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Phenotypic Variations of TRAIL Sensitivity in Cloned Populations of Prostate Cancer Cells

N.A. Cross,* E.A. Waterman, N. Jokonya, A. Fowles, C.H. Buckle, J. Phillips, I. Holen, F.C. Hamdy, and C.L. Eaton

Academic Urology Unit, University of Sheffield Medical School, Sheffield, UK

Abstract Factors that regulate the induction of apoptosis of tumour cells are potential candidates for therapeutic intervention for the majority of cancers. Studying modifiers of apoptotic responses, such as members of the tumour necrosis factor receptor superfamily, may give clues as to how induction of apoptosis in tumours could be maximized to enhance the benefit of treatment regimes. Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising anti-tumour molecule since its activity is specific for tumour cell populations. TRAIL binds to death receptors, inducing apoptosis in susceptible cells. The mechanisms which determine whether tumour cells are susceptible to TRAIL are unclear, and several mechanisms have been proposed, including expression of osteoprotegerin (OPG), decoy receptors, and factors that affect intracellular signalling of pro-apoptotic molecules, such as c-FLIP. Here we show that experiments to modulate the activity of one of these factors, OPG, by over-expression and also by stable knockdown of OPG expression, alters the TRAIL sensitivity of PC3 prostate cancer cells. However we show that some observed effects, which appear to support the hypothesis that OPG prevents TRAIL-induced apoptosis of tumour cells, may be due to variation of the TRAIL response of sub-clones of tumour cells, even within a cloned population. These results highlight potential limitations of experiments designed to test contribution of factors affecting intrinsic apoptosis susceptibility using cloned tumour cell populations. J. Cell. Biochem. 104: 1452–1464, 2008.

Key words: osteoprotegerin; RANKL; TRAIL; apoptosis; clonal variation; prostate cancer; osteolytic; bone metastasis

Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the tumour necrosis factor (TNF)-superfamily [Wiley et al., 1995; Pitti et al., 1996] and is a potent inducer of apoptosis. TRAIL has potential as an anti-tumour agent since its activity appears to specifically target tumour cells while not affecting most normal cell types [Lawrence et al., 2001]. Induction of apoptosis in susceptible cells in response to TRAIL is mediated by its binding to specific Death Receptors, DR4 and DR5 [Sheridan et al., 1997; Pan et al., 1997a,b] initiating apoptosis via an established signalling cascade. Activation of the latter by TRAIL-Death Receptor complexes is abrogated by decoy receptors on the cell surface, which bind TRAIL but do not contain functional intracellular signalling domains [Sheridan et al., 1997; Pan et al., 1997b]. TRAIL sensitivity is tightly regulated and can be affected both by intracellular and extracellular factors. For example intracellular signalling of TRAIL responses may be inhibited by over-expression of c-FLIP, an inhibitor of initiator caspases [Zhang et al., 2004]. Secretion of osteoprotegerin (OPG), which acts as a soluble decoy receptor for TRAIL, prevents binding of TRAIL to the Death Receptors [Emery et al., 1998]. OPG also acts as a soluble decoy receptor for Receptor Activator of NFκB (RANK) Ligand (RANKL), preventing osteoblast-derived RANKL binding and activating RANK on the surface of osteoclasts, thus affecting bone turnover [Simonet et al., 1997].

Our recent studies have shown that prostate cancer cells and breast cancer cells are able to secrete biologically relevant levels of OPG

Abbreviations used: OPG, osteoprotegerin; RANK, receptor activator of NFκB; TRAIL, tumour-necrosis factor-related apoptosis-inducing ligand.

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*Correspondence to: N.A. Cross, Academic Urology Unit, Du10, University of Sheffield Medical School, Beech Hill Road, Sheffield S10 2RX, UK. E-mail: n.a.cross@shef.ac.uk

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in vitro, reducing the pro-apoptotic effects of TRAIL [Holen et al., 2002, 2005], an effect that is reversed by soluble RANKL. Similar findings have been observed in other tumour types, suggesting that OPG secretion from tumour cells may represent a mechanism allowing tumour cells to bypass the pro-apoptotic effects of TRAIL [Naumann et al., 2004]. Furthermore OPG derived from cells of the bone marrow, which represents the most common single metastatic site for breast cancer, prostate cancer and multiple myeloma, secrete high levels of OPG in vitro which can inhibit TRAIL-induced apoptosis of tumour cells [Shipman and Croucher, 2003; Neville-Webbe et al., 2004; Nyambo et al., 2004]. OPG is expressed in the bone marrow by stromal cells, osteoblasts and potentially by endothelial cells. Work in our laboratories and elsewhere has also shown that serum levels of OPG are raised in patients with advanced prostate cancer compared to patients with organ confined disease [Brown et al., 2001; Jung et al., 2001; Eaton et al., 2004] and that elevated OPG levels may be an early indication of relapse in those patients receiving androgen ablation treatments [Eaton et al., 2004]. Taken together, these studies highlight OPG as a marker of progression in prostate cancer that may reflect disturbances in bone turnover associated with skeletal metastasis. Equally, raised serum OPG in prostate cancer patients, could also promote escape from anti-tumour mechanisms such as TRAIL-induced apoptosis resulting in disease progression.

Recent studies using the LNCaP prostate cancer cell line suggest that over-expression of OPG in these cells can alter the bone microenvironment when implanted in bone [Corey et al., 2005]. In contrast to PC3 cells, which induce osteolytic tumours in vivo, LNCaP cells induce osteosclerotic lesions. PC3 cells induce lytic bone disease most probably as a result of high DKK1 expression [Hall et al., 2005], which would be predicted to inhibit osteoblast maturation. In PC3 xenografts it is therefore likely that osteoclast-mediated bone resorption remains unopposed resulting in predominantly lytic lesions. Indeed, this concept has been recently tested and it has been shown that in the absence of DKK1, PC3 cells induce osteosclerotic lesions [Hall et al., 2005]. Over-expression of OPG in LNCaP cells increases the bone mineral density of tumour-affected bone, and decreases active osteoclast numbers, suggesting that OPG derived from prostate tumours can affect the bone marrow [Corey et al., 2005]. However LNCaP cells are largely resistant to TRAIL, and therefore correlates between OPG secretion and protection from TRAIL-induced apoptosis were not conclusive from this study.

