

Keywords: Insulin-like growth factor; IGFBP; xenograft; breast cancer; vascularisation

Exogenous administration of protease-resistant, non-matrix-binding IGFBP-2 inhibits tumour growth in a murine model of breast cancer

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Background: Insulin-like growth factors (IGF-I and IGF-II) signal via the type 1 IGF receptor (IGF-1R) and IGF-II also activates the insulin receptor isoform A (IR-A). Signalling via both receptors promotes tumour growth, survival and metastasis. In some instances IGF-II action via the IR-A also promotes resistance to anti-IGF-1R inhibitors. This study assessed the efficacy of two novel modified IGF-binding protein-2 (IGFBP-2) proteins that were designed to sequester both IGFs. The two modified IGFBP-2 proteins were either protease resistant alone or also lacked the ability to bind extracellular matrix (ECM).

Methods: The modified IGFBP-2 proteins were tested *in vitro* for their abilities to inhibit cancer cell proliferation and *in vivo* to inhibit MCF-7 breast tumour xenograft growth.

Results: Both mutants retained low nanomolar affinity for IGF-I and IGF-II (0.8–2.1-fold lower than IGFBP-2) and inhibited cancer cell proliferation *in vitro*. However, the combined protease resistant, non-matrix-binding mutant was more effective in inhibiting MCF-7 tumour xenograft growth and led to inhibition of angiogenesis.

Conclusions: By removing protease cleavage and matrix-binding sites, modified IGFBP-2 was effective in inhibiting tumour growth and reducing tumour angiogenesis.

The insulin-like growth factor (IGF) system is complex and includes two ligands, IGF-I and IGF-II, which bind and activate the type 1 IGF receptor (IGF-1R). IGF-II also binds with high affinity to the structurally similar tyrosine kinase receptor, the alternate splice variant of the insulin receptor (IR) known as the IR isoform A (IR-A). Activation of these receptors elicits a myriad of cellular responses including promotion of cellular proliferation, survival, differentiation and migration (Denley *et al*, 2005; Belfiore and Malaguarnera, 2011). As a result, the IGF system has important roles in normal mammalian growth and development. Under normal conditions, IGF bioavailability is tightly modulated by a group of six conserved high-affinity IGF-binding proteins (IGFBP-1–6) (Bach *et al*, 2005; Forbes *et al*, 2012).

IGFBPs consist of 216–289 amino acids with masses ranging from 24 to 50 kDa. They possess three distinct domains, with the structured N- and C-domains connected by an unstructured linker domain. The major IGF-binding determinants are located in the N- and C- domains and they interact with residues on the IGFs that are also important for IGF-1R binding (and IR-A binding in the case of IGF-II). Thus interaction with IGFBPs prevents interaction of IGFs with their receptors. IGFBPs also possess IGF-independent activities dependent on structural motifs mainly located within the linker and C-domains, which confer the ability to bind integrins (in the case of IGFBP-1 and -2), extracellular matrix and other binding partners and to enter the nucleus (Firth and Baxter, 2002; Forbes *et al*, 2012).

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Received 2 March 2014; revised 6 April 2014; accepted 8 April 2014; published online 22 May 2014

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IGFBP proteolysis by a range of different proteases has been shown to regulate IGF bioavailability (Firth and Baxter, 2002) with cleavage leading to fragments of lower IGF affinities (Carrick *et al*, 2001). Interestingly the majority of protease cleavage sites are located within the IGFBP linker domains (Forbes *et al*, 2012). A second mechanism by which IGF is released from IGF:IGFBP complexes involves extracellular matrix (ECM) binding. Binding to ECM lowers the affinity of IGFBPs for IGF and results in increased concentrations of bioavailable IGF (Jones *et al*, 1993; Arai *et al*, 1994). IGFBP-2, -3, -5 and -6 are able to associate with various ECM components and ECM-binding sites can be found within their C-domains adjacent to residues important for IGF binding (Forbes *et al*, 2012). IGFBP-2 is unique, as it has been reported to have two ECM-binding sites (Conover and Khosla, 2003; Russo *et al*, 2005).

There is considerable evidence for the involvement of the IGF system in cancer (Pollak, 2012; Yee, 2012). Epidemiological studies have shown an increased risk of breast, prostate, lung and colorectal cancers in individuals with high serum levels of IGF-I and IGF-II (Pollak, 2012; Yee, 2012). Also, reduction of circulating IGF-I serum levels in mice delays tumour onset and progression (Takahara *et al*, 2013). In addition, upregulation of the IGF-1R has been observed in many cancers, and has been suggested to contribute to accelerated tumour growth (reviewed in Frasca *et al*, 2008). IGF signalling via the IGF-1R promotes tumour cell growth, proliferation, survival and migration (Rosenzweig and Atraya, 2010; Pollak, 2012).

The IR-A is also known to have a role in tumour growth (Denley *et al*, 2003; Belfiore and Malaguarnera, 2011). The relative abundance of IR-A is markedly higher in eight different breast cancer cell lines including MCF-7 (Sciaccia *et al*, 1999). Furthermore, IGFBP-2 expression has recently been associated with a variety of cancers (Mehrian-Shai *et al*, 2007; So *et al*, 2008; Degraff *et al*, 2009; Foulstone *et al*, 2013) and the mode of IGFBP-2 action in tumour biology is likely to involve IGF-dependent and IGF-independent actions. Besides overexpressing components of the IGF system, tumour cells also often upregulate expression of various IGFBP-specific proteases to promote tumour cell proliferation and survival (Morgan and Hill, 2005; Hadler-Olsen *et al*, 2013). Upregulated levels of IGFs and their respective receptors, coupled with increased rate of IGF release from the IGF:IGFBP complex inevitably leads to unmitigated activation of the IGF-specific mitogenic pathways.

Several strategies to inhibit IGF-1R signalling are being developed including anti-receptor monoclonal antibodies, which directly inhibit IGF binding in xenograft models of a variety of cancers (Cohen *et al*, 2005; Attias-Geva *et al*, 2011; Pollak, 2012; Yee, 2012), and small molecule inhibitors (SMI) of the IGF-1R tyrosine kinase. Both lack of efficacy and emerging resistance to monoclonal antibody treatments in clinical trials have been reported (Yee, 2012). One SMI, picropodophyllin, has been shown to disrupt substrate phosphorylation, hence effectively inhibiting signal transduction (Menu *et al*, 2006). However, the major challenge of using SMI is the cross reactivity with the insulin receptor isoform B due to the conserved tyrosine kinase domain structures between the IGF-1R and IR (Clemmons, 2007).

