Van Lonkhuyzen, Derek R. and Hollier, Brett G. and Shooter, Gary K. and Leavesley, David I. and Upton, Zee (2007) Chimeric vitronectin:insulin-like growth factor proteins enhance cell growth and migration through co-activation of receptors. *Growth Factors* 25(5):pp. 295-308.

Chimeric vitronectin:insulin-like growth factor proteins enhance cell growth and migration through co-activation of receptors.

DEREK R. VAN LONKHUYZEN, BRETT G. HOLLIER, GARY K. SHOOTER, DAVID I. LEAVESLEY & ZEE UPTON.

Tissue Repair and Regeneration Program, Institute of Health and Biomedical Innovation, Queensland University of Technology, 60 Musk Avenue, Kelvin Grove, 4069, Queensland, Australia.

Running Title: Functional chimeric Vitronectin:IGF-I proteins

Address correspondence to: Derek Van Lonkhuyzen, 60 Musk Avenue, Kelvin Grove, 4069, Queensland, Australia; E-mail: d.vanlonkhuyzen@qut.edu.au

Key Words: Vitronectin, IGF-I, mimetic, IGF-1R, αv-integrin, cell growth, cell migration.

Complexes comprised of IGF-I, IGF-binding proteins and the ECM protein vitronectin (VN) stimulate cell migration and growth and can replace the requirement for serum for the *ex vivo* expansion of cells, as well as promote wound healing *in vivo*. Moreover, the activity of the complexes is dependent on co-activation of the IGF-I receptor and VN-binding integrins. In view of this we sought to develop chimeric proteins able to recapitulate the action of the multiprotein complex within a single molecular species. We report here the production of two recombinant chimeric proteins, incorporating domains of VN linked to IGF-I, which mimic the functions of the complex. Further, the activity of the chimeric proteins is dependent on co-activation of the IGF-I- and VN-binding cell surface receptors. Clearly the use of chimeras that mimic the activity of growth factor:ECM complexes, such as these, offer manufacturing advantages that ultimately will facilitate translation to cost-effective therapies.

Introduction

The insulin-like growth factors (IGFs) have well-established roles as potent mitogens and cell survival factors. As such, the IGFs have been actively investigated as therapeutics and as critical ingredients in many cell culture media (Morris et al. 1994; Sunstrom et al. 2000). We recently discovered novel links between IGFs and the ECM protein vitronectin (VN) and have reported that IGF-II can bind directly to VN (Upton et al. 1999), while IGF-I binds indirectly via IGF-binding proteins (IGFBP)-2, -3, -4 and -5 (Kricker et al. 2003), resulting in dimeric and trimeric complexes, respectively. These interactions are potentially very significant as VN is a protein that is abundant not only in tissues, but also in the circulation (0.4 mg/ml) (Schvartz et al. 1999); hence it is also a major constituent of serum that is commonly required to maintain the survival and expansion of mammalian cells *in vitro* and *ex vivo*. Indeed, our studies investigating the outcomes of the interaction of IGFs with VN have revealed that these complexes significantly enhance migration and proliferation in a variety of cells (in the absence of serum) including human breast epithelial (Noble et al. 2003), osteoblast-like (Schleicher et al. 2005), and skin and corneal epithelial cell lines (Hyde et al. 2004; Hollier et al. 2005). Importantly, studies using keratinocytes derived from adult human skin and the limbal region of adult cornea, as well as osteoblast-progenitor cells derived from alveolar bone chips, have validated these findings (Hyde et al. 2004; Hollier et al. 2005; Schleicher et al. 2005; Ainscough et al. 2006; Dawson et al. 2006). Moreover, we have demonstrated that these complexes can substitute for serum, enabling the serum-free *ex vivo* serial expansion of cells used in cell-based therapies (Ainscough et al. 2006; Dawson et al. 2006) and can also promote wound healing *in vivo* (Upton et al. 2007). These data led us to hypothesise that VN may have a role in facilitating more effective “delivery”, or “presentation” of growth factors to cells. This represents a paradigm shift; however, it more closely mimics the situation that cells encounter in the body. Indeed, the importance of interactions between growth factors and proteins other than "classic signalling" receptors in the extracellular milieu is now generally considered to be a process critical to a range of cellular functions (Giancotti and

Ruoslahti 1999). Furthermore, recent investigations within our research program have demonstrated that functional responses to these novel complexes are dependent on the co-activation of the IGF-1R and the αv-integrins (Hollier et al. 2005; Hollier et al. 2007; Upton et al. 2007).

In view of the significant functional responses induced by these novel complexes *in vivo* and *in vitro* we believe the VN:IGFBP:IGF-I complex technology has the potential to be translated into defined culture reagents and effective clinical treatments. The translation process would, however, be significantly constrained by the complex methodology and cost required to generate and incorporate 3 recombinant proteins into one bioactive therapy. Thus we investigated whether the VN:IGFBP:IGF-I complex-induced cellular responses, particularly cell growth and migration, could be reproduced using chimeric proteins that incorporate the essential features of both the VN and IGF proteins. Such a mimetic protein would in turn facilitate the translation of the VN:IGF-I approach into approved clinical and industrial products more cost-effectively compared to the multi-protein complex. We report here the generation of recombinant proteins, including full-length and truncated forms of VN linked to IGF-I, to form four novel chimeric proteins. Further, we have confirmed that two of these chimeric proteins elicit similar cellular responses to that found with the multimeric VN:IGFBP:IGF-I complexes and provide evidence that co-activation of both the VN-binding αv-integrins and the IGF-1R is critical to these responses.