To further elucidate the relevance of secreted OPG to the prevention of TRAIL-induced apoptosis of tumour cells, we designed experiments that would allow us to test the hypothesis that tumour-derived OPG protects prostate tumour cells from the effects of TRAIL both in vitro and in vivo. We engineered PC3 prostate cancer cells, which are sensitive to TRAIL and secrete sufficient levels of OPG to inhibit TRAIL-induced apoptosis in vitro, to express high levels of OPG and tested whether these cells now displayed enhanced resistance to TRAIL. Conversely we used shRNA knockdown of endogenously produced OPG in PC3 cells and tested whether TRAIL sensitivity was increased in these cells. We show that the generation of stable cell lines with either over-expression, or knockdown of OPG expression by shRNA, results in some clones of cells with phenotypes consistent with our hypothesis that PC3 tumour-derived OPG can protect cells from TRAIL-induced apoptosis. However we also show that the phenotypes observed may occur via mechanisms independent of secreted OPG.

METHODS

Cell Lines and Cultures

The human metastatic, androgen independent cell line PC3 was obtained from ATCC. Cells were cultured in DMEM medium containing Glutamax (Invitrogen, Paisley, UK) supplemented with 10% Fetal calf serum (Invitrogen) and routinely grown in 5% CO_2 at 37°C. Prior to transfection experiments, single clones of PC3 cells were isolated by serial dilution. These cells were shown to express OPG and were TRAIL sensitive. All cultures were confirmed negative of mycoplasma spp. infection using EZ-PCR mycoplasma system (Geneflow, Staffordshire, UK). In addition to studies with PC3 cell lines and sub-clones, two breast cancer cell lines, MDA-MB-231 and MDA-MB-436, were used in some experiments. Both of these cell lines have been shown to produce OPG and be sensitive to TRAIL [Holen et al., 2005].

Over-Expression of OPG

A cloned population of PC3 cells was stably transfected with the pTracer vector (Invitrogen) containing full length OPG (nucleotides 168–1,644, NM_002546.2), or the empty vector containing no insert. The sequence of the insert was confirmed by DNA sequencing to match the reference sequence for OPG. Cells were stably transfected using the profection transfection system (Promega, Southamptom, UK). Stable transformed cell populations were selected using 50 μg/ml Blasticidin (Invitrogen). Individual colonies were picked using cloning disks (Sigma, Poole, UK).

Knockdown of Endogenous OPG

Knockdown of endogenous OPG was performed using the pSilencer vector system (Ambion, Huntingdon, UK). shRNA inserts were designed to OPG using the shRNA design tools (www.ambion.com) and were synthesised by MWG-Biotech (Ebersberg, Germany) as follows: Forward 5′-GAT CCC ACA GCT CAC AAG AAC AGA TTC AAG AGA TCT GTT CTT GTG AGC TGT GTT A-3′ and reverse 5′-AGC TTA ACA CAG CTC ACA AGA ACA GAT CTC TTG AAT CTG TTC TTG TGA GCT GTG G-3′. Inserts were cloned into pSilencerCMV4.0 Neo^R vector and transfected into cloned PC3 cells using the Profection transfection system (Promega). Stable transformed cells were selected using 0.5 mg/ml Geneticin (Invitrogen) and stable transfected clones were isolated as described earlier.

Assessment of OPG Secretion Into Medium

Cells were plated at 10,000 cells/cm^2 in 24-well plates, and after 2 days, the medium was changed. Cell numbers were determined by Coulter counter and conditioned medium collected at 24-h intervals for up to 4 days. Conditioned medium was stored at −20°C until analysis as follows. Briefly, ELISA plates were coated with 2 μg/ml mouse monoclonal antihuman OPG (MAB 8051, R&D Systems, Abingdon, Oxfordshire). The OPG standard curve was generated using recombinant human OPG (R&D Systems) at concentrations from 2,000 to 31.25 pg/ml. The secondary antibody was biotinylated antihuman OPG (BAF 805, R&D Systems) at 200 ng/ml, and detection was done using streptavidin-horseradish peroxidase (R&D Systems) in combination with a 3,3′,5,5′-tetramethylbenzidine substrate (Sigma). The reaction was stopped after 5–20 min incubation in the dark by addition of 50 μl 2 M H_2SO_4. The plate was read at 450 nm on a Dynex plate reader using Revelation® software (Dynex, Worthing, Sussex, UK).

Evaluation of OPG Expression by Western Blotting

OPG was immuno-precipitated from conditioned medium from PC3 cells and PC3-OPG/EV clones as follows. Two micrograms of MAB8051 mouse anti-OPG (R&D systems) was added to 1 ml of conditioned medium for 2 h at 4°C. To this, 40 μl of hydrated Protein A sepharose (Pharmacia Biotech) was added and incubated at 4°C for 18 h. After washing three times in PBS, samples were added to SDS–PAGE loading buffer and run on NuPAGE 4–12% Bis-TRIS polyacrylamide gels (Invitrogen). Proteins were then blotted onto PVDF membrane at 50 V for 1 h. To ensure even transfer, membranes were stained with *Ponceau S* (Sigma). Antigens were detected on the casein-blocked membrane using mouse monoclonal primary antibodies against human OPG (MAB 8051). Detection was carried out using a peroxidase labelled anti-mouse secondary antibody (Amersham Biosciences, Buckinghamshire, UK) with ECL detection kit (Amersham Biosciences).

Assessment of Gene Expression by Real-Time RT-PCR

Total cellular RNA was isolated using Tri-Reagent (Sigma) which was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) and random primers (Invitrogen). Real-time RT-PCR was performed on an ABI7900 (Applied Biosystems, Warrington, UK) using Universal PCR mastermix (Applied Biosystems). Briefly, each reaction consisted of 5 μl of 2× Universal Taqman mastermix, 3.5 μl H_2O, 0.5 μl Assay-on-Demand primer/probe mix (Applied Biosystems). Assays used were GAPDH (Hs99999905 m1), OPG (Hs00171068 m1) DR5 (Hs00187196 m1) and (OAS1 Hs00242943 m1). All reactions were performed in triplicate in 384-well plates.