In light of the potential problems of anti-IGF-1R monoclonal antibodies and SMIs outlined above and the fact that neither strategy targets IGF-II signalling via the IR-A, we have developed a modified IGFBP as a potential therapeutic agent that will potentially inhibit signalling through both receptors. The use of soluble receptors, anti-ligand monoclonal antibodies or IGFBPs to neutralise IGF action has previously been suggested (Clemmons, 2007; Pollak, 2012) and validated in xenograft studies using either endogenously expressed (Ojima *et al*, 2012; Zhang *et al*, 2012; Wang *et al*, 2013) or exogenously administered (Dransfield *et al*, 2010; Oh *et al*, 2012; Bid *et al*, 2013) receptor/anti-ligand monoclonal antibodies/IGFBPs.

However, we took a novel approach to develop protease resistant (PR) and protease resistant/non-matrix-binding (PR/NMB) variants of IGFBP-2 as potential tumour growth inhibitors. We hypothesise that lack of linker domain protease and matrix-binding sites render the IGFBP-2 devoid of the ability to promote IGF-dependent action (through release of IGFs to the receptors) and IGF-independent action (through ECM binding). The advantages of using IGFBP-2 over other IGFBPs were its ability to bind both IGF-I and IGF-II with almost equal affinity and also its apparent lack of posttranslational modification, thereby simplifying its recombinant expression. In this study, we demonstrated that a protease-resistant, non-matrix-binding IGFBP-2 has the potential to inhibit cancer cell proliferation, tumour growth and angiogenesis.

MATERIALS AND METHODS

Materials and cells. FreeStyle 293-F (FS293F) cells and the mammalian expression vector pcDNA3.1 were from Invitrogen (Mt Waverley, VIC, Australia). Anti-IGFBP-2 polyclonal antibody was generated in-house and anti-rabbit HRP antibody was from Pierce (Scoresby, VIC, Australia). Plasmin, MMP-1 and -7 were from Roche (Dee Why, NSW, Australia), Merck Millipore and Chemicon Millipore (Kilsyth, VIC, Australia), respectively. Heparin salt and fibronectin were purchased from Sigma (Castle Hill, NSW, Australia) and vitronectin was from Promega (Madison, WI, USA). Antibodies used in immunohistochemistry were anti-endomucin antibody (Santa Cruz Biotechnology Inc., Dallas, TX, USA) and anti-rabbit Dylight649 antibody (Jackson Immuno Research, West-Grove, PA, USA). T84 (CCL-248) and SW480 (CCL-228) colon cancer, DU145 (CRL-2698) and LnCaP (CRL-1740) prostate cancer and the MCF-7 (HTB-22) breast cancer cell lines were cultured as recommended by the ATCC at 37 °C in 5% CO₂. ITS (insulin 10 mg ml⁻¹, transferrin 5.5 mg ml⁻¹, sodium selenite 5 µg l⁻¹, Sigma) was a supplement for serum-free cell cultures. Reagents for immunohistochemistry included Histolene from Fronine Laboratory Supplies and ProLong Gold antifade reagent from Life Technologies (Mulgrave, VIC, Australia).

IGFBP-2 and IGBP-2 mutant expression and purification. Human IGFBP-2 cDNA (GenBank Accession no. NP_000588) and amino acid mutants of IGFBP-2 were codon-optimized for human expression and synthesised by Geneart (Life Technologies) each with a Kozak consensus sequence (GCCACC) immediately upstream of the initiating methionine (+1), a heterologous signal peptide (MNPLLLITFVAAALA) and a C-terminal 6 × Histidine-tag. Human IGFBP-2 (WT IGFBP-2) and variant proteins (protease-resistant mutant (PR):des(114–170) Ala115 human IGFBP-2 and PR + non-matrix-binding mutant PR/NMB:des(114–170) Ala115, K180A, K181A, K227A, K234A, K237A human IGFBP-2) were generated using standard PCR-based mutagenesis techniques (Figure 1).

Proteins were transiently expressed in FS293F cells, culture supernatants were filtered and IGFBP-2 or mutants were purified by tandem nickel and size exclusion chromatography. Post-elution samples were applied directly to a Superdex 200PG 26/60 column (GE Healthcare Life Sciences) at 4 ml min⁻¹ in PBS and fractions collected. Peak fractions containing IGFBP-2 fractions were pooled after additional size exclusion analysis and sterile-filtered followed by rHPLC analysis.

IGF-binding analysis. IGF-binding affinities of IGFBP-2 and variants were determined by surface plasmon resonance as previously described (Galea *et al*, 2012). Kinetic data were analysed and fitted to the 1:1 Langmuir binding interaction model. Values for IGFBP-2 binding were the average of three independent experiments and were similar to those reported previously (Galea *et al*, 2012).

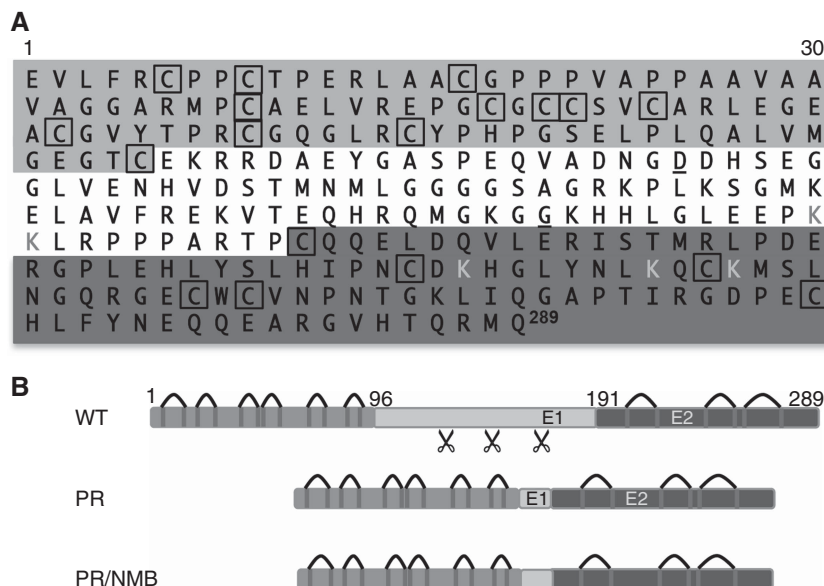


Figure 1. Designing protease resistant and non-matrix-binding IGFBP-2. **(A)** The amino acid sequence of IGFBP-2 highlighting cysteine residues (boxed), N-, C- and linker domains (light grey, dark grey and white backgrounds, respectively). **(B)** Schematic diagram of WT, PR and PR/NMB IGFBP-2. Black lines represent the disulphide bonds between cysteine residues in the N- and C-domains. Scissor icons represent protease cleavage sites, whereas E1 and E2 indicate the two known ECM binding sites within the WT IGFBP-2 molecule. The linker domain was truncated from residue Asp114 to Gly170 (underlined in **(A)**) in the PR and PR/NMB mutants. Lys residues substituted by Ala in the PR/NMB variant (silver) are also indicated in **(A)**.

