Regulation of apoptosis by long non-coding RNA GAS5 in breast cancer cells: implications for chemotherapy

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Abstract

Purpose: The putative tumour suppressor and apoptosis-promoting gene, *growth arrest-specific 5* (*GAS5*), encodes lncRNA and snoRNAs. Its expression is down-regulated in breast cancer, which adversely impacts patient prognosis. In this preclinical study, the consequences of decreased GAS5 expression for breast cancer cell survival following treatment with chemotherapeutic agents are addressed. In addition, functional responses of triple-negative breast cancer cells to GAS5 lncRNA are examined, and mTOR inhibition as a strategy to enhance cellular GAS5 levels is investigated. Methods: Breast cancer cell lines were transfected with either siRNA to GAS5 or with a plasmid encoding GAS5 lncRNA and the effects on breast cancer cell survival were determined. Cellular responses to mTOR inhibitors were evaluated by assaying culture growth and GAS5 transcript levels. Results: *GAS5* silencing attenuated cell responses to apoptotic stimuli, including classical chemotherapeutic agents; the extent of cell death was directly proportional to cellular GAS5 levels. Imatinib action in contrast, was independent of GAS5. GAS5 lncRNA promoted the apoptosis of triple-negative and estrogen receptor-positive cells but only dual PI3K/mTOR inhibition was able to enhance GAS5 levels in all cell types. Conclusions: Reduced *GAS5* expression attenuates apoptosis induction by classical chemotherapeutic agents in breast cancer cells, providing an explanation for the relationship between *GAS5* expression and breast cancer patient prognosis. Clinically, this relationship may be circumvented by the use of GAS5-independent drugs such as imatinib, or by restoration of *GAS5* expression. The latter may be achieved by the use of a dual PI3K/mTOR inhibitor, to improve apoptotic responses to conventional chemotherapies.

Keywords: GAS5, ncRNA, apoptosis, breast, cancer, chemotherapy.

Abbreviations:

A490	Absorbance at 490 nm
C_T	Threshold cycle
EST	Expressed sequence tag
5-FU	5-Fluorouracil
GAS5	Growth arrest-specific 5
HER2	Human epidermal growth factor receptor 2
lncRNA	Long ncRNA
MCT	Multiple comparison test
NC	Negative control
ncRNA	Non-coding RNA
NMD	Nonsense-mediated decay
RT-qPCR	Real time reverse transcription polymerase chain reaction
5′-TOP	5′-Terminal oligopyrimidine tract
TNBC	Triple-negative breast cancer
UV-C	Ultraviolet-C.

Introduction

Long ncRNAs (size range: 0.2 - 100 kb) are emerging as key regulators of fundamental cellular processes and are increasingly recognized as of importance in the pathogenesis, diagnosis and treatment of major cancers, including breast cancer [1-5]. Apart from ribosomal RNAs, lncRNAs comprise a plethora of mRNA-like transcripts (long intergenic ncRNAs, pseudogene-derived transcripts and sequences that are intronic or antisense to protein coding genes) [1, 6, 7], which can act as signals, scaffolds, guides, decoys or tethers to modulate, for example, the maintenance of subcellular structures, chromatin modification and transcriptional/post-transcriptional regulation of gene expression [7, 8].

The lncRNA and small nucleolar RNA (snoRNA) host gene, *growth arrest-specific 5* (*GAS5*), is of particular interest in breast cancer, since its expression is down-regulated in tumour tissue [9], and patient prognosis is related to GAS5 transcript levels [10]. *GAS5* is a 5′-terminal oligopyrimidine tract (5′-TOP) gene, and comprises 12 exons which are alternatively spliced to yield two possible mature lncRNAs [11]. *GAS5* intronic sequence additionally encodes ten distinct box C/D snoRNAs [11]; three of which (U44, U74 and U78) may serve as miRNA precursors [12]. Multiple other *GAS5* expressed sequence tags (ESTs) have also been identified, many of which contain retained introns, indicating that post-transcriptional processing of GAS5 RNA is complex.

GAS5 transcripts accumulate in growth-arrested cells [11, 13] due to interplay between the mTOR pathway (through its regulation of translation of RNAs carrying the 5′-TOP sequence) and the nonsense-mediated decay (NMD) pathway (due to the *GAS5* short reading frame) [11, 14]. Thus, in actively growing cells, where mTOR activity is high, translation of the

short reading frame is promoted, resulting in degradation of transcripts by the NMD pathway, and consequently low cellular GAS5 lncRNA levels. Suppression of cell growth and mTOR activity prevents the active translation of GAS5 transcripts and their consequent degradation by NMD, resulting in the accumulation of GAS5 lncRNA [11, 13, 14]. Direct inhibition of mTOR activity by rapamycin and rapalogues produces the predicted increase in cellular GAS5 lncRNA [11, 15, 16].

In breast and other cell types, GAS5 inhibits cell proliferation and/or stimulates apoptosis [9, 17-21], *i.e.*, key determinants of cell survival that are often disrupted in cancer [22]. Indeed, elevated *GAS5* expression inhibits human breast tumour growth in a xenograft mouse model [21], consistent with a tumour suppressor role for this gene. Moreover, the apoptosis-promoting activity of GAS5 has implications for cancer therapies, since the action of many chemotherapeutic drugs ultimately depends on engagement of the cellular apoptotic machinery [23, 24]. However, the consequences of *reduced GAS5* expression for chemotherapeutic drug action in breast cancer cells have not been examined to-date.

In this study we have focussed on the chemotherapeutic implications of reduced *GAS5* expression in breast cancer. Firstly, we have investigated the hypothesis that *GAS5* silencing impairs the responses of breast cancer cells to apoptotic stimuli. Secondly, we have examined whether functional responses to GAS5 remain intact in triple-negative breast cancer (TNBC) cells. Finally, we have investigated mTOR inhibition as a strategy to enhance cellular GAS5 levels in breast cancer cells and consequently to suppress cancer growth through the induction of apoptosis and/or cell cycle arrest.

Materials and methods

Material

Cell lines were from ATCC-LGC Promochem (Teddington, UK). Cell culture materials and classical chemotherapeutic drugs were from Sigma-Aldrich Company Ltd (Gillingham, UK). Sources of other drugs were: rapamycin (Millipore, Watford, UK); temsirolimus and everolimus (LC Laboratories, Woburn, MA); BEZ235 and AZD8055 (Stratech Scientific, Newmarket, UK); and imatinib (mesylate) (Cayman Chemical, Ann Arbor, MI). TRIzol, reverse transcription reagents, TaqMan assays and Silencer Select siRNAs were from Life Technologies Ltd (Paisley, UK). SensiFast Probe Hi-ROX kit was from Bioline (London, UK), RQ1 RNase-free DNase and the MTS assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay) were from Promega (Southampton, UK), RNAiFect was from Qiagen (Crawley, UK) and nucleofector solution V was from Lonza Biosciences (Verviers, Belgium).