RT-PCR data were analysed using SDS 2.0 software (Applied Biosystems). Basal gene expression was first expressed as ΔC_T, namely the cycle threshold (C_T) at which fluorescence was detectable above background for the internal standard (GAPDH) minus C_T of the test

gene. Data for OPG levels and OAS1 levels were expressed as relative expression calculated by the comparative C_T or $\Delta\Delta C_T$ method (Applied Biosystems recommendations). Relative expression was determined by applying the formula $2^{-\Delta\Delta C_T}$, where $\Delta C_T = C_T$ of test gene—C_T of reference gene (GAPDH) and $\Delta\Delta C_T = \Delta C_T$ of test gene (knockdown cells)—ΔC_T of test gene (control cells). To enable the use of the comparative C_T calculation, all primer/probe sets were shown to have an efficiency of 100% as determined by template dilution experiments (Applied Biosystems recommendations) in which a 10-fold dilution of cDNA resulted in a 3.3-fold (± 0.1) increase in C_T over at least three orders of magnitude.

Assessment of Apoptosis

Apoptosis levels in response to the presence or absence of TRAIL was determined by assessment of nuclear morphology following DAPI staining. Briefly, cells were seeded into 24-well plates as described previously. Cells were allowed to grow for 4 days, then medium was changed and the cells allowed to grow for a further 4 days. Cells were then challenged with 25 ng/ml TRAIL (R&D Systems) for OPG over-expression studies, and 10 ng/ml TRAIL for OPG knockdown studies in fresh media or in the presence of 4-day conditioned medium for 24 h. Cells were harvested and fixed in 4% formaldehyde. In some experiments, cells were challenged with TRAIL in conditioned media in the absence and presence of at least a 10-fold molar excess of sRANK ligand (PeproTech, London, UK). The fixed cells were cytospun onto glass slides and stained with DAPI (1 μg/ml; Sigma). The slides were mounted using Citiflour (Agar Scientific, Essex, UK) and cells were counted using UV light microscopy. In other experiments, cells were plated at low density in 100 mm dishes and discrete colonies allowed to grow until 2–3 mm diameter, allowing assessment of the range of TRAIL sensitivities in a given cell culture. Colonies were marked prior to challenge with TRAIL (25 ng/ml) in fresh medium, and apoptosis in individual colonies was assessed by adding 1 μg/ml Hoechst 33342 (Sigma) prior to assessment of apoptosis by fluorescence microscopy.

Intratibial Implantation of PC3-OPG Cells

PC3-OPG and PC3-EV cells were harvested when 80% confluent and detached using trypsin/EDTA All procedures were performed on male MF1 athymic mice (aged 6–8 weeks; Harlan laboratories, Bicester, UK). Intratibial injections were performed using a modification of the method of Corey et al. [2002]. Prior to and during surgery, mice were anaesthetised using 1–2% Isofluorane. An incision was made in front of the right tibia and two holes drilled into the bone marrow cavity using a 27-gauge needle. The bone marrow cavity was then flushed with sterile saline prior to injection of 10 μl of culture medium containing 1×10^5 cells. Finally, holes in the bone were filled with bone wax. Prior to closure, the area washed with sterile water to kill any tumour cells that spilled out of the holes in the bone, thus reducing the incidence of sub-cutaneous tumour growth in the leg.

Tartrate Resistant Acid Phoshphatase Staining of Activated Osteoclasts

After sacrifice of animals, legs were fixed in 4% paraformaldehyde followed by decalcification for 96 h in 0.15 M EDTA at 45°C. Tissues were then assessed by standard histology. TRAP-staining was performed on sections using standard histological procedures.

RESULTS

Over-Expression of OPG in PC3 Cells

A cloned population of PC3 cells was transfected with pTracer vector containing full length human OPG. Stable cell clones were isolated and OPG ELISA performed as shown in Figure 1a. OPG levels in conditioned medium from empty vector clones and untransfected PC3 cells were less than 1 ng/ml after 4 days in culture. In conditioned medium from clones of cell over-expressing OPG, over 35 ng/ml was detected. Over-expression of OPG did not correlate with altered proliferation of transfected cell clones, although some variation in proliferation rates of individual OPG clones was evident, however this did not correlate with the amount of OPG secreted (Fig. 1b). Real-time RT-PCR analysis of PC3-OPG clones showed approximately 1000-fold increase in OPG transcript levels compared to untransfected PC3 cells and PC3 cell clones transfected with empty vector (Fig. 1c). Conditioned medium from PC3 cell cultures was analysed for the presence of OPG by western blotting (Fig. 1d). Conditioned

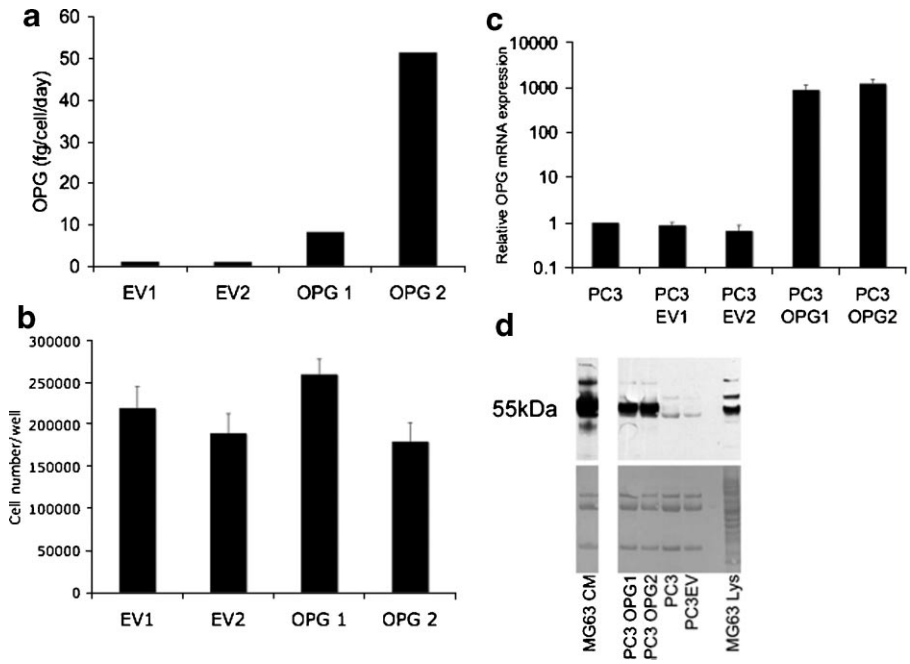

Fig. 1. Detection of OPG in cells transfected with full length OPG sequences. **a**: Detection of increased levels of OPG in cells transfected with pTracer-OPG, compared to cells transfected with pTracer-empty vector (EV). Accumulated levels of OPG in PC3-OPG1 and PC3-OPG2 were 8 and 37 ng/ml respectively, compared with less than 1 ng/ml in control cells. **b**: Effect of OPG over-expression on proliferation. OPG expression levels did not correlate with altered proliferation in PC3 cells. **c**: Relative expression of OPG mRNA in PC3-OPG cell clones and control cells, as determined by real-time RT-PCR (note logarithmic scale). **d**: Detection of a 55 kDa immunoreactive species of OPG in conditioned medium from PC3 cells transfected with full length OPG cells by immunoprecipitation followed by Western blotting. MG63 (osteosarcoma cell line) is used as a positive control and shows the presence of a 55 kDa species in both conditioned medium and cell lysate. Non-specific bands are likely to relate to IgG bands (see Ponceau S stained loading control gel below).