Enzyme cleavage assays and western immunoblotting

Conditioned medium cleavage. Cancer cell lines of different tissue type and stage were cultured to 60% confluence, washed and grown under serum-free conditions (medium plus ITS) for 4 days. Conditioned media were centrifuged to remove cell debris, concentrated $10\times$ (Amicon 10 kDa cutoff ultrafiltration) and stored at -20°C . WT or PR IGFBP-2 (250 ng per $2\mu\text{l}$) was added to $12\mu\text{l}$ conditioned medium and incubated at 37°C for 3 or 24 h. Cleavage was stopped by addition of $2\mu\text{l}$ 0.1 M acetic acid.

Plasmin cleavage. Purified binding protein (100 ng of WT, PR or PR/NMB IGFBP-2) in PBS was cleaved by $9\mu\text{U}$ human plasminogen in a total volume of $19\mu\text{l}$ at 37°C . The reaction mixtures were then terminated with $1\mu\text{l}$ of freshly prepared 10 mM phenylmethanesulfonyl fluoride (PMSF) at $T=0$, $T=20$ min, $T=3$ h, $T=8$ h and $T=24$ h.

Matrix metalloproteinase 1 and 7 (MMP-1/7) cleavage. Matrix metalloproteinase 1 and 7 (MMP-1/7) was activated using 50 mM p-aminophenylmercuric acetate (APMA) solution at 10:1 (enzyme: APMA) volume ratio and incubated at 37°C for 3 h. A total 500 ng of recombinant WT IGFBP-2 and mutants were incubated with $1.5\mu\text{U}$ and $0.5\mu\text{U}$ activated MMP-1 and MMP-7, respectively, in a total volume of $40\mu\text{l}$ cleavage buffer (10 mM HEPES, 150 mM NaCl, 5 mM CaCl_2) pH 7.4 at 37°C . An aliquot ($8\mu\text{l}$) was taken at various time points (0 min to 24 h) and proteolysis were terminated with $2\mu\text{l}$ 0.1 M acetic acid.

SDS-PAGE and western blotting. The cleavage reaction mixtures were boiled with $10\mu\text{l}$ protein non-reducing load dye for 5 min. Proteolysed fragments of IGFBP-2 (100 ng per lane) were separated using 12% Tris-tricine gels followed by transfer onto nitrocellulose membranes. The membranes were then blocked with 1% BSA in PBS, and probed with anti-IGFBP-2 polyclonal (primary) and anti-rabbit HRP (secondary) antibodies. Blots were then quantitated using ImageJ (version 1.47c). Proteolysis experiments were conducted at least three times for each protease.

Matrix binding by WT, PR and PR/NMB IGFBP-2. To determine the ability of IGFBP-2 and its mutants to bind extracellular matrix heparin salt ($10\mu\text{g ml}^{-1}$) fibronectin ($10\mu\text{g ml}^{-1}$) and vitronectin ($6\mu\text{g ml}^{-1}$) diluted in PBS were coated onto 96-well Maxisorp plates ($100\mu\text{l}$ per well) overnight at 4°C . Plates were washed with SB buffer (20 mM sodium phosphate pH 6, 125 mM NaCl, 2 mM CaCl_2 , 2 mM MgCl_2 , 0.1% BSA, 0.02% Tween 20) and then blocked 2 h with 1% BSA in SB buffer. Meanwhile, WT IGFBP-2 or mutants (3.2 nM or 160 nM) were complexed for 2 h with an equimolar concentration of IGF-I in SB buffer at 37°C . Subsequently, the IGF-I:IGFBP-2 complex was added into each well (washed first with SB buffer three times) and incubated for 2 h at 37°C . Next, the wells were washed and probed with $100\mu\text{l}$ per well 1:1000 anti-IGFBP-2 polyclonal antibody and then 1:5000 anti-rabbit HRP antibody diluted in SB buffer each for 1 h at 37°C with a washing step in between. TMB solution was added ($100\mu\text{l}$ per well) and the reaction was stopped with 1 N sulphuric acid ($100\mu\text{l}$ per well) after 10 min. The O.D. of the plates was read at 450 nm. Assays were performed three times with triplicates for each data point.

Cell viability assays. HT29 colon cancer cells were serum-starved for 5 h at 37°C in 5% CO_2 before being treated with 0.1% BSA in 5 mM sodium butyrate in the presence of increasing concentrations of IGF-I. IGFBP-2 or mutants were added to test their ability to inhibit IGF-I rescue of cells from butyrate-induced apoptosis. After 48 h, ATP levels were measured as an indication of cell viability with a Cell-Titer Glo Cell Viability Luminescent Assay according to the manufacturer's instructions (Promega) as previously described (Denley *et al*, 2006).

Breast cancer xenograft model. Female nude BALB/C 6–8-week-old mice were implanted with β -estradiol-17-acetate pellets (1.7 mg) on the day before inoculation with MCF-7 breast cancer cells (3×10^6 cells per $50\mu\text{l}$ diluted in $50\mu\text{l}$ ice-cold RPMI/matrigel). Tumour dimensions (length and width) were measured twice weekly with vernier callipers and tumour volume calculated according to the formula of prolate spheroid volume $0.5(L\times W^2)$, where L is the length and W is the width. Once tumour volumes

reached 0.2 cm^3 , mice ($n=8-10$ per group) were treated by intraperitoneal injection with PBS, IGFBP-2 or mutants (6.5×10^{-9} moles kg^{-1} per day in PBS) for 28 days. This dose was chosen on the basis of a similar study using IGFBP-3 which has a similar affinity for IGFs to WT IGFBP-2 (Alami *et al*, 2008). Four groups were treated for 5 days per week with tamoxifen ($100\ \mu\text{l}$ of 2.5 mg ml^{-1} solution) alone or with WT IGFBP-2 or mutants (as above). The day after the last treatment, samples of blood ($0.5-1.0\text{ ml}$) were collected and tumours were dissected, weighed and formalin fixed. The study was reviewed and approved by the Animal Ethics Committee of the Institute for Medical and Veterinary Science, Adelaide, South Australia (AEC# 123/10).

Immunofluorescence analysis and quantitation of angiogenesis.

Tumours ($n=4$) from each MCF-7 xenograft treatment group were sectioned ($7\ \mu\text{m}$) from various positions across the tumours, deparaffinised in two changes of Histolene, followed by rehydration in decreasing concentrations of methanol (from 100% to 50%). Slides were then subjected to heat-induced epitope retrieval for 30 min in $10\ \text{mM}$ citrate buffer pH 6 and cooled for 30 min. Following washing, slides were incubated 30 min in blocking solution (1% BSA, 10% horse serum in PBS). Next, the tumour sections were incubated overnight at 4°C with anti-endothelin antibody (Santa Cruz Biotechnology Inc.), 1:500 dilution in blocking solution. Subsequently, the tissues were incubated in 1:800 anti-rabbit Cy5-labelled secondary antibodies (Jackson Immuno Research) at room temperature for 2 h. Tissues were then washed, mounted and stained with DAPI using ProLong Gold antifade reagent.

