level of DNMT1 in MCF-7 (Fig. S2B), although RNF20 expression was unaffected under these conditions (Fig. 1A).

We next tested whether the transcriptional activation of RNF20 is accompanied by local epigenetic changes, rather
than by changes in the enzymes themselves. We initially analysed DNA methylation at the promoter and transcribed regions of RNF20 upon resveratrol treatment (50 μM). However, we failed to observe any significant changes in DNA methylation in either cell line (data not shown). We then examined the occupancies of several histone post-translational modifications which are linked to gene activation, namely H3K4me3, H3K9K14Ac, H4K16Ac and H4tetraAc (Reid, Gallais, & Metivier, 2009), in five regions of RNF20 (Fig. 2A) using chromatin immunoprecipitation (ChIP). Our analyses revealed that resveratrol treatment causes a significant increase of all histone markers tested at most analysed regions in MDA-MB-231 (Fig. 2B). Conversely, only H3K4me3 was increased at all regions examined upon resveratrol treatment in the MCF-7 breast cancer cell line (Fig. 2C). These findings are consistent with the observation that resveratrol treatment increased RNF20 mRNA and protein in MDA-MB-231, but not MCF-7 cells (Fig. 1A and B).

3.3. RNF20 induction by resveratrol is linked to suppression of cell growth

Since RNF20 depletion has been reported to augment the expression of pro-oncogenic genes in cancer cells (Shema et al., 2011), we examined the effect of resveratrol on cell growth in MDA-MB-231. Cell viability assays revealed that resveratrol significantly inhibited MDA-MB-231 breast cancer cell growth in a time- and dose-dependent manner, with 46% inhibition observed following 72 h of treatment (50 μM resveratrol) (Fig. 3A). The decrease of cell viability may result from induction of apoptosis or disruption of cell cycle progression. However, a previous study demonstrated that resveratrol (25–200 μM) does not induce apoptosis in MDA-MB-231 cells (Pozo-Guisado, Alvarez-Barrion, Mulero-Navarro, Santiago-Josefat, & Fernandez-Salgueiro, 2002). Thus, we examined whether resveratrol treatment (10–50 μM, 24 h) of MDA-MB-231 cells affects the expression of two cell cycle
regulators: cyclin D1, encoding a G1/S transition cyclin (Murray, 2004), and p21WAF1 (p21), encoding a cyclin-dependent kinase inhibitor (Oglyzko, Wong, & Howard, 1997). We found that cyclin D1 expression was unaffected at any dose of resveratrol tested (10–50 µM) (Fig. 3B), while p21 expression was increased by resveratrol in a dose-dependent manner in MDAMB-231 cells (Fig. 3B).

To determine whether the change in gene profile has any impact on cell cycle distribution in MDAMB-231, we analysed cell cycle distribution under 50 µM resveratrol by flow cytometry. Cells in S phase were increased after 24 h of resveratrol treatment, as compared with the control group (vehicle) (Fig. 3C). The proportion of S phase cells increased further with time (Fig. 3C). Furthermore, resveratrol exposure also induced a small fraction of subG1 (dying) cells after 48 h (Fig. 3C). Thus, these results suggest that resveratrol inhibits cell growth through inducing S phase arrest, which ultimately leads to cell death.

3.4. RNF20 induction by resveratrol is associated with p21 activation

In order to verify that RNF20 expression is correlated with p21 expression in MDAMB-231 breast cancer cells, cells were treated with 50 µM resveratrol for a longer period of time (72 h), and expression of RNF20 and p21 was monitored every 24 h. We observed that RNF20 expression increased steadily during a 72-h period (Fig. 4A) and this was paralleled by an increase in p21 mRNA (Fig. 4A). The correlation between the relative gene expression of RNF20 and p21 in MDAMB-231 cancer cells is 0.893 (p < 0.05) (Fig. 4B).