medium was immunoprecipitated using the mouse anti-OPG antibody (MAB8051, R&D systems) and the immunoprecipitated OPG was detected by western blotting using the goat anti-OPG antibody BAF805 (R&D systems). A strong immunoreactive signal of approximately 55 kDa was detected in PC3-OPG clones, but not in PC3-EV clones or untransfected cells. Both conditioned medium and cell lysate from the MG63 osteosarcoma cell line, which expresses very high levels of OPG (>1,000 ng/ml), is shown as a positive control, with immunoreactive species at 55 kDa.

Effect of OPG Over-Expression on TRAIL-Induced Apoptosis

To assess whether OPG affected TRAIL-induced apoptosis in PC3-OPG cells, PC3-EV and PC3-OPG cell clones were challenged with TRAIL (25 ng/ml). In previous experiments, we have shown that cultured PC3 cells can secrete biologically relevant levels of OPG into conditioned medium over 4 days (up to 2.5 ng/ml), and these levels are sufficient to suppress TRAIL-induced apoptosis. We tested the TRAIL sensitivity of PC3-OPG and PC3-EV cells in response to 25 ng/ml TRAIL for 24 h. Figure 2a shows that when PC3-OPG cells and PC3-EV cells are challenged with TRAIL in fresh medium, that is before accumulation of protective levels of OPG, these clones have widely varying responses to TRAIL. These clones of cells were isolated from an isogenic cloned parent population of cells, and not from a heterogenous starting population as present in many cell lines. To determine whether this variation in TRAIL sensitivity was reproducible in PC3-EV clones, transfections were repeated, using a different cloned population of PC3 cells, and TRAIL sensitivity assessed as previously determined. Again, several clones of PC3-EV cells were isolated and these clones also had widely varying TRAIL sensitivities when challenged with 25 ng/ml TRAIL for 24 h in fresh medium.

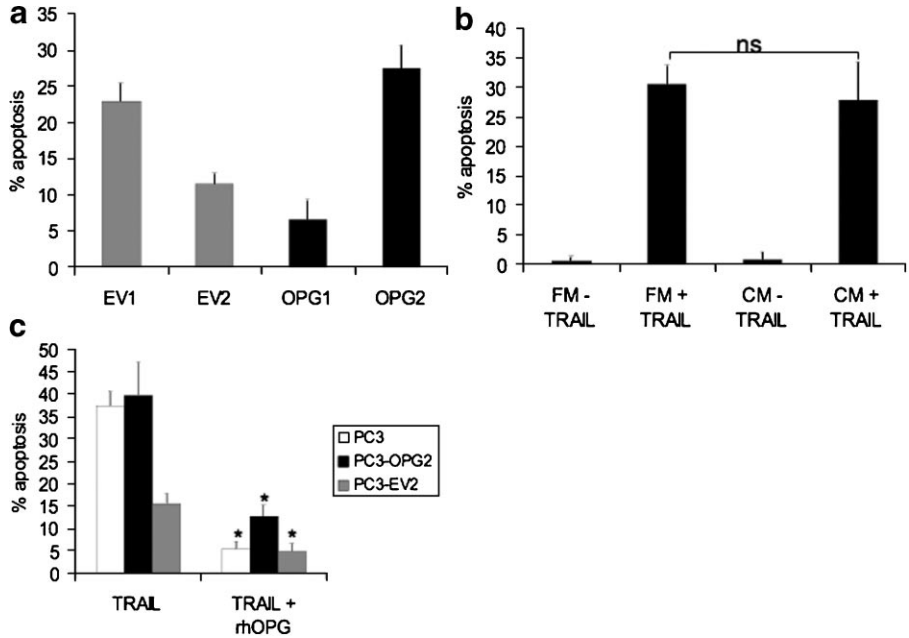

Fig. 2. **a**: PC3-EV and PC3-OPG clones were challenged with 25 ng/ml TRAIL for 24 h in fresh medium. Marked variation between clones of cells was evident and no correlation between OPG expression and TRAIL sensitivity was observed. **b**: Challenge of PC3-OPG2 cells with 25 ng/ml TRAIL in fresh medium and in medium conditioned (over PC3-OPG2 cells for 48 h). Conditioned medium containing high levels (approximately 25 ng/ml) of accumulated OPG did not protect the cells from TRAIL. **c**: Challenge of PC3 cells, PC3-OPG2 and PC3-EV2 cells with TRAIL in the presence or absence of rhOPG (20 ng/ml). rhOPG protected all cells from TRAIL ($P < 0.05$ *t*-test). All data shown are representative experiments of three independent analyses.

In these experiments, TRAIL sensitivity did not correlate with OPG levels, potentially indicating the expression of an inappropriate form of OPG from these cells when expression is forced at high levels. To determine whether OPG was able to prevent TRAIL-induced apoptosis, as shown previously for the PC3 cell line [Holen et al., 2002], PC3-OPG clones were challenged with TRAIL in the presence of fresh medium or growth medium that had been conditioned over these cells for up to 4 days (Fig. 2b). In this system, OPG over-expression did not further prevent TRAIL-induced apoptosis. Data are show for the PC3-OPG2 clone, but similar effects were observed in numerous PC3-OPG clones, suggesting that this secreted OPG is unable to prevent TRAIL-induced apoptosis in these clones.

To establish that cloned populations of cells were responding to TRAIL in a similar manner to previous experiments, PC3 cells, PC3-OPG cells and PC3-EV cells were challenged with TRAIL in the presence or absence of 20 ng/ml recombinant human OPG (Fig. 2c). All three strains of cells were significantly protected from TRAIL by the addition of rhOPG, consistent with our previous studies [Holen et al., 2002].