Representative images were captured on a Zeiss Axioplan 2 confocal microscope (North Ryde, NSW, Australia) at a magnification of $\times 200$. To quantitate the images of five different sectors of each tumour, ImageJ (version 1.47c) was used to count the number of lumina and blood vessels for each experimental condition. The data were expressed as the average number of blood vessels and lumina per experimental condition.

Statistical analyses. All statistical analyses were done using Graphpad Prism 6.01. Data collected from IGF binding, xenograft tumour growth and angiogenesis quantitation were analysed by one-way analysis of variance (ANOVA) followed by Dunnett's post test. Data from protease cleavage experiments were analysed by both one-way ANOVA followed by Dunnett's post test and two-way ANOVA followed by Tukey's multiple comparisons post test. One-way ANOVA with Tukey's multiple comparisons test was used to analyse the data from the matrix-binding assay whereas two-way ANOVA with Dunnett's post test was done on the data from the cell viability assay.

RESULTS

Expression and characterisation of recombinant WT IGFBP-2 and its mutants. rHPLC inspection of recombinant WT IGFBP-2 and its mutants (PR and PR/NMB) showed that purified binding proteins (Supplementary Figure S1a) were $>95\%$ pure. Mass spectrometry data indicated that WT, PR and PR/NMB variants were of the expected masses of 32198.6 Da , 26277.9 Da and 26106.6 Da , respectively. Moreover, circular dichroism spectra of PR and PR/NMB IGFBP-2 were very similar to WT (Supplementary Figure S1b) suggesting that the mutants adopted a similar fold to WT IGFBP-2.

Using surface plasmon resonance, it was determined that the purified proteins retained high affinity for both IGF-I and -II. WT bound IGF-I and IGF-II with similar affinities ($1.38\ \text{nm}$ and $1.37\ \text{nm}$, respectively; Table 1 and Supplementary Figure S1c), as we have previously demonstrated (Galea *et al*, 2012). Compared with WT IGFBP-2, the PR variant had a 1.9-fold lower affinity for IGF-I but the same affinity for IGF-II. The PR/NMB-binding protein had

Table 1. Binding affinities of WT IGFBP-2 and mutants

	IGF-I			IGF-II		
	K_D (nm)	s.e.m.	Relative K_D	K_D (nm)	s.e.m.	Relative K_D
WT	1.38	0.13	1.00	1.37	0.18	1.00
PR	3.74	0.50	1.94 ^a	1.15	0.12	0.86 ^a
PR/NMB	3.16	0.38	1.99 ^a	2.17	0.07	2.10 ^a

Abbreviations: K_D = dissociation constant (kd/ka, dissociation rate/association rate); Relative $K_D = K_D$ mutant/ K_D WT; PR = protease resistant; PR/NMB = protease resistant/non-matrix-binding; WT = wild-type.
^aNot statistically significant.

slightly lower affinities than WT for both IGF-I ($3.16\ \text{nm}$, 1.9-fold) and II ($2.17\ \text{nm}$, 2.1-fold), respectively.

IGFBP-2 mutants are resistant to proteolysis

Conditioned media proteolysis. To examine the efficacy of the mutant IGFBP-2s as inhibitors of MCF-7 breast cancer xenograft growth, we first assessed their susceptibility to protease cleavage using serum-free conditioned media (CM) collected from a range of cell lines of different tissue type and stage: T84 (CCL-248) and SW480 (CCL-228) colon cancer cell lines, LnCaP (CRL-1740) and DU145 (CRL-2698) prostate cancer cell lines and MCF-7 (HTB-22) breast cancer cell line. These proteolysis experiments were conducted using WT and PR mutant as a preliminary assessment of the protease resistance achieved upon removal of the linker domain. WT binding protein ($37\ \text{kDa}$) was partially cleaved by enzymes secreted by cancer cell lines, with low molecular weight fragments appearing with time (Supplementary Figure S2). It was evident from the different extent of cleavage and the range of fragment sizes generated that the conditioned media contained different mixtures of secreted proteases at various concentrations. In contrast to WT IGFBP-2, the PR variant was resistant to proteolysis by proteases present in T84, SW480 and MCF-7 CM. Proteases present in the CM collected from DU145 and LnCaP cultures promoted limited cleavage of PR IGFBP-2, generating lower molecular weight cleavage products. Therefore we can conclude that the PR mutant is resistant to some but not all the proteases secreted by this range of colon, prostate and breast cancer cell lines.

Plasmin and matrix metalloproteinase cleavage. To establish whether the IGFBP-2 mutants were resistant to plasmin, MMP-1 and MMP-7 (proteases known to be secreted by breast cancer cells (Morgan and Hill, 2005; Hadler-Olsen *et al*, 2013)), we incubated all three binding proteins with the purified proteases for 24 h and performed immunoblots to assess the extent of cleavage.

By the end of the 24 h incubation with plasmin (Figure 2A), only 11% of the WT binding protein remained intact whereas there was no detectable degradation of the two mutants (with the statistical difference from WT being $P < 0.05$ at $T = 8\ \text{h}$ for PR/NMB IGFBP-2; $P < 0.01$ and $P < 0.0001$ at $T = 24\ \text{h}$ for PR and PR/NMB IGFBP-2, respectively).

When exposed to MMP-7, the cleavage of WT IGFBP-2 was clearly detectable at $T = 3\ \text{h}$, with only 59% of intact protein remaining, followed by only 31% and 3.6% intact protein remaining at $T = 8\ \text{h}$ and $T = 24\ \text{h}$, respectively (Figure 2B). In contrast, both PR and PR/NMB IGFBP-2 remained intact after 8 h ($P < 0.0001$) and cleavage of both PR and PR/NMB IGFBP-2 was only detected at 24 h with 15% ($P < 0.01$) and 44% ($P < 0.0001$) intact protein remaining, respectively. This indicates that some MMP-7 cleavage sites had been removed or become less accessible