Methods

Cell culture

The breast cancer cell lines MCF7, T-47D and MDA-MB-231 were generated from secondary stocks of cells which had been frozen down within two weeks receipt from the ATCC. Cells were cultured in R-10 medium (RPMI-1640 supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 10% fetal bovine serum and 50 µg/ml gentamicin) at 37 °C in a humidified incubator with 5% CO_2. Cell lines were replaced with fresh stocks after a maximum culture period of two months.

RNA interference by siRNA

Cells were transfected with Ambion Silencer Select siRNAs using RNAiFect reagent, according to a standard protocol [20]. Up to 4 different GAS5 siRNAs were employed, termed siRNAs #1 – 4 (product codes/targeted exons are: n272334/exon 2; n272337/exon 8; n272340/exon 12 and n272331/exon 12, respectively); controls received negative control (NC) siRNA (code AM4611).

Plasmid DNA transfection

Plasmids were: pcDNA3/GAS5.O1 (encodes GAS5 O1 EST); pcDNA3/GAS5.AE (encodes mature GAS5 lncRNA); and pcDNA3 vector (for controls) [20]. Cells were nucleofected with plasmids (2 µg per 2 x 10^6 cells in 0.1 ml nucleofector solution V) using programmes E-014 and X-013 for MCF7 and MDA-MB-231, respectively, and cells were plated in 3 ml R-10 medium in 6-well plates.

Induction of cell death and cell survival assays

At 72 h *post*-siRNA transfection or at 20 h *post*-plasmid nucleofection, cells were trypsinized, sampled for RNA, then seeded (0.8 x 10^5 cells for siRNA-transfected cells; 1.6 x 10^5 cells for plasmid-transfected cells) into 12-well plates. Ultraviolet-C (UV-C) irradiation was performed prior to plating [20]; doses were 40 J/m^2 for MCF7 and MDA-MB-231 cells, and 60 J/m^2 for T-47D cells; controls were mock-irradiated. For drug treatments, cells were cultured for 20 h, before addition of 5-fluorouracil (5-FU; 175 µM), docetaxel (10 µM),

imatinib (50 μM) or vehicle (0.25% dimethyl sulphoxide). Cells were cultured for 48 h *post*-treatment, then adherent cells were trypsinized and combined with non-adherent cells for analysis of cell survival.

Apoptosis was routinely determined by assessment of nuclear morphology by fluorescence microscopy after staining with acridine orange (25 μg/ml); cells containing condensed or fragmented chromatin were scored as apoptotic. Cell viability was determined by counting of nigrosin blue (0.1% (w/v)) stained samples using a haemocytometer and light microscopy. For clonogenic assays, cells were re-plated in culture medium supplemented with 10% (v/v) cell-conditioned medium in 6-well plates, cultured for 3 weeks, then the number of colonies was counted after staining with crystal violet.

Assessment of mTOR inhibitor sensitivity

Cells were plated in 96-well plates (500 cells in 0.1 ml R10 medium) and in 12-well plates (1.6×10^5 cells in 0.8 ml R10 medium). After 4 h, an equal volume of medium containing the appropriate drug or vehicle (final concentrations: 1 μM rapamycin, 10 nM everolimus, 10 nM temsirolimus, 100 nM BEZ235, 50 nM AZD8055 or 0.1% dimethyl sulphoxide) was added. Experiments were terminated after a further 20 h (12-well plates; for GAS5 determination) or 70 h (96-well plates; for MTS assay). For the latter, sample absorbance readings at 490 nm (A490) were corrected for the appropriate medium plus drug blank values.

Effect of cell density-induced growth arrest on cellular GAS5 levels

For low cell density, logarithmic phase cultures, cells were plated at 2 x 10^5 per well (6-well plate) then harvested after 24 h culture. For high cell density, stationary phase cultures, cells plated at 10 x 10^5 cells per well (6-well plate) then harvested after 96 h culture.

Real time RT-PCR (RT-qPCR)

TaqMan Gene Expression Assays (assay codes Hs99999901_m1 for 18S and Hs03464472_m1 for GAS5) were employed with cDNA prepared by random hexamer priming, as described previously [20]. Assays (25 µl) contained 10 ng sample cDNA or 0.2 – 60 ng standard cDNA (prepared from MCF7 cells). Input amounts of samples were calculated from their respective threshold cycle (C_T) values, using standard curves generated with each assay. Data were expressed relative to 18S rRNA.

Statistical analyses

Data are presented as the mean ± SEM; the number of observations (n) refers to different transfected samples (each from separate cultures) or separate cultures. Data analysis was either by an unpaired Student's *t*-test (when comparing two groups only) or by one-way analysis of variance with either Bonferroni's multiple comparison test (MCT) or Dunnett's MCT (when comparing multiple groups *versus* a single group), as indicated. Homogeneity of variance was checked by Bartlett's test and, where necessary, data were transformed (log or square root) prior to analysis. The relationship between survival parameters and GAS5 levels in cells was analyzed by linear regression. Statistical analyses were performed using GraphPad Prism v4.03.

Results

GAS5 silencing attenuates apoptosis induction in breast cancer cells

To examine the effects of reduced *GAS5* expression on breast cancer cell survival, GAS5 siRNAs were employed to silence endogenous *GAS5* expression in two cell lines, MCF7 and T-47D; a range of siRNAs were employed to reduce the likelihood of 'off-target' effects. The influence of *GAS5* silencing on cell survival was examined under basal conditions and after apoptosis induction by a range of stimuli.

In MCF7 cells, GAS5 siRNAs reduced GAS5 transcript levels by upto one third of control levels (Fig. 1a). This had no statistically significant effect on basal apoptosis (Fig. 1b), short-term viability (Fig. 1d), viable cell number (Fig. 1f) or clonogenic activity (Fig. 1h), as judged from mock-irradiated cells. However, GAS5 knockdown attenuated apoptosis induction by UV-C irradiation (Fig. 1c) and the consequent reduction in culture viability (Fig. 1e) and clonogenic activity (Fig. 1i). Growth arrest due to UV-C treatment appeared less markedly affected however, as judged from viable cell counts (Fig. 1g).

Consistent with these observations, GAS5 knockdown in T-47D cells (Fig. 2a) diminished apoptosis induction by UV-C irradiation (Fig. 2c), as well as the associated losses in culture viability (Fig. 2e) and clonogenic activity (Fig. 2i); less robust effects on UV-C-induced growth arrest were also noted for this cell line (Fig. 2g). Basal apoptosis was also reduced by GAS5 silencing in T-47D cells (Fig. 2b), in contrast to findings in MCF7 cells (Fig. 1b). This

was associated with increased culture viability (Fig. 2d), but basal clonogenic activity was unaffected (Fig. 2h).

These findings were confirmed (Figs. 3a & b) in control experiments examining the effects of *GAS5* silencing on chemotherapeutic drug action. *GAS5* silencing diminished docetaxel-induced apoptosis and the associated loss of culture viability in both MCF7 (Fig. 3c) and T-47D cells (Fig. 3d). Similar effects were observed regarding 5-FU action in MCF7 (Fig. 3e) and T-47D (Fig. 3f) cells, whereas, imatinib action was unaffected by GAS5 silencing (Figs. 3g & h).