Assessment of OPG-Over Expression on Tumour Growth In Vivo

To elucidate why OPG derived from PC3-OPG cells did not protect PC3 cells from TRAIL-induced apoptosis, we tested whether this OPG could interfere with RANK-RANKL interactions. Previous studies have shown that over-expression of OPG in LNCaP prostate cancer cells can reduce osteoclast activation in bone by disrupting RANK–RANKL interactions and hence, osteoclast activation [Corey et al., 2005]. PC3 cells induce osteolytic tumours in bone, which are associated with activated osteoclasts. PC3-OPG2 cells were injected directly into the tibia of nude mice and bone sections assessed by staining for Tartrate resistant acid phosphatase (TRAP) to determine whether the tumour cells supported osteoclast differentiation. Figure 3 shows that PC3-OPG2 cells supported osteoclast activation in bone, suggesting that that OPG derived from these cells was not functional.

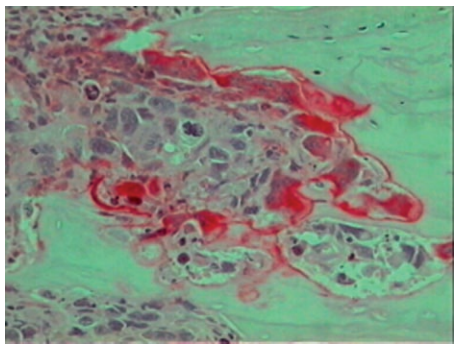

Fig. 3. TRAP staining of activated osteoclasts surrounding PC3-OPG2 cells implanted into the tibia of nude mice, showing the presence of numerous activated osteoclasts (red) in areas of eroded bone.

Knockdown of Endogenous OPG in PC3 Cells

We tested whether knockdown of endogenous OPG affected TRAIL sensitivity using stable expression of shRNA directed to OPG. Again, starting from a cloned population of PC3 cells which expressed OPG and was TRAIL sensitive, stable clones of cells were isolated with approximately 50% reduction of OPG secretion as determined by OPG ELISA (Fig. 4b). Real-time RT-PCR confirmed knockdown of OPG mRNA in these clones of cells (Fig. 4a).

Effect of OPG Knockdown on TRAIL-Induced Apoptosis

To test whether knockdown of OPG affected TRAIL sensitivity, cells were challenged with 10 ng/ml TRAIL for 24 h in fresh medium (i.e., before accumulation of OPG) and apoptosis levels determined by evaluation of nuclear morphology following DAPI staining (Fig. 5). The level of OPG knockdown in the clones was very similar, however TRAIL sensitivity was increased in knockdown clone 1 (OPG KD1), whereas no increase in TRAIL sensitivity was observed in knockdown clone 2 (OPG KD2). Increased TRAIL-sensitivity was also observed in several other OPG knockdown clones when challenged in fresh medium. To assess whether differential expression of the TRAIL receptor DR5 could account for differences in TRAIL

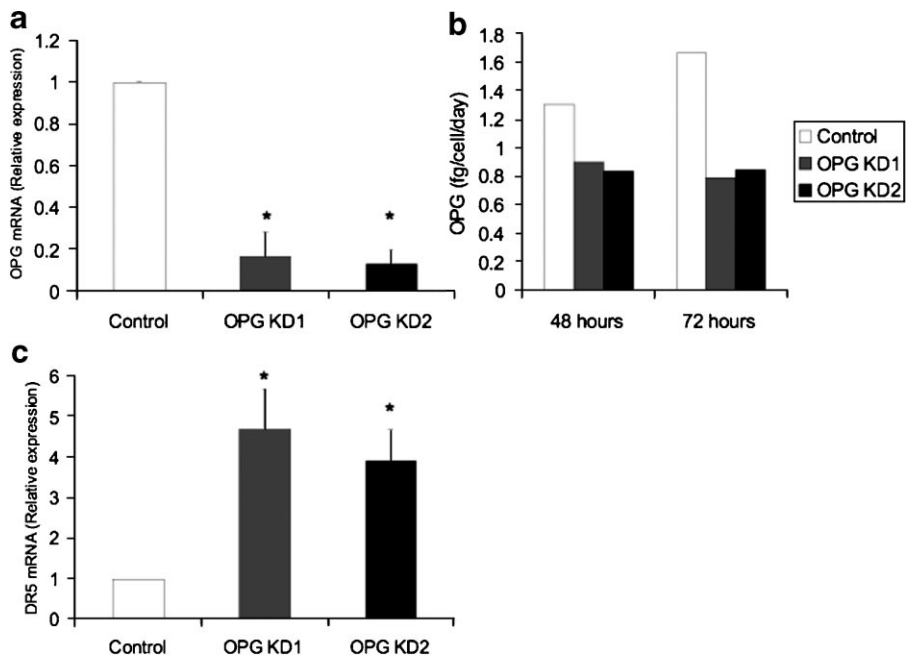

Fig. 4. **a**: Knockdown of OPG mRNA expression by shRNA as determined by real-time RT-PCR. Analyses were performed using triplicate wells and data obtained from duplicate independent experiments. **b**: Knockdown of OPG protein secretion in PC3 cells. Cells were cultured for up to 4 days and OPG levels in conditioned medium determined by ELISA. Reduced levels of secreted OPG was not due to alterations to cell proliferation. After 72 h, conditioned medium from control cells contained 835 pg/ml of accumulated OPG, whereas knockdown cells expressed 400 pg/ml. **c**: Analysis of Death Receptor 5 mRNA levels in OPG-knockdown cells. OPG-knockdown cells demonstrated raised DR5 mRNA levels as determined by real-time RT-PCR.