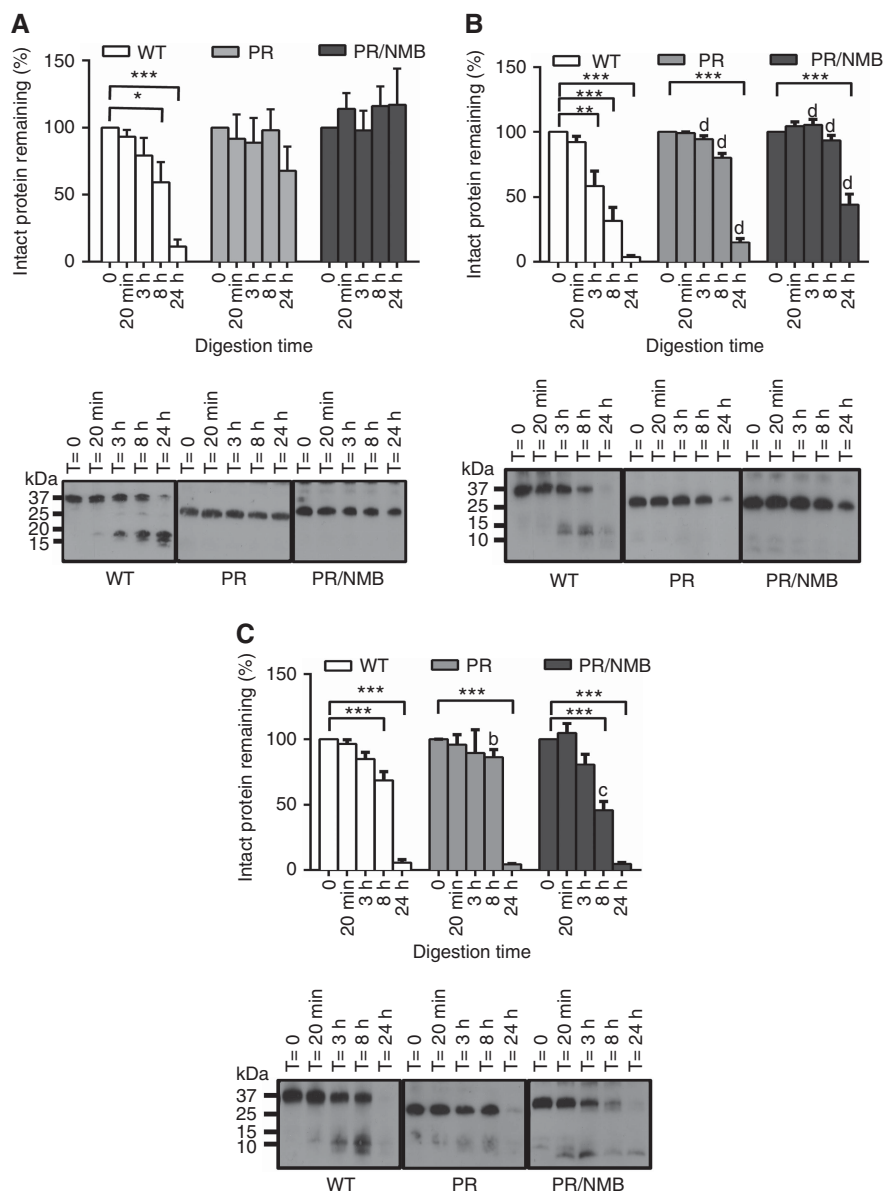


Figure 2. WT, PR and PR/NMB IGFBP-2 cleavage by **(A)** plasmin, **(B)** MMP-7 and **(C)** MMP-1. Intact IGFBP-2 (37 kDa band), PR and PR/NMB IGFBP-2 (25 kDa bands) and their fragments were detected with a rabbit anti-IGFBP-2 polyclonal antibody. A representative blot is shown in each case. Amount of intact IGFBP-2 or mutants remaining was quantitated using ImageJ (version 1.47c) and expressed as percent (%) intact protein remaining \pm s.e.m. of at least $n=3$ blots in graphs above. For each IGFBP, data were analysed by one-way ANOVA and Dunnett's post test when comparing cleavage at different time points to $T=0$; * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$. Two-way ANOVA followed by Tukey's multiple comparisons post test was used to compare cleavage of PR and PR/NMB to WT IGFBP-2 at a single time point; a = *, b = **, c = ***, d = ****.

in both mutants and that they acquired some resistance to MMP-7 proteolysis.

Interestingly, all three IGFBP-2 proteins were almost completely degraded by MMP-1 at $T=24$ h (Figure 2C). However, it is evident that PR IGFBP-2 was less susceptible to MMP-1 degradation than WT IGFBP-2. At $T=8$ h, WT had 64% intact protein remaining but PR IGFBP-2 was mostly uncleaved ($P<0.01$). In contrast, PR/NMB IGFBP-2 was cleaved more significantly ($P<0.001$) by MMP-1 than WT IGFBP-2 at this time point. We can therefore conclude that one or more MMP-1 cleavage sites were removed in the PR mutant. However, introduction of the five Lys to Ala mutations in the PR/NMB variant has somehow indirectly increased the rate of MMP-1 cleavage.

In conclusion, we have demonstrated that the removal of residues 114–170 of the linker domain in PR and PR/NMB IGFBP-2 results in different degrees of resistance to plasmin, MMP-1 and MMP-7

cleavage. Given that the mutants are still cleaved to a certain extent by both MMP-1 and MMP-7, these results suggest that some MMP cleavage sites remain within these IGFBP-2 mutants.

Mutation of extracellular matrix (ECM) binding sites disrupts ECM binding. The two ECM-binding sites located in the linker and C-domains, respectively were mutated in the PR/NMB variant by substituting Lys with Ala at positions 180, 181, 227, 234 and 237. At 3.2 nM, there is no significant difference in vitronectin, fibronectin and heparin binding when comparing WT IGFBP-2 with PR or PR/NMB (Figure 3). However, there is a difference ($P<0.05$) between the PR and PR/NMB variant in binding vitronectin at this concentration.

At a higher concentration of IGFBPs (160 nM), the difference in ECM binding is more discernible. There is a significant decrease in vitronectin binding to PR/NMB IGFBP-2 ($P<0.0001$) when

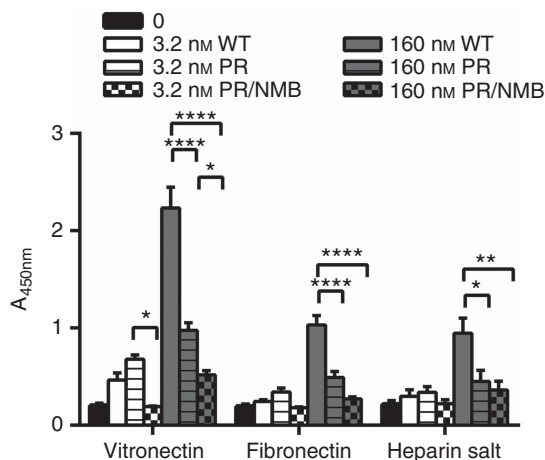


Figure 3. IGFBP-2 mutants bind with low affinity to fibronectin, vitronectin and heparin. Binding proteins (3.2 nM or 160 nM in complex with equimolar concentrations of IGF-I) were allowed to bind to fibronectin, vitronectin and heparin. Amount of WT IGFBP-2 or mutants bound was determined at 450 nm after probing with polyclonal anti-IGFBP-2 antibody and anti-rabbit HRP and is expressed as mean O.D. \pm s.e.m. with $n=3$. One-way ANOVA followed by Tukey's multiple comparisons post test was performed when comparing ECM binding of IGFBP variants against WT at 3.2 nM and 160 nM IGFBPs, respectively. * $P<0.05$, ** $P<0.01$, **** $P<0.0001$.

compared with WT. Similarly, binding of PR/NMB IGFBP-2 to fibronectin and heparin are significantly reduced ($P<0.0001$ and $P<0.01$, respectively). This is because mutation of the ECM-binding sites has significantly perturbed the ability of PR/NMB to bind to all three ECM components.