As RNF20 is a transcription co-activator (Kim et al., 2005; Zhu et al., 2005), we hypothesised that it may regulate p21 activity in MDAMB-231 breast cancer cells. Histone H2Bub, which is regulated by RNF20, has been found to trigger methylation on lysines (K) 4 and 79 of histone H3, and such methylation is a marker of actively transcribed genes (Briggs et al., 2002; Sun & Allis, 2002). Using chromatin immunoprecipitation, we detected an increase of H3K4me3 in the promoter and 5' coding region of p21 after resveratrol administration (50 µM) (Fig. 4C). Thus, the results suggest that RNF20-mediated modification of chromatin may have occurred at the p21 locus.

3.5. RNF20 deletion attenuates p21 expression and the effects of resveratrol on cell cycle distribution

Finally, we investigated whether RNF20 contributes to p21 activation and the effect of resveratrol on cell cycle progression. First, RNF20 was depleted by RNA interference, and the
expression level of p21 was measured after gene knockdown (Fig. S4). As shown in Fig. 5A, the level of RNF20 protein was reduced by almost 70% at 24 h post-siRNA transfection, and was reduced by 90% at 72 h. As expected, RNF20 depletion significantly reduced the amount of H2Bub for up to 72 h (Fig. 5A, lower panel). The mRNA level of p21 was also significantly reduced (by up to 25%) (Fig. 5B) after 48 h, at which time RNF20 and H2Bub were also diminished. Next, we investigated how resveratrol treatment affected the cell cycle distribution of cells in which RNF20 had been abolished for at least 24 h (Fig. S4). Consistent with the previous finding (Fig. 3C), resveratrol treatment increased the proportion of S phase (36.41% in RES 50 μM vs. 30.13% in vehicle) and subG1 (22.96% in RES 50 μM vs. 20.19% in vehicle) cells in MDA-MB-231 (Fig. 5C). Crucially, RNF20 knockdown in MDA-MB-231 breast cancer cells attenuated the accumulation of subG1 and S phase cells induced by resveratrol treatment (Fig. 5C). Taken together, these results suggest that RNF20 may participate in regulating p21 activity and inhibiting cell cycle progression in MDA-MB-231 cells upon resveratrol treatment.

4. Discussion

It has become increasingly apparent that the chemopreventive function of resveratrol on breast cancer is modulated through epigenetic enzymes, such as DNMT1 and SIRT1 (Paluszczak et al., 2010; Papoutsis et al., 2010; Wang et al., 2008). Our study revealed that expression of RNF20, a gene encoding a histone H2B ubiquitin ligase, is significantly up-regulated by resveratrol in MDA-MB-231 (ER−) but not in MCF-7 (ER+) cells; thus, resveratrol may modulate this particular histone modifying enzyme in more aggressive breast cancer cells.

RNF20 mediates H2B ubiquitylation (Kao et al., 2004; Zhang, 2003) and acts as a transcriptional co-activator to assist the proper expression of certain genes (Kim et al., 2005; Zhu et al., 2005). In addition, it functions as a tumour suppressor (Shema et al., 2008; Shema et al., 2011). Resveratrol exerts several beneficial anti-inflammatory and cancer chemopreventive effects through multiple targets, some of which overlap with RNF20. First, resveratrol and RNF20 both down-regulate inflammation-related genes through inhibiting the signal transducer and activator of transcription 1 (STAT1) transcription factor (Buro, Chipumuro, & Henriksen, 2010; Chung et al., 2010). Second, they both repress epidermal growth factor (EGF)-responsive genes, which are usually linked to tumourigenesis (Bishayee, 2009; Le Corre, Chalabi, Delort, Bignon, & Bernard-Gallon, 2005; Lee, Pan, Chioi, Cheng, & Huang, 2011; Shema et al., 2008). Taking these similarities into account, we postulate that RNF20 is likely an epigenetic target of resveratrol, and that RNF20 may partially...
underlie the chemopreventive functions of resveratrol in MDA-MB-231 breast cancer cells.