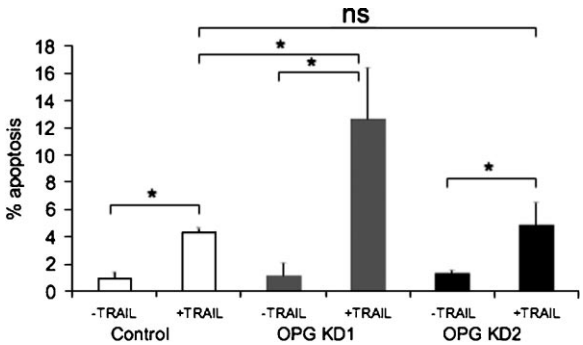

Fig. 5. TRAIL-induced apoptosis of cells with reduced OPG expression. Cells were challenged with 10 ng/ml TRAIL for 24 h in fresh medium. Lower levels of TRAIL were used in these experiments, compared to OPG over-expression studies, to allow observation of small changes in TRAIL sensitivity, which may be due to alterations of low levels of endogenous OPG. All clones demonstrated a significant induction of apoptosis in response to TRAIL ($P < 0.05$, *t*-test). OPG Knockdown clone 1 demonstrated significantly increased TRAIL sensitivity compared control cells. Knockdown clone 2, with comparable OPG knockdown to clone 1, demonstrated similar TRAIL sensitivity to control cells. All data shown are representative experiments of three independent analyses.

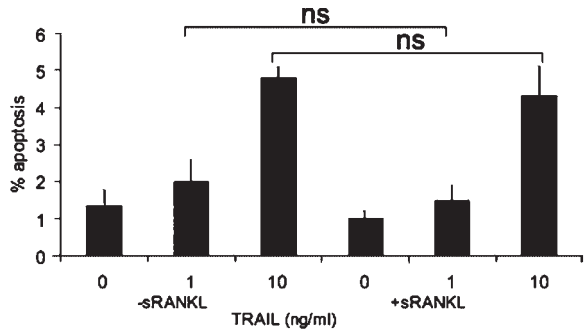

Fig. 6. Increased TRAIL-sensitivity of cells with reduced OPG expression in fresh medium is not due to secreted OPG. PC3 cells were cultured challenged with 0, 1, or 10 ng/ml TRAIL for 24 h in the presence or absence of sRANKL (100 ng/ml). sRANKL did not affect apoptosis in the absence of TRAIL, and did not affect TRAIL sensitivity in fresh medium, suggesting that OPG levels in fresh medium after 24 h are insufficient to affect TRAIL-induced apoptosis in normal PC3 cells. These findings confirm that the increased TRAIL sensitivity of PC3 OPG KD clone 1 in fresh medium cannot be attributed to decreased OPG expression.

sensitivity, real-time RT-PCR for DR5 mRNA levels was performed. OPG KD clones 1 and 2 exhibited raised DR5 levels (Fig. 4c), however since only OPG KD clone 1 demonstrated increased TRAIL-sensitivity, DR5 levels alone were unable to fully explain the differences in TRAIL sensitivity between two knockdown clones with very similar OPG knockdown.

Our previous studies show that the accumulation of OPG in conditioned medium protects PC3 cells against TRAIL-induced apoptosis, and this activity is inhibited using sRANKL [Holen et al., 2002]. To confirm that OPG levels were insufficient to protect PC3 cells when challenged in fresh medium, that is increased TRAIL sensitivity of OPG-KD clones was not due to reduced OPG secretion, parental PC3 cells were challenged with TRAIL in fresh medium in the presence and absence of sRANKL, which binds OPG preventing anti-TRAIL activity of OPG (Fig. 6). sRANKL had no effect on TRAIL-induced apoptosis of parental PC3 cells in fresh medium and had no effect on apoptosis in the absence of TRAIL, confirming that altered OPG levels cannot explain altered TRAIL sensitivity when challenged in fresh medium, and that increased TRAIL-sensitivity in OPG knockdown cells must be independent of secreted OPG levels.

Interferon Response Is Not Induced by OPG shRNA Expression

Some studies using RNA interference have encountered problems due to an interferon response [Bridge et al., 2003; Fish and Kruithof, 2004]. To test whether interferon response was the likely cause of increased TRAIL-induced apoptosis in OPG KD clone 1, cells were tested for the expression of Oligo Adenylate Synthase-1 (OAS1), a marker of interferon response [Pebernard and Iggo, 2004]. Using quantitative RT-PCR, all clones of cells were assessed for OAS1 levels. Figure 7 shows that all clones of

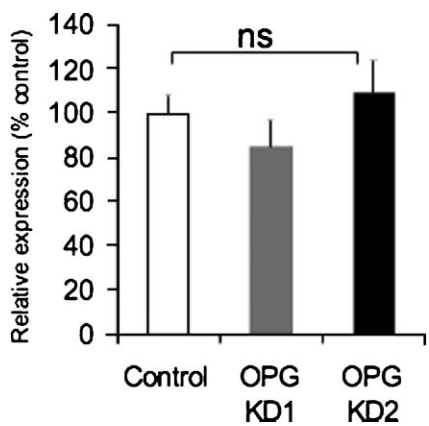

Fig. 7. Real-time RT-PCR determination of OAS1 levels in cells with reduced OPG expression. OAS1, a marker of interferon response, is not significantly altered between clones of cells with reduced OPG expression but with altered TRAIL sensitivities, suggesting that increased TRAIL sensitivity of Clone 1 is not due to induction of interferon by expression of shRNA.

cells expressed very similar levels of OAS1, suggesting that increased TRAIL-sensitivity of OPG knockdown clones was not likely to be due to an interferon response.

Assessment of TRAIL Sensitivity of Individual Cell Colonies

To assess the range of TRAIL-sensitivities of individual cell populations within cell lines and clonal cell lines, cells were plated at low density and discrete colonies allowed to develop. Colonies were photographed and position referenced. These colonies were challenged with TRAIL and apoptosis determined by Hoechst 33342 staining and assessment of nuclear morphology. Using untransfected PC3 cells, colonies with widely varying TRAIL sensitivities were observed, ranging from as high as 35% apoptosis to less than 5% apoptosis over 24 h (Fig. 8a). In parallel experiments, cells were treated with TRAIL for longer periods and colonies that were unaffected by TRAIL readily proliferated, whereas neighbouring colonies were completely killed. Similar variations in TRAIL sensitivity were also observed with single cell clones of PC3 cells, demonstrating that even within cloned cell lines, there is considerable variation in TRAIL-sensitivity between individual cells (Fig. 8b). Similar variation was observed in the PC3-OPG clonal cell lines (Fig. 8c). To establish whether this phenomenon was restricted to PC3 cells, we tested two breast cancer cell lines, MDA-MB-231 and MDA-MB-436, both shown to be TRAIL sensitive in our previous studies [Holen et al., 2005]. Similarly, cloned cell strains from both of these cell lines demonstrated a wide range of TRAIL-sensitivities in individual colonies (Fig. 8d,e).