Somewhat unexpectedly, the PR mutant (at 160 nM) also had a lower affinity for vitronectin ($P<0.0001$), fibronectin ($P<0.0001$) and heparin ($P<0.05$) than WT IGFBP-2, although PR bound to vitronectin more effectively than the PR/NMB mutant ($P<0.05$). It is possible that the removal of residues adjacent to the linker domain ECM-binding site has disrupted binding via this site whereas binding of the C-domain ECM site still occurs. Therefore, we have demonstrated that both IGFBP-2 mutants are high-affinity IGF binders while being partially resistant to protease cleavage and lacking matrix binding *in vitro*.

Mutant binding proteins inhibit proliferation in cancer cells. The ability of WT, PR and PR/NMB IGFBP-2 to prevent IGF-stimulated rescue of HT29 colon cancer cells from butyrate-induced apoptosis was measured using the Cell-Titer Glo viability assay. Cells were rescued from apoptosis with increasing doses of IGF-I (0.1 nM to 0.65 nM; 0.3 nM $P<0.01$, 0.65 nM $P<0.0001$) (Figure 4). WT IGFBP-2 at a concentration of 3.1 nM (but not 0.31 nM) was able to inhibit the IGF-I induced rescue of butyrate-induced apoptosis ($P<0.0001$) for all IGF-I concentrations. In contrast, both PR and PR/NMB IGFBP-2 at both concentrations were able to potently inhibit IGF-I action ($P<0.0001$). Interestingly, the number of viable cells in the presence of both mutants was much lower than that seen for cells grown in the presence of butyrate alone. Although it could be suggested that this effect of the mutants may be due to an IGF-independent mechanism, it seems unlikely as we have removed motifs responsible for some of IGFBP-2 IGF-independent actions (ECM binding). A more likely explanation is that the IGFBP-2 mutants are inhibiting the action of endogenously produced IGF-I or IGF-II. HT29 cells have previously been shown to secrete IGF-II (Culouscou *et al*, 1990). As IGFBP-2 has similar affinities for both IGFs, we assume that it will also inhibit IGF-II actions to a similar extent.

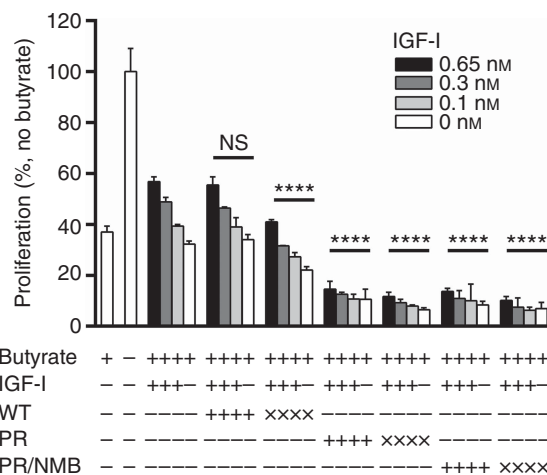


Figure 4. PR and PR/NMB IGFBP-2 inhibition of IGF-I stimulated proliferation after sodium butyrate-induced apoptosis in HT29 cells. Butyrate treated cells were incubated in the presence of increasing concentrations of IGF-I with or without WT, PR or PR/NMB IGFBP-2 (+ = 0.31 nM, \times = 3.1 nM IGFBP). Results are expressed as percentage (%) of proliferating cells in the absence of sodium butyrate \pm s.e.m. ($n=3$). Statistical significance was determined by comparing cells treated with IGFBPs to cells treated only with IGF-I using two-way ANOVA with Dunnett's multiple comparisons post test. NS = not significant, **** $P<0.0001$.

MCF-7 breast cancer xenograft model. To investigate the effects of PR and PR/NMB IGFBP-2 *in vivo*, we established 0.2 cm³ xenografts (from subcutaneous injection of single-cell suspensions of MCF-7 cells). Compared with vehicle-treated controls, tumours of mice treated for 28 days with PR/NMB IGFBP-2 (10 mg kg⁻¹ per day) failed to grow across the entire treatment period ($P<0.0001$; Figure 5A). Although WT and PR IGFBP-2 appeared to inhibit tumour growth, this effect was not statistically significant. In addition, tumours of mice treated with tamoxifen (Tam) (25 μ g, 5 days per week) alone grew to \sim 60% of vehicle control ($P=0.001$ to 0.01) whereas treatment with WT + Tam ($P<0.01$) and PR + Tam ($P<0.05$) had a similar effect to treatment with tamoxifen alone (Figure 5B). However, the size of tumours of PR/NMB + Tam treated mice appears unchanged across the entire treatment period ($P<0.0001$). Importantly, treatments throughout the xenograft study had no significant effect on mouse weight (data not shown). Also, IGF-I and IGFBP-3 levels were the same in all treatment groups 24 h after the final treatment (Supplementary Figure S4a and S4b).

PR/NMB IGFBP-2 treatment disrupts tumour angiogenesis. Most solid tumours rely on angiogenesis for growth as it helps provide nutrition to the tumour as well as a route for dissemination and metastasis (Bhat and Singh, 2008). In addition, the IGF system has been implicated previously in tumour angiogenesis under hypoxic environments (Contois *et al*, 2012; Zhang *et al*, 2012). Hence, we decided to investigate the effects of administering WT IGFBP-2 and mutants on tumour vascularisation using the biological samples collected from the mouse model.

Presence of blood vessels was determined by measuring the amount of endomucin (a mucin-like sialoglycoprotein found specifically on endothelial cells of blood and lymphatic vessels (Liu *et al*, 2001)) on tumour sections using immunohistochemical methods. The anti-endomucin antibody used in this study has previously been used by others to monitor angiogenesis (Roland *et al*, 2009; Stefater *et al*, 2011). Initially, we found that there was no significant difference between the number of blood vessels found in tumours from each experimental condition (Figure 6I).