Breast cancer cell survival is related to cellular GAS5 levels

If impaired responses to chemotherapeutic drugs underlie the clinical relationship between patient survival and breast cancer GAS5 levels [10], then parameters of breast cancer cell survival should show quantitative relationships with cellular GAS5 levels. Regression analysis was therefore performed on data from the *GAS5* silencing experiments; cellular GAS5 levels were expressed relative to those in negative control siRNA-treated cultures to facilitate comparisons between the two cell lines.

Under basal conditions, apoptosis and viability were related to cellular GAS5 levels in T-47D cells (Fig. 4b) but not in MCF7 cells (Fig. 4a). Upon treatment with either UV-C irradiation (Figs. 4c & d), docetaxel (Figs, 4e & f) or 5-FU (Figs. 4g & h), consistent and statistically significant relationships were observed for MCF7 and T-47D cells; apoptosis was characterized by a direct relationship—and viability by a corresponding inverse

relationship—with cellular GAS5 levels (Figs. 4c – 4h). Parameters of cell survival after imatinib treatment were unrelated to cellular GAS5 levels in both cell lines (data not shown).

GAS5 lncRNA is sufficient to induce apoptosis in MCF7 cells

The transfection of breast cancer cells with GAS5 ESTs promotes apoptosis [9], but it is unclear whether mature GAS5 lncRNA is sufficient to mediate this action, as all the effective GAS5 ESTs contained retained snoRNAs. Therefore, the cell death-promoting activities of mature GAS5 lncRNA (*i.e.*, exons 1 – 12 only) and the GAS5-O1 EST (comprises snoRNA U74, 3′ sequence of the first intron and exons 2 – 12) were compared in MCF7 cells.

Transfection of MCF7 cells with each GAS5 construct increased cellular GAS5 levels to a similar extent (Fig. 5a). As expected [9], the GAS5-O1 construct increased the proportion of apoptotic cells and decreased culture viability (Fig. 5b) at 24 h *post*-nucleofection; the GAS5-AE construct, which encodes mature lncRNA, produced a similar quantitative response (Fig. 5b). Likewise, GAS5-O1 and GAS5-AE constructs stimulated UV-C-induced apoptosis and loss of culture viability (Fig. 5c) to a similar extent. Thus, mature GAS5 lncRNA is sufficient to promote apoptosis in MCF7 cells.

GAS5 lncRNA also induces apoptosis in triple-negative MDA-MB-231 cells

Endogenous levels of GAS5 transcripts are reduced in the TNBC cell line, MDA-MB-231, relative to estrogen receptor-positive cell lines [9, 21]. To examine if TNBC cells retain sensitivity to GAS5 lncRNA, parameters of cell survival were assessed following transfection

of MDA-MB-231 cells with the plasmid encoding mature GAS5 lncRNA. Increased GAS5 levels (Fig. 6a) were associated with increased basal apoptosis and reduced culture viability (Fig. 6b) from 24 h *post*-nucleofection, and also enhanced the death of MDA-MB-231 cells upon UV-C-irradiation (Fig. 6c). Thus, triple-negative MDA-MB-231 cells are sensitive to GAS5 lncRNA, as for MCF7 cells.

mTOR inhibition modulates GAS5 expression in breast cancer cell lines

Clinically, the use of mTOR inhibitors may offer a simple method to enhance cellular GAS5 levels in order to promote the death of breast cancer cells. Therefore, we initially examined the effects of rapamycin and related first generation mTOR inhibitors on breast cancer cell lines. These agents inhibited the growth of all cell lines by between *ca*. 20 – 50%; the extent of growth inhibition was similar for MCF7 and T-47D cells, but was noticeably smaller for MDA-MB-231 cells, irrespective of the applied rapalogue (Fig. 7a). Cellular GAS5 levels were enhanced by >50% ($P < 0.05$; one-way ANOVA and Bonferroni's MCT) by rapalogue treatment of MCF7 and T-47D cells, whereas they were unchanged in MDA-MB-231 cells (Fig. 7a). In view of this resistance of MDA-MB-231 cells to first generation mTOR inhibitors (which act selectively on mTORC1), we next examined the effects of inhibitors with a broader specificity, i.e. the dual mTORC1/mTORC2 inhibitor, AZD8055, and the dual PI3K/mTOR inhibitor, BEZ235. The pattern of cell line response to AZD8055 was similar to that of rapalogues, whereas BEZ235 markedly inhibited growth and robustly elevated GAS5 levels in all cell lines (Fig. 7b).

Consistent with this insensitivity to rapalogues and AZD8055, MDA-MB-231 cells failed to up-regulate cellular GAS5 levels upon cell density-induced growth arrest (Fig. 7c). In contrast, both MCF7 and T-47D cells were characterized by increased GAS5 expression in stationary *versus* logarithmic phases of growth (Fig. 7c). Further analysis of these data confirmed that, in actively growing cultures, levels of GAS5 expression were lower ($P < 0.05$; one-way ANOVA and Bonferroni's MCT) in MDA-MB-231 cells relative to both MCF7 and T-47D cells, as previously reported [9].

Discussion

The development of targeted therapies has revolutionised the treatment of hormone receptor-positive and human epidermal growth factor receptor 2 (HER2)-positive breast cancer, but resistance to these therapies remains problematic [25, 26]. Furthermore, there currently exists no approved targeted therapy for TNBC (lacks estrogen receptor, progesterone receptor and HER2 amplification), which frequently exhibits highly malignant behaviour and resistance to conventional chemotherapies [27]. The identification of novel therapeutic targets for multiple breast cancer subtypes is therefore important, and lncRNAs may offer new opportunities in this regard, given their emerging roles as regulators of fundamental biological processes [1-5]. Indeed, *GAS5* is already of particular interest in relation to breast cancer, since its expression is down-regulated in tumour tissue [9], and patient prognosis is significantly related to GAS5 transcript levels [10]. Current findings in preclinical models demonstrate here, for the first time, that breast cancer cell responses to several apoptotic stimuli are quantitatively related to cellular GAS5 levels, suggesting a potential functional basis for the latter clinically important relationship. The cytotoxic action of certain drugs, such as imatinib, is shown to be GAS5–independent, thereby suggesting a way to bypass the

relationship to improve patient outcomes. Importantly, a possible therapeutic role for GAS5 lncRNA in TNBC is identified and, in this regard, dual PI3K/mTOR inhibition is demonstrated to be effective in increasing GAS5 levels in such cells.

Although functional studies have previously reported that GAS5 transcripts exert apoptosis-promoting effects in breast cancer cells and in a range of other cell types [9, 15, 18-21], the consequences of reduced *GAS5* expression for breast cancer cell survival have not been addressed. Here we demonstrate that reductions in *GAS5* expression of *ca*. 50 - 70% in MCF7 and T-47D cells are consistently associated with attenuated cell death in response to a range of apoptosis-inducing agents (UV-C irradiation, 5-FU and docetaxel). Crucially, as in recent experiments with prostate cancer cells [20], both the extent of apoptosis induction and the associated loss of culture viability are quantitatively related to the extent of *GAS5* silencing. Thus, even small reductions in endogenous *GAS5* expression may adversely impact upon the responses of breast cancer cells to certain death-inducing stimuli, including conventional chemotherapeutic agents, and this resistance to therapy may explain why breast cancer patient survival is related to tumour GAS5 levels [10].