Consistent with the above hypothesis, we observed that (1) induction of the cyclin-dependent kinase inhibitor, p21, correlated with induction of RNF20 upon resveratrol treatment in MDA-MB-231; (2) H3K4me3, an active histone mark and down-stream signal of H2Bub, increased in the promoter and 5’-ORF (open reading frame) of p21; and (3) RNF20 depletion significantly reduced p21 mRNA expression. Thus, these findings suggest that RNF20 likely participates in the transcriptional regulation of the p21 gene in MDA-MB-231. The expression of p21 is controlled by the tumour suppressor, p53 (el-Deiry et al., 1993), and in some cases, through p53-independent pathways (Abbas & Dutta, 2009). Although resveratrol has been reported to regulate the p53-p21 pathway in breast cancer cells (Bishayee, 2009; Kundu & Surh, 2008; Le Corre et al., 2005), p53 is mutated in MDA-MB-231 cells (Katayose et al., 1995). Therefore, the results imply that resveratrol modulates p21 expression in an RNF20-dependent, but p53-independent manner in MDA-MB-231.

A recent study identified that H2Bub may be a novel direct or indirect epigenetic target of resveratrol in glioma cells (Gao, Xu, Barnett, & Xu, 2011). It demonstrated that resveratrol (20–60 μM) induces cellular senescence in glioma cells and attenuated levels of H2Bub (Gao et al., 2011). Acute (4 h) treatment with a high dose (500 μM) of resveratrol had similar effects in several cancer (including MCF-7) and normal cell lines (Gao et al., 2011). The apparent contradictions between these findings and those of our study may be due to differences in cell type and genetic background, as resveratrol has been found to exert its anti-tumour functions through different molecular pathways in different cancers (Aggarwal et al., 2004; Bishayee, 2009; Liu et al., 2010; Mao et al., 2010; Vanamala et al., 2010). In addition, the effects of resveratrol on breast cancer cells expressing ER have been reported to depend on the dose of the treatment (Le Corre et al., 2005). Resveratrol (3–10 μM) acts as a super agonist to oestrogen, while it acts as an antagonist of oestrogen-mediated cell function and suppresses ER activation at higher doses (>5–100 μM) (Gehm, McAndrews, Chien, & Jameson, 1997; Lu & Serrero, 1999). The
ER expression pattern may also determine the response of cancer cells to resveratrol. It has been previously demonstrated that resveratrol has different effects on MDA-MB-231 (ER–) and MCF-7 (ER+) breast cancer cells (Paluszczak et al., 2010; Pozo-Guisado et al., 2002). For example, resveratrol was found to transiently induce expression of cell cycle regulators in MCF-7 cells; expression peaked at 50 μM, and decreased at higher concentrations. On the contrary, it inhibited the activities of cell cycle regulators in MDA-MB-231 breast cancer cells in a dose-dependent manner (Pozo-Guisado et al., 2002). Thus, our findings and those of the previous study (Gao et al., 2011) may be reconciled by the dose–effect theory of resveratrol in ER+ cells, and we may conclude that resveratrol (10–50 μM) induces H2Bub in a dose-dependent manner in the MDA-MB-231 breast cancer cell line.

Metastatic breast cancer (such as ER-negative MDA-MB-231 cancer cells) often fail to respond to hormone therapy, and have a higher rate of recurrence (Foulkes, Smith, & Reis-Filho, 2010). Since abnormal epigenetic events are widely implicated in breast cancer development and progression (Jovanovic et al., 2010), epi-drugs are a powerful addition to our arsenal of anti-cancer treatments (Mai & Altucci, 2009). The ability of several epigenetic drugs to revert resistance gained from traditional anti-cancer drugs (Mai and Altucci, 2009) make their combinatorial treatment with traditional therapy highly effective. Our study demonstrates that RNF20 is involved in the anti-proliferative activity of resveratrol, specifically in MDA-MB-231 (ER–) cells. Hence, RNF20 may be a promising target for the development of epi-drugs targeting metastatic breast tumours. Further studies are required to increase our understanding of how resveratrol modulates RNF20 and the levels of H2Bub in different cancers, and its relevance to cancer chemoprevention.

5. Conclusion

Although the mechanisms are not yet fully understood, the present study indicates that resveratrol may carry out its anti-cancer function in part through up-regulating the putative tumour suppressor gene RNF20 in MDA-MB-231 breast cancer cells. This suggests that RNF20 may be an effective therapeutic target for epigenetic drugs, and its relative expression level in tumour cells may serve as an indicator of responsiveness to chemopreventive drugs.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jff.2013.01.025.

REFERENCES