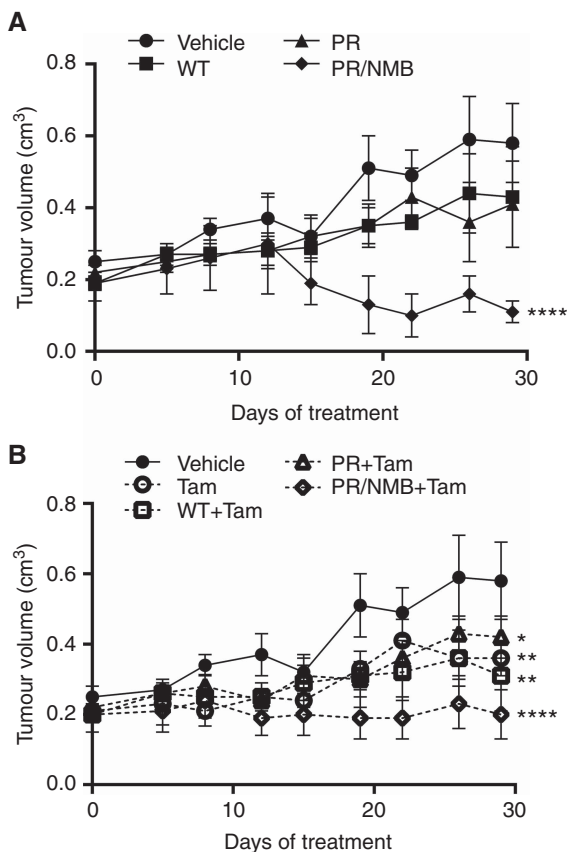


Figure 5. PR/NMB with or without tamoxifen significantly inhibited MCF-7 tumour xenograft growth. Tumours were established by subcutaneous injection of MCF-7 cells and treatment began on day 0 when tumours reached approximately 0.2 cm³. Mice were treated with (A) vehicle, WT, PR and PR/NMB (10 mg IGFBP-2 or mutants per kg per day) or (B) tamoxifen (25 µg per mouse 5 days a week), WT + Tam, PR + Tam, and PR/NMB + Tam ($n = 8-10$ per group). One-way ANOVA followed by Dunnett's multiple comparisons post test were used to determine statistical significance when comparing groups treated with vehicle and the various treatments previously mentioned. * $P < 0.05$, ** $P = < 0.01$, **** $P < 0.0001$. (Statistical analyses not shown on the graph: Tam vs PR/NMB **, Tam vs PR/NMB + Tam *, PR/NMB vs PR/NMB + Tam NS).

However, by analysing the captured images (Figure 6A–H), we determined that tumours being treated with PR/NMB ($P = 0.01$ to 0.05) and PR/NMB + Tam ($P < 0.01$) have fewer visible lumina (where the term 'No. of visible lumen' has previously been used to describe angiogenesis in prostate cancer tumours (Mucci *et al*, 2009; Gustavsson *et al*, 2010)) compared with tumours treated with the vehicle (negative control) and tamoxifen (positive control) (Figure 6J). In addition, those vessels with visible lumina were of smaller diameter than those seen in all other treatment groups. This correlates well with the tumour growth inhibition data collected in the mouse xenograft model in which the mice treated with PR/NMB variant (with and without tamoxifen) had the smallest tumour size at the end of the treatment period. These data suggest that PR/NMB IGFBP-2 inhibits tumour growth by impeding the process of tumour angiogenesis.

We also measured tumour cell proliferation using immunohistochemistry to detect Ki67 (Supplementary Figure S3), as a reduced proliferation rate can directly influence the tumour size (Cohen *et al*, 2005). However, there appeared to be no significant difference in proliferation across all treatment groups.

DISCUSSION

To inhibit tumour growth promoted by both IGF-I and IGF-II, we designed two novel IGFBP-2 analogues, PR IGFBP-2 and PR/NMB IGFBP-2. These were expressed, purified and their IGF-binding affinities were measured. Both PR and PR/NMB IGFBP-2 bound the ligands with high affinity and had only a 1.9-fold lower affinity for IGF-I than WT IGFBP-2. PR IGFBP-2 had a similar affinity for IGF-II whereas PR/NMB IGFBP-2 bound IGF-II with only 2.1-fold lower affinity than WT IGFBP-2. We can conclude that mutation of the five lysine residues to alanine in PR/NMB IGFBP-2 (K180A, K181A, K227A, K234A, K237A) does not have a major effect on overall binding affinity and therefore these residues do not play a significant role in IGF binding. Previous structural and truncation mutant studies identified the region encompassing residues 227–241 of the C-domain as being important for IGF binding (Forbes *et al*, 1998), which suggests that residues other than K227, K234 and K237 within that region are involved in IGF binding. Interestingly, R185 in IGFBP-4 (equivalent to K227) was identified in the IGFBP-4:IGF-I crystal structure as interacting with the C-domain (Sitar *et al*, 2006) but the equivalent residue in IGFBP-6 was not seen to be involved in IGF-II binding in NMR studies of the C-BP-6 (Headey *et al*, 2004). Obviously, there are subtle differences in the binding interfaces between the different IGFBPs.

By removal of most of the linker domain, we have created a mutant IGFBP-2 that is somewhat resistant to a range of proteases. PR IGFBP-2 was not cleaved by most but not all proteases in conditioned media of a range of colon, prostate and breast cancer cell lines (Supplementary Figure S2), suggesting that deletion of residues 114–170 removed the majority of protease IGFBP-2 cleavage sites. There was evidence for protease cleavage at sites outside the truncated region of the IGFBP-2 linker domain as some cleavage of PR IGFBP-2 was identified upon incubation with conditioned medium from the prostate cancer cell lines LnCaP and DU145 (Supplementary Figure S2).

Analysis of protease susceptibility towards cleavage by pure proteases known to be secreted by breast tumours (Morgan and Hill, 2005; Hadler-Olsen *et al*, 2013) revealed that PR and PR/NMB variants remained mostly intact when exposed to plasmin and MMP-7, whereas WT IGFBP-2 was rapidly degraded. IGFBP-2 cleavage by plasmin (Menouny *et al*, 1997) and MMP-1 (Rajah *et al*, 1999) was first reported many years ago, although the respective cleavage sites have not been mapped. Our data provide evidence that the main cleavage sites for these enzymes are located in the linker domain. Surprisingly the rate of PR/NMB cleavage by MMP-1 was greater than the rate for PR and WT IGFBP-2. Consistent with our observations, Miyamoto *et al* showed that MMP-7 cleaves WT IGFBP-2 in the linker domain and has a preference for hydrophobic residues, cleaving N-terminal to Leu152, Leu176 and Leu182 (Miyamoto *et al*, 2007). However, our data suggest that further mutation of the C-domain ECM-binding domain in some way rendered the mutant more susceptible to MMP-1 proteolysis.

We hypothesised that removal of protease cleavage sites and the ability to bind ECM would remove the two mechanisms leading to IGF release from IGFBP-2 and thus improve the ability of IGFBP-2 to inhibit IGF action. Indeed we saw a greater ability of both mutants to inhibit IGF-stimulated proliferation *in vitro* (Figure 4). All three IGFBP-2 treatments inhibited tumour growth, although, interestingly, PR/NMB IGFBP-2 was more effective than PR IGFBP-2 in inhibiting tumour growth (Figure 5), despite similar protease resistance and fibronectin- and heparin-binding affinities *in vitro*. The mechanism underlying the different *in vivo* activities of the mutants is not clear at this stage although it is possible that the difference in activities may be explained by their different vitronectin-binding affinities.