Imatinib, which provides targeted therapy through inhibition of receptor and non-receptor tyrosine kinases (*e.g.,* PDGF receptors, c-kit, c-Abl and Arg) in contrast, efficiently induced apoptosis in both MCF7 and T-47D cells, irrespective of cellular GAS5 levels. Thus *GAS5* selectively modulates the action of chemotherapeutic agents, perhaps related to their differing mechanisms of engagement of the apoptotic machinery, as suggested by the additive or synergistic nature of imatinib action with a range of conventional chemotherapeutic agents [28-30]. From a therapeutic perspective, the use of *GAS5*-independent drugs may offer an

improved strategy for breast cancer patients with reduced *GAS5* expression, albeit further work is required to identify additional chemotherapeutic agents which act in a *GAS5*-independent manner in breast cancer cells.

An alternative approach may be to enhance cellular GAS5 levels prior to, or concomitant with, the administration of conventional chemotherapies, in order to improve cytotoxic drug action. Current findings confirm that GAS5 lncRNA (rather than *GAS5*-derived snoRNA sequence) is sufficient for apoptosis-promoting activity, and further demonstrate that GAS5 lncRNA is active in the TNBC cell line, MDA-MB-231, which exhibits lower endogenous GAS5 levels than estrogen receptor-positive cell lines. Consequently, the proposed approach may be beneficial in TNBC, the treatment of which remains especially challenging [27]. Such a therapeutic goal could be achieved by a direct approach, involving, for example, gene therapy with vectors encoding GAS5 lncRNA, but the clinical application of such therapies in general, remains a long term prospect. Since a conventional pharmacological approach would be preferable in the short term, we focussed here on the mTOR pathway, which is involved in the physiological regulation of cellular GAS5 levels [11, 14]. Only the dual PI3K/mTOR inhibitor, BEZ235, elevated GAS5 levels in all cell lines.

The reasons for the resistance of GAS5 transcript levels to mTOR inhibition in MDA-MB-231 cells is unknown. This cell line also failed to increase GAS5 transcript levels upon cell density-induced growth arrest, suggesting a defect in the physiological mechanism of mTOR-mediated regulation of *GAS5*. Mutation of the 5’-TOP sequence can be excluded however, since the sequence of *GAS5* exon 1 (contains the 5’-TOP) in MDA-MB-231 cells is identical to that of the NCBI Reference Sequence, NR_002578.2 (data not shown). The mTOR

inhibitors are in increasing clinical use and resistance to such agents is becoming increasingly evident. The growth of MDA-MB-231 cells has been noted to exhibit reduced sensitivity to rapalogues [31-33], as also noted in our study. Nevertheless, basal-like cell lines are not totally unresponsive to mTOR inhibitors, since their death can be synergistically enhanced by combining such agents with cisplatin [34]. A striking finding in our study, however, was that GAS5 levels in MDA-MB-231 cells showed complete resistance to mTORC1 inhibition by rapalogues and to dual mTORC1/mTORC2 inhibition by AZD8055. In contrast, they displayed a significant increase in response to the dual PI3K/mTOR inhibitor, BEZ235, suggesting that mTOR-independent induction of GAS5 may prove a more productive approach to improving the therapy of advanced breast cancers.

Conclusions

The extent of breast cancer cell death consequent upon exposure to certain chemotherapeutic drugs and other apoptotic stimuli is directly related to cellular GAS5 levels, implicating regulation of apoptosis as the functional role that links *GAS5* expression levels with patient survival. The use of novel targeted therapies which act independently of GAS5, or combination therapies comprising conventional chemotherapeutics coupled with agents to enhance cellular GAS5 lncRNA levels, in breast tumours could prove beneficial in improving patient outcomes. In this regard, a wide range of mTOR inhibitors may prove useful in patients with receptor-positive disease, whereas treatment options may be limited to dual PI3K/mTOR inhibitors in TNBC patients. Findings here in breast cancer cell lines may be relevant to the therapy of a wide range of other cancers, including head and neck squamous cell carcinoma [10], glioblastoma multiforme [35], renal clear cell carcinoma [36], bladder

cancer [37], pancreatic cancer [38] and non-small-cell lung cancer [39], all of which are characterized by deficient *GAS5* expression.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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Figure legends

Fig. 1 Effect of GAS5 siRNA on basal survival and UV-C-induced cell death in MCF7 cells. MCF7 cells (n = 5 cultures) were transfected with the indicated GAS5 siRNA or negative control (NC) siRNA and harvested at 72 h *post*-transfection. Cells were treated ± UV-C irradiation and replated for assessment of cell survival after a further 48 h. RT-qPCR analysis confirmed decreased cellular GAS5 levels at 72 h *post*-transfection in cells treated with GAS5 siRNAs (**a**). Silencing of *GAS5* has no statistically significant effect on basal apoptosis in MCF7 cells (**b**), whereas it attenuates UV-C-induced apoptosis (**c**). Correspondingly, viability is not significantly affected in control cells (**d**), but it is enhanced in UV-C-treated cells (**e**). Viable cell numbers are not significantly affected in both control (**f**) and UV-C-treated (**g**) cells. Clonogenic activity is also unaffected in control cells (**h**) but it is enhanced in UV-C-treated cells (**i**). $^{*}P < 0.05$ & $^{**}P < 0.01$ *versus* cells transfected with NC siRNA (one-way ANOVA and Dunnett's MCT)

Fig. 2 Effect of GAS5 siRNA on basal survival and UV-C-induced cell death in T-47D cells. T-47D cells (n = 4 cultures) were transfected with the indicated GAS5 siRNA or negative control (NC) siRNA and harvested at 72 h *post*-transfection. Cells were treated ± UV-C irradiation and replated for assessment of cell survival after a further 48 h. RT-qPCR analysis confirmed decreased cellular GAS5 levels at 72 h *post*-transfection in cells treated with GAS5 siRNAs (**a**). Silencing of *GAS5* reduces both basal (**b**) and UV-C-induced apoptosis (**c**). Correspondingly, viability is enhanced in control (**d**) and UV-C-treated cells (**e**). Viable cell numbers are not significantly affected in both control (**f**) and UV-C-treated (**g**) cells. Clonogenic activity is unaffected in control cells (**h**) but it is enhanced in UV-C-treated cells (**i**). *$P < 0.05$, **$P < 0.01$ & ***$P < 0.001$ *versus* cells transfected with NC siRNA (one-way ANOVA and Dunnett's MCT)