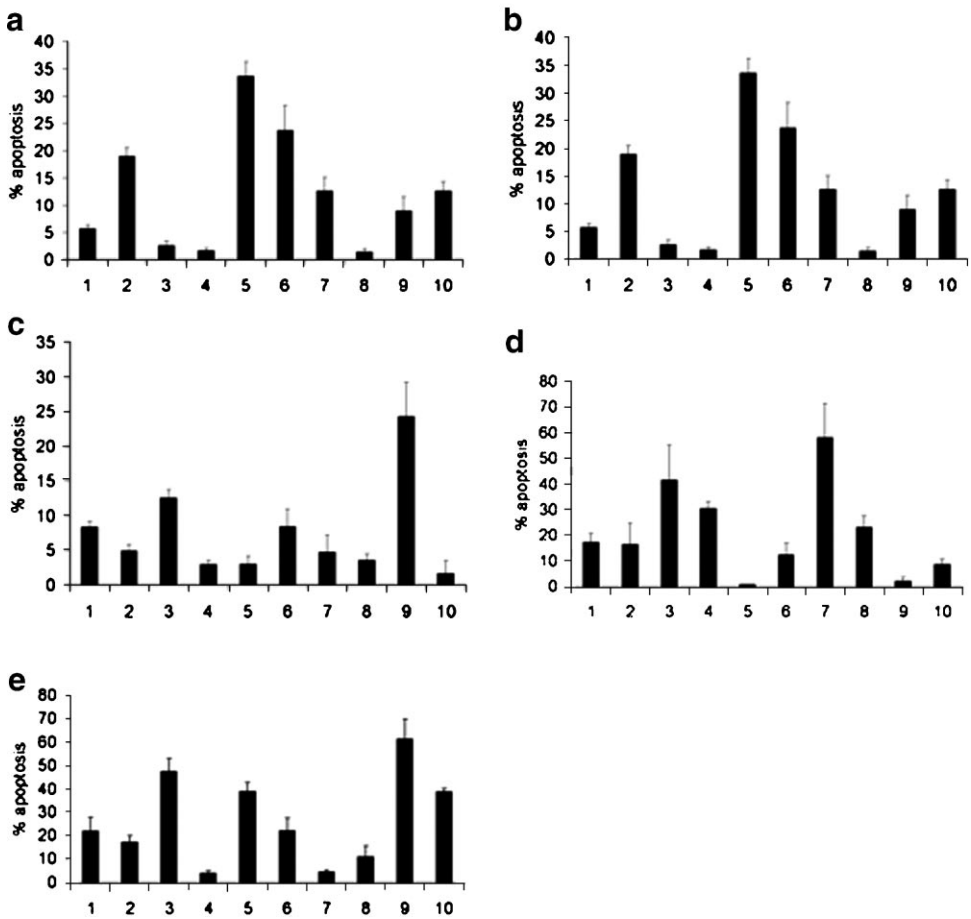

Fig. 8. Determination of the range of TRAIL-sensitivities within clonal and non-clonal cell lines. **a**: PC3 (parental cell line, uncloned). **b**: PC3 (cloned). **c**: PC3-OPG (cloned). **d**: MDA231 breast cancer cells (cloned). **e**: MDA436 breast cancer cells (cloned). Considerable variation of TRAIL sensitivity exists with both cloned and uncloned cell lines.

DISCUSSION

Bone and regional lymph nodes are the most common sites for prostate cancer metastasis, and it is likely that interactions between tumour cells and bone marrow cells make bone a suitable environment for the growth and survival of prostate cancer and other tumour types. OPG is a bone-derived factor that could potentially contribute to prostate cancer growth and survival in bone by two mechanisms: (1) by modulation of the bone remodelling, and (2) by promoting prostate cancer cell survival. The aim of the present study was to develop a suitable system that would allow the testing of the hypothesis that endogenously produced OPG from prostate cancer cells could prevent TRAIL-induced apoptosis in vivo, as a continuation of in vitro findings with this and other OPG-producing cell lines [Holen et al., 2002, 2005]. By modulating OPG expression in PC3 cells by over-expression and knockdown, followed by challenge with TRAIL in vivo, the effects of tumour-derived OPG, including that produced endogenously, would be assessed on preventing the cytotoxic effects of TRAIL. To minimise any effects of clonal variation, all transfections were carried out using cloned populations of PC3 cells. This would reduce the random isolation of TRAIL sensitive and insensitive cells strains that develop in heterogeneous populations of PC3 and other cell lines. We successfully isolated strains of cells from the cloned parent population with either OPG over-expression, or OPG knockdown by stable expression of shRNA directed to OPG. These cells were tested for TRAIL sensitivity and we found in both cases, evidence for clonal variation of TRAIL responses. We found wide variations of TRAIL sensitivities between strains of cells transfected with empty vectors, suggesting that variation could not be as a result of OPG secretion. We also found variations in TRAIL sensitivity between strains of cells with apparently similar levels of OPG knockdown, again suggesting that increased TRAIL-sensitivity was not necessarily due to decreased OPG expression. Furthermore, experiments using sRANKL to inactivate OPG provided evidence that OPG levels did not determine altered TRAIL-sensitivity in OPG knockdown cells.

Observations in the current study are consistent with clonal variation of TRAIL sensitivity in cloned populations of PC3 cells. The use of cloned populations of cells was specifically designed to reduce the occurrence of such effects, however clonal cell populations may evolve during the time between initial clonal isolation, transfection and subsequent analysis. Clearly, the differing TRAIL-sensitivities of empty vector cell strains provides the potential for considerable variation between PC3-OPG cells and PC3-EV cells, variation that could be misinterpreted as effects due to gene expression.