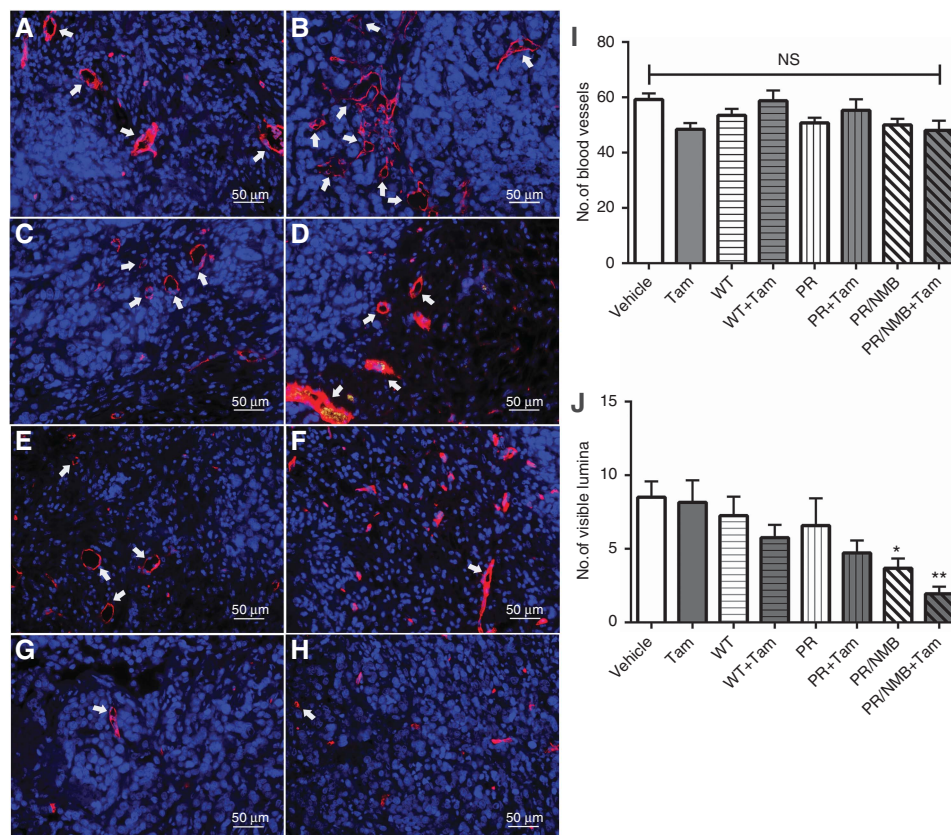


Figure 6. PR/NMB significantly reduces the number of visible lumina in MCF-7 tumour xenografts. Tumour sections collected from the xenograft model were probed with anti-endomucin antibody and then visualised with Dylight645 secondary antibody. Images of tumours treated with (A) vehicle, (B) Tamoxifen, (C) WT, (D) WT + Tam, (E) PR, (F) PR + Tam, (G) PR/NMB, and (H) PR/NMB + Tam were captured with a Zeiss Axioplan 2 confocal microscope. The mean number of (I) blood vessels and (J) lumina per tumour section are shown (analysis of $n=4$ tumours per group \pm s.e.m.). One-way ANOVA followed by Dunnett's multiple comparisons post test were used to determine statistical significance when comparing groups treated with vehicle and the various treatments previously mentioned. * $P<0.05$, ** $P<0.01$.

In support of this, both vitronectin and IGFs have recently been shown to have an important role in breast cancer survival and migration (Kashyap *et al*, 2011).

Interestingly, Ryan *et al* developed a protease-resistant IGFBP-4 by mutating the PAPP-A cleavage site and neighbouring positively charged residues located within the IGFBP-4 linker domain to alanine (Ryan *et al*, 2009). IGFBP-4 does not bind ECM as it lacks ECM-binding sites equivalent to those found in IGFBP-2, -3 or -5 (Forbes *et al*, 2012) and therefore only has one IGF release mechanism. Like our PR and PR/NMB mutants, this IGFBP-4 mutant retained high IGF-binding affinity. However, xenografts arising from 4T1.2 mammary adenocarcinoma cells overexpressing the protease-resistant IGFBP-4 grew significantly slower than 4T1.2 xenografts expressing WT IGFBP-4 or the empty vector (Ryan *et al*, 2009), which is in contrast to our PR variant that had an equal effect to the WT IGFBP-2. This highlights the different mechanisms by which the IGFBPs control IGF action *in vivo*.

There has been an accumulation of data suggesting that components of the IGF system have a major role in vascularisation (Delafontaine *et al*, 2004; Azar *et al*, 2011; Bid *et al*, 2012). Hence, we have examined the MCF-7 tumours harvested from our animal study for signs of disrupted vascularisation. Initially, we found that the total number of blood vessels detected by endomucin staining per tumour section was similar for all treatment groups. We found that treatment with PR/NMB with and without tamoxifen significantly reduced the number of visible lumen and size of the blood vessels. This suggests that the mutant IGFBP-2 might be inhibiting tumour growth by exerting an anti-angiogenic effect.

Inhibition of tumour angiogenesis appears to be a common outcome of all inhibitors blocking IGF signalling. For example, SCH-717454 (an anti-IGF-1R monoclonal antibody) perturbed capillary-like tube formation in a murine matrigel plug experiment (Bid *et al*, 2012). In addition, it was found that treatment with NVP-AEW5H (an IGF-1R/IR SMI) abrogated orthotopic pancreatic cancer growth and angiogenesis (Moser *et al*, 2008). Also, the strategy of sequestering IGFs using IGFBPs (Liu *et al*, 2007) resulted in inhibition of angiogenesis. While in most cases these treatments reduced the number of tumour blood vessels, the IGF-II specific antibody DX-2647 decreased the tumour blood vessel size (Dransfield *et al*, 2010), as we observed with PR/NMB IGFBP-2. Interestingly, DX-2647 did not significantly affect tumour cell proliferation as was also observed in our own study (Supplementary Figure S3), raising the possibility that PR/NMB is inhibiting IGF-II action in the MCF-7 xenografts.

In conclusion, we have developed a protease-resistant and non-matrix-binding mutant of IGFBP-2 that is able to inhibit tumour growth possibly by inhibition of angiogenesis. Through development of the IGFBP-2 mutants we have confirmed the importance of IGFBP-2 proteolysis and matrix binding in the control of IGF action. While we are aware that IGFBP-2 is overexpressed in some cancers and it has been suggested that it may promote tumorigenesis (Mehrian-Shai *et al*, 2007; So *et al*, 2008; Degraff *et al*, 2009; Foulstone *et al*, 2013), our findings suggest that IGFBP-2 lacking IGF-dependent (proteolysis) and IGF-independent (matrix binding) activities may be effective for the treatment of cancers in the future.

ACKNOWLEDGEMENTS

We acknowledge the contributions of M Marcinkiewicz and PV Gordon, (University of Virginia Children's Hospital) for preliminary observations of IGFBP-2 proteolysis, Ms J Cook and Ms D Turner for technical assistance, Dr P McCarthy for technical discussions and Drs A Rofe and D Gancarz, (SA Pathology) for the animal studies. Dr Forbes was supported by National Health and Medical Research Council (project and Development grants) and Australian Research Council Linkage grant funding.

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