Fig. 3 Effect of *GAS5* silencing on chemotherapeutic drug-induced death of breast cancer cell lines. MCF7 and T-47D cells (n = 5 cultures) were transfected with the indicated GAS5 siRNA or negative control (NC) siRNA and, after 72 h, exposed to chemotherapeutic agents or vehicle for 48 h. For all figure parts, apoptosis is displayed in the left-hand panel and culture viability in the right-hand panel. For vehicle controls, *GAS5* silencing had negligible effects overall on apoptosis and culture viability in MCF7 cells (**a**), whereas it reduced apoptosis and increased culture viability in T-47D cells (**b**). For docetaxel treatment, prior *GAS5* silencing resulted in consistent and corresponding effects on apoptosis and culture viability in both MCF7 (**c**) and T-47D cells (**d**). Similar effects were observed for 5-FU treatment of MCF7 (**f**) and T-47D (**g**) cells, whereas induction of cell death by imatinib was unaffected by *GAS5* silencing in both cell lines (**h** and **i**, respectively). *$P < 0.05$ & **$P < 0.01$ *versus* cells transfected with NC siRNA (one-way ANOVA and Dunnett's MCT)

Fig. 4 Parameters of cell survival are related to cellular GAS5 levels in breast cancer cell lines. Regression analysis was performed for data from Figs. 1 – 3 against cellular GAS5 levels normalized relative to control (NC siRNA) values; for all figure parts, apoptosis is displayed in the left-hand panel and culture viability in the right-hand panel. In the absence of any exogenous apoptotic stimulus, apoptosis and culture viability were unrelated to GAS5 levels in MCF7 cells (**a**), whereas apoptosis was directly related to—and culture viability was inversely related to—cellular GAS5 levels in T-47D cells (**b**). Apoptosis and culture viability were also related to GAS5 levels in both MCF7 and T-47D cells upon exposure to the apoptotic stimuli: UV-C irradiation (**c** & **d**); docetaxel (**e** & **f**); and 5-FU (**g** & **h**)

Fig. 5 Comparison of the effects of GAS5 ncRNA constructs on the survival of MCF7 cells, both basally and after UV-C-induced apoptosis. Cells (n = 4) were transfected with GAS5 constructs corresponding to mature lncRNA (exons 1 – 12; AE), the GAS5.O1 EST (snoRNA U74 plus exons 2 – 12; O1), or empty pcDNA3 vector (V) as control. After 24 h, cells were treated ± UV-C irradiation then re-sampled after a further 48 h. **a**) Cellular GAS5 levels at 24 h *post*-transfection are increased to a similar extent by GAS5 O1 and AE constructs. **b**) At 24 h *post*-transfection, the two GAS5 constructs result in similar increases in basal apoptosis (left-hand panel) and similar decreases in basal culture viability (right-hand panel). **c**) The two GAS5 constructs exhibit similar potency in enhancing both UV-C-induced apoptosis (left-hand panel) and the associated loss in culture viability (right-hand panel). *$P < 0.05$ & ***$P < 0.001$ *versus* cells transfected with pcDNA3 alone (one-way ANOVA and Bonferroni's MCT)

Fig. 6 GAS5 lncRNA is pro-apoptotic in MDA-MB-231 cells. Cells (n = 4) were transfected with a construct encoding mature lncRNA (AE) or empty pcDNA3 vector (V) as control and, after 24 h, cells were treated ± UV-C irradiation, then re-sampled after a further 48 h. **a**) Cellular GAS5 levels at 24 h *post*-transfection are increased by the GAS5 AE construct. **b**) GAS5 lncRNA increases basal apoptosis (left-hand panel) and reduces culture viability (right-hand panel) at 24 h *post*-transfection. **c**) GAS5 lncRNA increases UV-C-induced apoptosis (left-hand panel) and the associated loss of culture viability. $*P < 0.05$ & $***P < 0.001$ *versus* cells transfected with pcDNA3 alone (Student's *t*-test [a & b] or one-way ANOVA and Bonferrroni's MCT [c])

Fig. 7 Effects of pharmacological and physiological inhibitors of mTOR activity in breast cancer cell lines. **a**) Effect of rapamycin (1 μM; R), everolimus (10 nM; E) and temsirolimus (10 nM; T) on culture growth (at 70 h; left-hand panel) and GAS5 levels (at 20 h; right-hand panel). Data are expressed relative to the vehicle control; $^{*}P < 0.05$ and $^{**}P < 0.01$ *versus* MDA-MB-231 cells (one-way ANOVA and Bonferrroni's MCT; n = 4 cultures). **b**) Effect of AZD8055 (50 nM) and BEZ235 (100 nM) on culture growth (at 70 h; left-hand panel) and GAS5 levels (at 20 h; right-hand panel). Data are expressed relative to the vehicle control; $^{**}P < 0.01$ and $^{***}P < 0.001$ *versus* MDA-MB-231 cells and $^{++}P < 0.01$ *versus* MCF7 cells (one-way ANOVA and Bonferrroni's MCT; n = 4 cultures). **c**) Effect of cell density-induced growth arrest on cellular GAS5 levels; cells were harvested at logarithmic (log) and stationary (stat) phases of growth; $^{**}P < 0.01$ and $^{***}P < 0.001$ *versus* logarithmic phase cultures (one-way ANOVA and Bonferrroni's MCT; n = 4 cultures)

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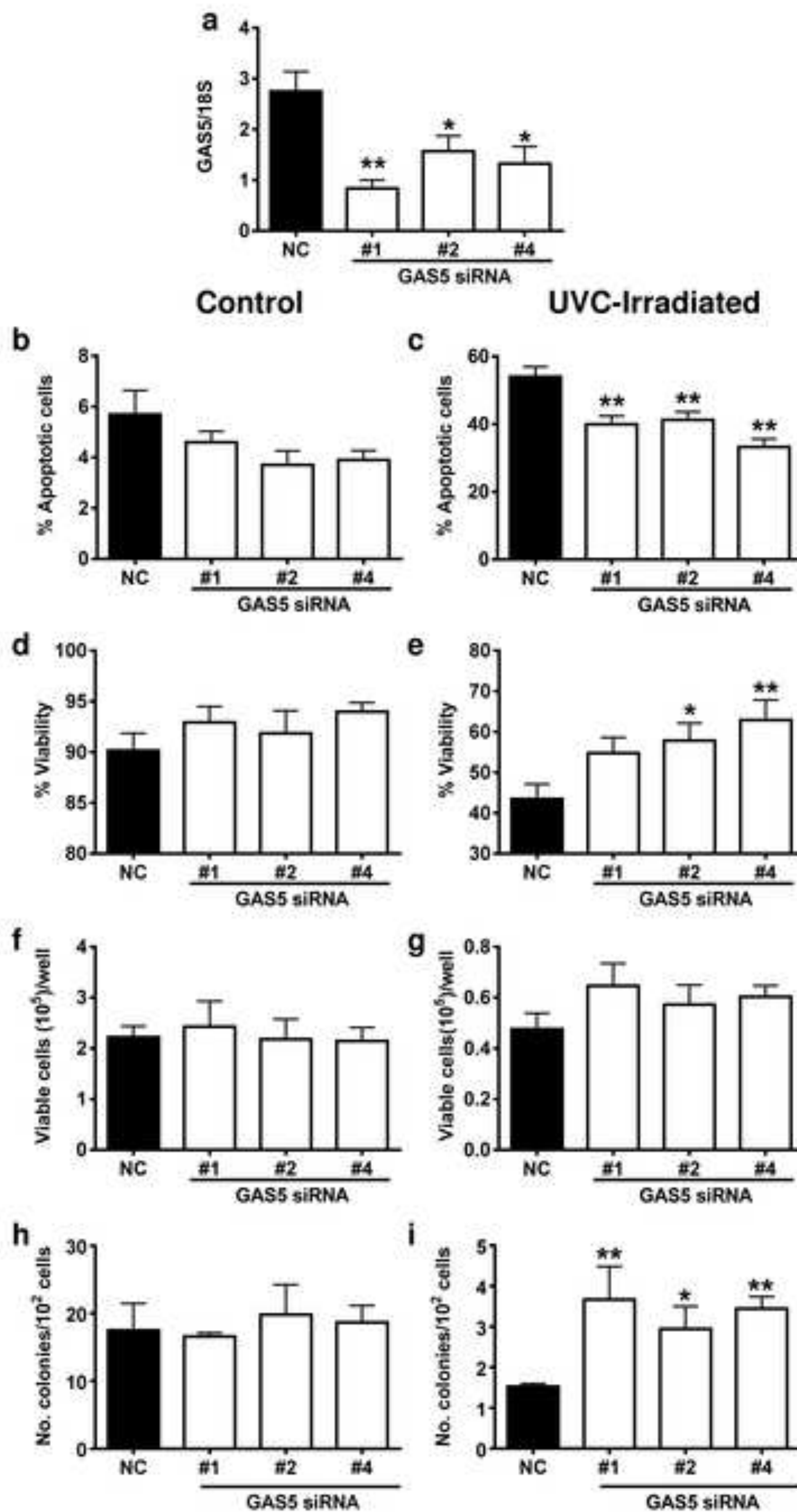