The predicted effect of OPG over-expression in TRAIL-sensitive cells would be a reduction in TRAIL sensitivity, which would be further enhanced in the presence of accumulated OPG. Our studies suggest that over-expressed OPG derived from PC3 cells did not function in the same way as recombinant OPG in modulating responses to TRAIL. In the present study, recombinant OPG was able to suppress TRAIL-induced apoptosis as reported previously [Holen et al., 2002], however OPG derived from PC3-OPG cells did not appear to have any TRAIL-suppressive activity, even when present at levels that would be expected to be strongly inhibitory based on studies with recombinant OPG. Furthermore, injection of PC3-OPG2 cells into the tibia of nude mice resulted in widespread osteolytic disease, with tumour cells surrounded by active osteoclasts. This situation observed in our study differs from the reported effects of rhOPG on prostate cancer cell growth in bone [Zhang et al., 2001], and also the effects in bone of over-expression of OPG from LNCaP cells [Corey et al., 2005], where OPG inhibited osteoclast activation in bone. We conclude from our studies that OPG over-expression from PC3 cells does not produce biologically active or available OPG. The findings of the current study mirror observations using breast cancer cells [Fisher et al., 2006], whereby over-expression of OPG in MCF-7 cells resulted in highly osteolytic tumours surrounded by increased numbers of activated osteoclasts, suggesting that OPG derived from tumour cells differs in its activity compared to recombinant OPG delivered therapeutically. Furthermore, over-expression of OPG in MCF-7 cells did not affect TRAIL-induced apoptosis in vitro [Fisher et al., 2006], suggesting clear differences between recombinant human OPG and OPG expressed at high levels from some tumour cell lines. Why secreted OPG possessed no apparent

biological activity when over-expressed in PC3 cells in the present study is unclear, however aberrant post-translational modification in PC3 cells is possible. Our own studies confirm that the same cloned OPG mRNA sequence used in the present study was able to produce biologically active OPG, as shown by decreased osteoclast activity, in an alternate biological system [Rabin et al., 2007].

Since the above studies suggested that elevated OPG production in PC3 cells did not affect TRAIL-induced apoptosis, we decided to knockdown endogenous OPG production in cloned PC3 cells. Endogenous OPG produced by the parent PC3 cell line had been shown previously to suppress TRAIL-induced apoptosis. Analysis of OPG production by cloned cells before transfection and control cells after transfection indicated that the level of production (maximum ~1 ng/ml) was substantially less than that produced by the heterogenous parent PC3 cell line (~2.5 ng/ml, Holen et al., 2002). Studies using recombinant OPG indicate that concentrations between 1 and 10 ng/ml are sufficient to inhibit TRAIL-induced apoptosis in PC3 cells. This being the case, it may be that even though the knockdown experiments successfully depressed OPG levels in clones, there was not sufficient OPG made by these cloned cells prior to knockdown or in control cells transfected with a control vector to inhibit TRAIL. However other non-OPG related differences in apoptosis regulation could again be responsible for the lack of consistent correlations between OPG secretion and TRAIL responses. We tested whether Death Receptor expression might account for observed differences between TRAIL-sensitive and less-sensitive OPG knockdown clones. Since Death Receptors are responsible for TRAIL-signalling, alterations to Death Receptor expression could potentially promote TRAIL-induced apoptosis in these cells. Death Receptor 5 expression was indeed raised in all knockdown clones tested, however not all OPG knockdown clones were more sensitive to TRAIL than control cells (Fig. 4c). Whereas it is possible that raised Death Receptor levels observed in OPG-knockdown cells could potentially be due to off-target effects of constitutive shRNA expression targeted to OPG, we cannot conclude that raised Death Receptor expression in OPG knockdown cells is correlated with increased TRAIL-sensitivity in these cells.

To establish whether the wide variation in TRAIL-sensitivity observed in transfected, cloned cell populations was due to the effects of modulation of OPG expression or a natural phenomenon in cloned cell population, the range of TRAIL-sensitivities was also determined for cloned populations of cells without modulation of OPG expression. In these studies, it was clear that considerable natural variation of TRAIL-sensitivity exists in cloned cell cultures. This observation was also observed in MDA-MB-231 and MDA-MB-436 breast cancer cells, confirming that it was not a phenomenon restricted to PC3 cells. Interestingly, the results demonstrate that whereas apoptosis frequencies of 10–40% are often reported when cells are challenged with TRAIL, certain populations are killed by TRAIL, whereas other colonies are not affected by TRAIL. These findings have important implications for studies of TRAIL-sensitive cell lines.

Comparisons of cell lines from different sources demonstrate widespread heterogeneity in genetically related cell lines, and in addition, heterogeneity exists within individual populations of cells [Nugoli et al., 2003]. Analysis of osteosarcoma cells over a considerable number of consecutive passages predictably reveals widespread changes of both genotype and phenotype [Hausser and Brenner, 2005]. The isolation of highly invasive populations from a uveal melanoma cell line over a much shorter period revealed populations of genetically related cells with highly unstable karyotypes, potentially accounting for their invasive phenotype [Cross et al., 2005]. Previous studies have shown that analysis of transfected populations of cells show biological variation in gene expression, as determined by oligonucleotide arrays of cell lines [Oh et al., 2003]. The use of cloned or isogenic populations of cells is commonly used in transfection studies to circumvent such phenotypic variations between selected clonal cell populations. However, evidence from the present study and elsewhere suggest that marked heterogeneity in the cloned populations can develop, over relatively short periods of time, with several potential mechanisms. The preferential isolation of clones of cells, during cloning, with high levels of genetic instability may be intrinsic to the process and may account for variation of observed phenotypes [Cross et al., 2005]. Alternatively, it was shown recently that adenocarcinoma cells,

including prostate cancer cells, contain intrinsic stem-like characteristics, facilitating asymmetrical cell division [Locke et al., 2005]. Phenotypic drift occurs in cloned populations of cells in the absence of selective pressure, even after several successive clonal isolations [Barnes et al., 2006]. Clonal variation could be reduced but not abolished after further rounds of clonal isolation, suggesting that isolation of cells with a more limited capacity for phenotypic drift is achievable, but isolation of phenotypically identical cells would not be possible [Barnes et al., 2006]. However, the inadvertent isolation of clones of cells with altered gene expression profiles, in many situations, may not adversely affect the outcomes of experiments, as altered gene expression may not relate to the biological outcome in question.

In summary, we have demonstrated marked biological variation of TRAIL sensitivity within transfected populations of cells in the absence of selection for this phenotype by TRAIL. These results have important implications for studies of this type. Although the use of cloned, transfected populations of cells has proven useful for determining the biological effect of gene overexpression and gene silencing in many situations, such studies are dependent on the use of stringent controls. This study demonstrates that adequate controls are not always possible using standard transfection procedures, and highlights how easily observations using transfected clonal cell populations can 'fit' the hypothesis being tested in the absence of testing sufficient numbers of control and test clones. Caution must therefore be observed in interpreting data where cloned populations of cells are assumed to have identical phenotypes.

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