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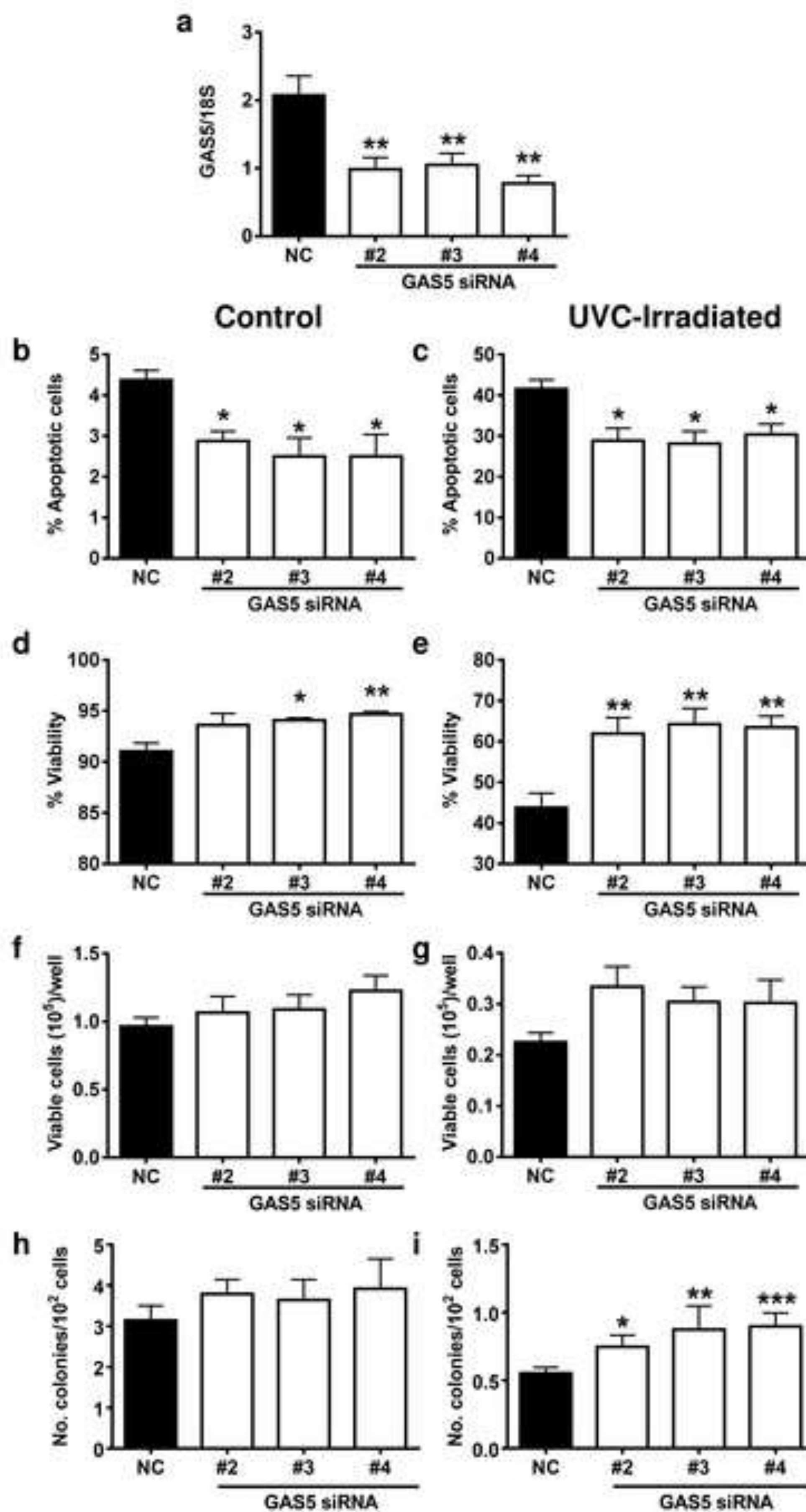

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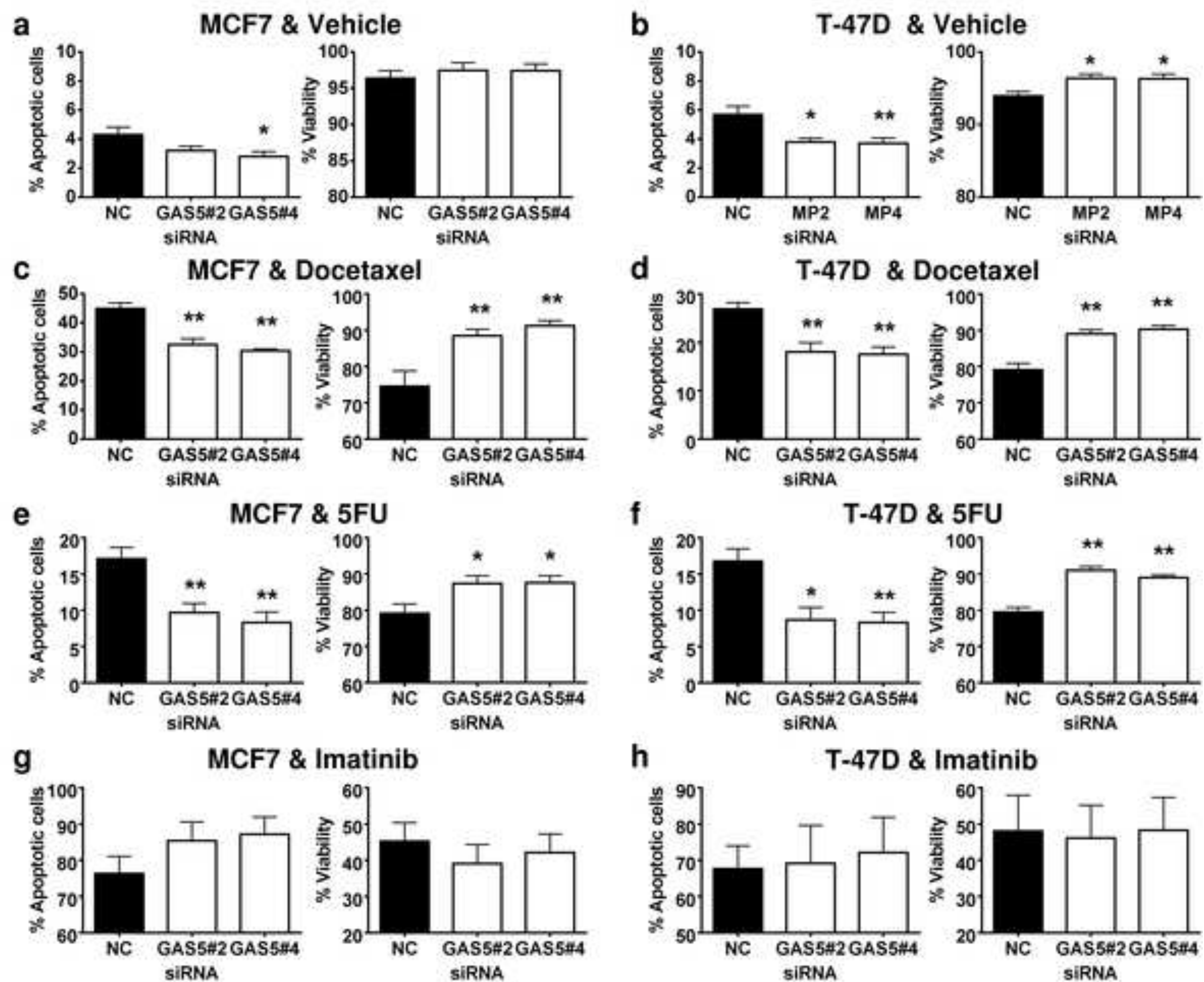

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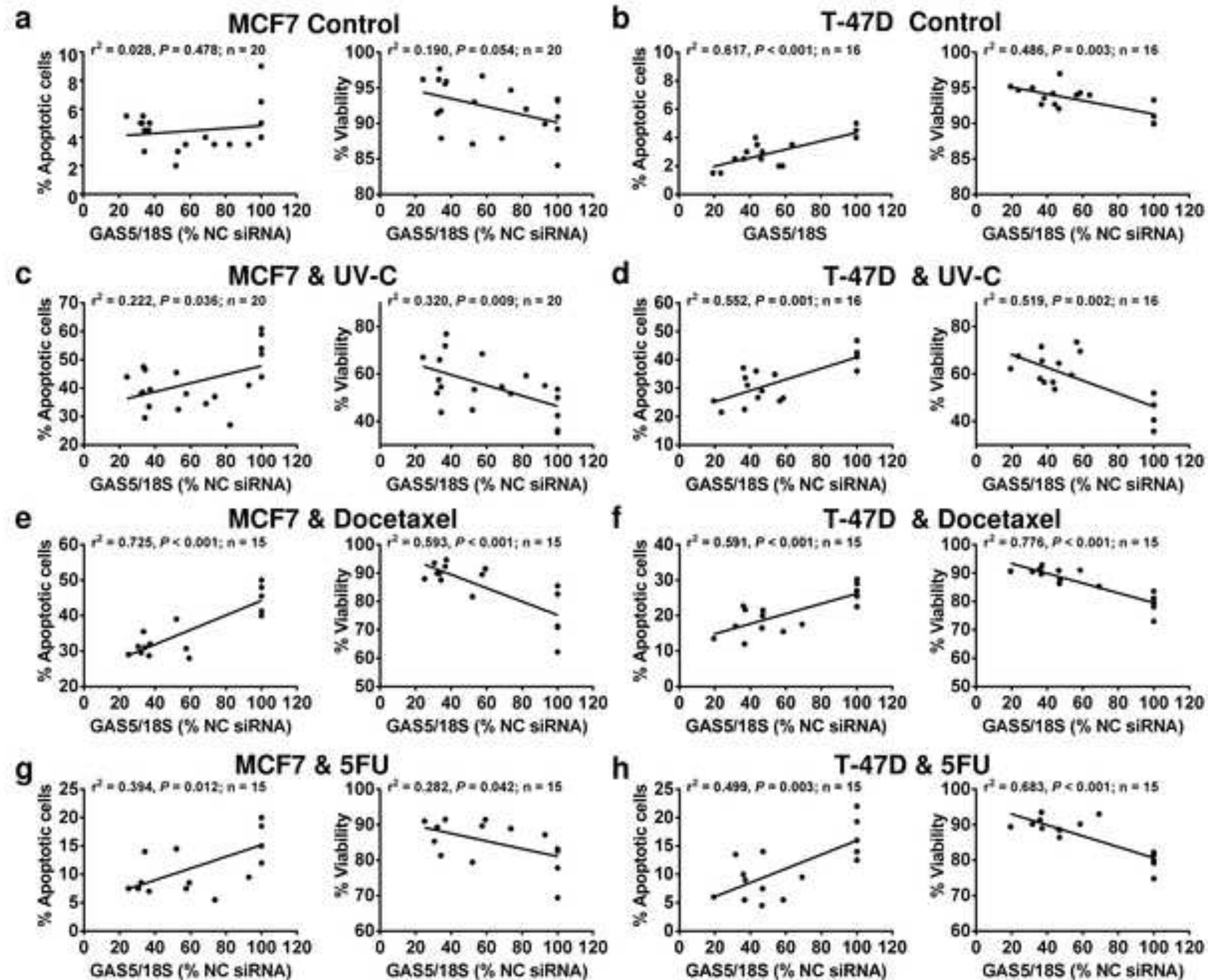

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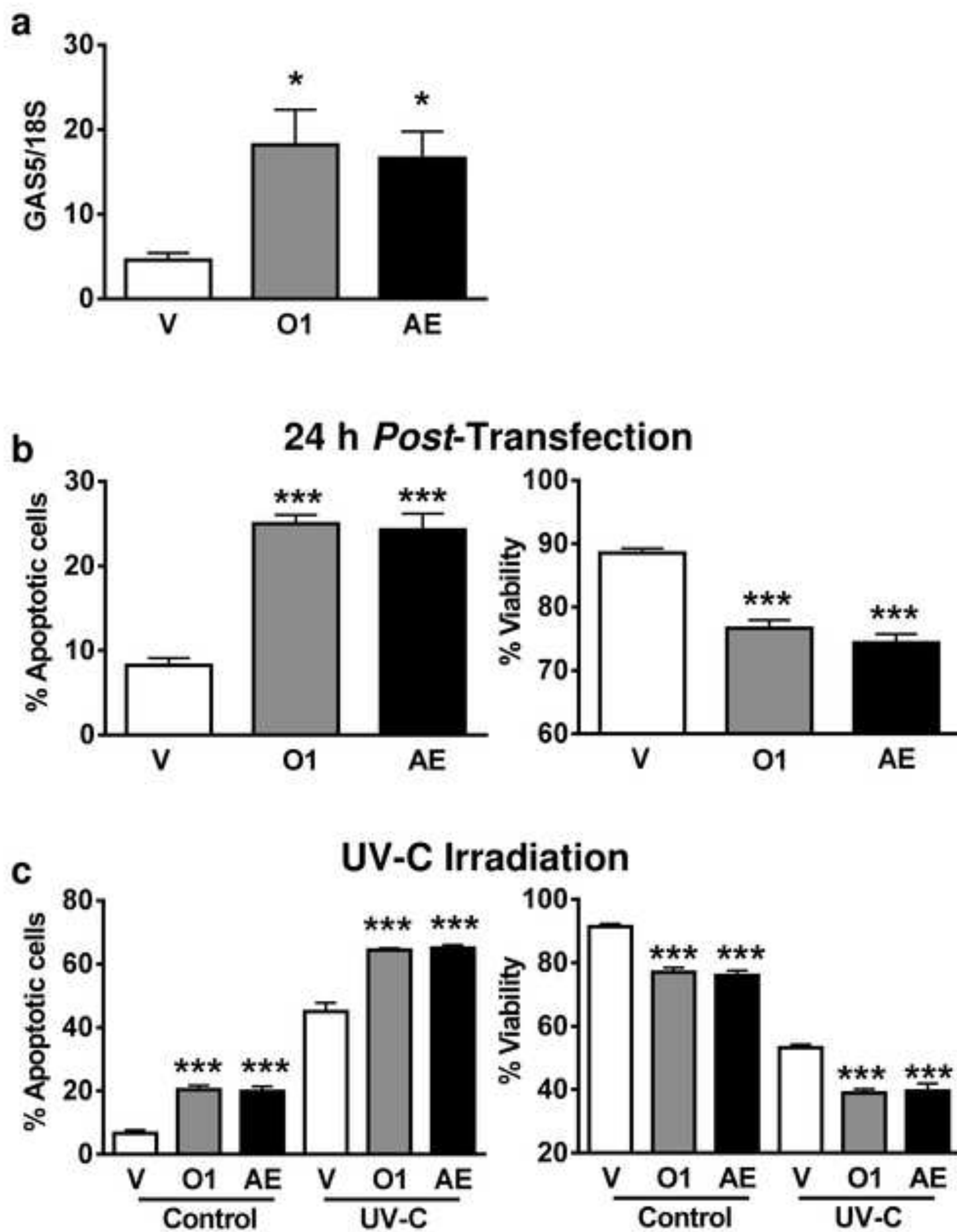

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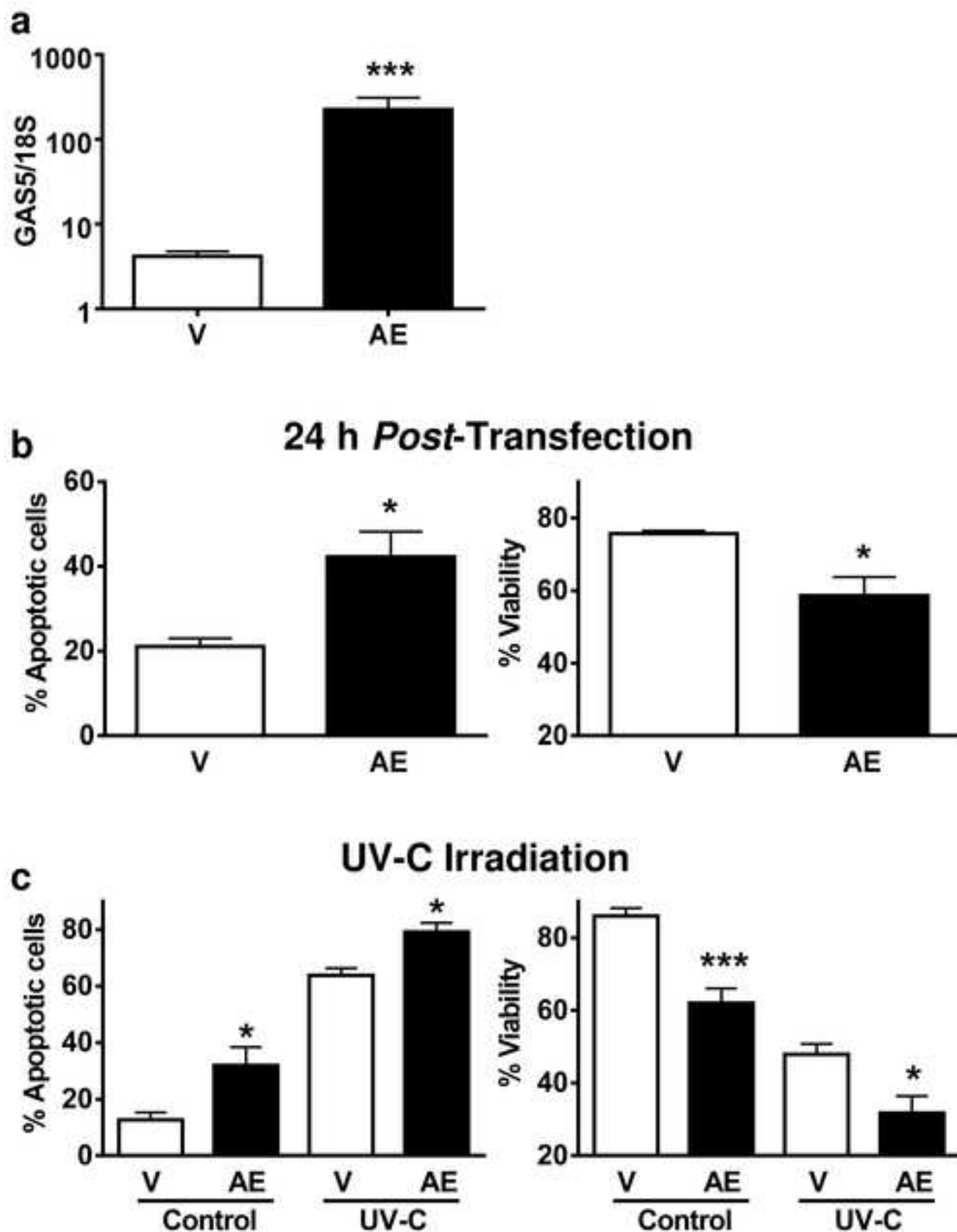

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Figure 7