Materials and methods

Reagents and materials. Human IGF-I was purchased from GroPep (Adelaide, SA, Australia), human VN was purchased from Promega (Annandale, NSW, Australia), while IGFBP-3 was purchased from Upstate Biotech (Lake Placid, NY, USA). For receptor blocking studies, monoclonal antibodies directed against the integrin αv-subunit (AV1) and the mouse IgG isotype matched control antibody were purchased from Chemicon (Temecula, CA, USA), while the monoclonal antibody against the IGF-1R (αIR3) was purchased from Merck Biosciences (Kilsyth, VIC, Australia). For immunoblotting studies, rabbit polyclonal anti-VN antibody was purchased from Calbiochem (San Diego, CA, USA), while anti-ERK 1/2 rabbit polyclonal, anti-phospho ERK1/2 MAPK (Thr 202/ Tyr 204) (E10), anti-AKT mouse monoclonal (2H10) and anti-phospho-AKT (Ser 473) (193H12) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). All other reagents were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia) unless otherwise stated. All manipulations using VN or IGFBP were performed using low protein adhesion or siliconised plastic-ware and tips.

Cell culture. The MCF-7 human breast carcinoma cell line (HTB-22, ATCC, Manassas, VA) used in this study was cultured in DMEM/Ham's F-12 (DMEM/F12) media (1:1) (Invitrogen, Mulgrave, VIC, Australia) containing 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA), 0.1 µg/mL streptomycin, 50 units/mL penicillin and 1 µg/mL gentamycin. All cultures were passaged every 2-3 days by trypsin/EDTA detachment. For use in bioassays, cells were serum-starved by incubation in serum-free and phenol-red free DMEM/F12 (1:1) (SFM) for 4 hours prior to trypsinization and seeding into tissue culture vessels. *Spodoptera frugiperda* (Sf9) insect cells were purchased from Invitrogen and were cultured in SF-900 II serum-free media (Invitrogen). Transfected cultures were maintained in shaker flasks of up to 0.5 L culture volume and were passaged to a viable density of 2 x 10^6 cells/mL every 2-3 days.

Engineering, expression and purification of the chimeric proteins. Human VN and IGF-I gene DNA sequences (NCBI accession # AF382388 and #X03563, respectively) were codon-optimised for expression in *Spodoptera frugiperda* and synthesised by GeneArt (Regensburg, Germany). The coding sequences were then cloned into the pIB/V5-His expression vector (Invitrogen) incorporating a poly-histidine affinity tag to aid in the purification of the chimeras. A nucleotide sequence encoding a $(Gly_4Ser)_4$Asn amino acid linker was inserted via site-directed mutagenesis polymerase chain reaction (PCR). The addition of an Asn to the C-terminus of the linker sequence was used to generate a Asn-Gly motif with Gly being the first amino acid of IGF-I. This motif enables hydroxylamine induced cleavage of the IGF-I protein from the chimeras. The resulting construct, referred to as Chimera A, encodes the full-length mature VN protein (amino acids 1-459) linked by $(Gly_4Ser)_4$Asn to the full-length mature IGF-I protein (amino acids 1-70). Three chimeras containing truncated portions of VN, chimeras B (VN amino acids 1-311), C (VN amino acids 1-125) and D (VN amino acids 1-64), were also produced by deletion of selected regions of the VN gene via additional rounds of site directed mutagenesis PCR (see **Table I** for primer sequences). The DNA sequence of all constructs were verified to ensure that the fidelity of the desired DNA sequences were maintained. Clones in the pIB/V5-His vector were used to transfect Sf9 insect cells and transiently-expressed secreted protein was detected in the conditioned media, as assessed by immunoblotting. Briefly, the samples were resolved on SDS-PAGE under reducing conditions (Laemmli 1970) and the proteins were transferred onto a nitrocellulose membrane using a semi-dry transfer method (Bjerrum and Schafer-Nielsen 1986). The membrane was interrogated with poly-clonal anti-VN antibodies and the target protein species were then visualized using enhanced chemiluminescence following the manufacturer's instructions (GE Healthcare, Buckinghamshire, UK).

[Insert Table I about here]

Purification of the chimeric proteins was based on Ni-NTA Superflow Agarose (QIAGEN, Australia) affinity chromatography performed according to the manufacturer's

instructions. The chimeric proteins were monitored throughout the purification process by SDS-PAGE and western blot analysis using a poly-clonal anti-VN antibody (Calbiochem). For Chimera A, clarified culture media was loaded onto a 2 mL Ni-NTA Superflow Agarose column equilibrated with 100 mM NaH_2PO_4, 10 mM Tris.Cl, pH 8.0 and material was eluted in the same buffer containing 8 M Urea at pH 4.5. The eluate was neutralised by dilution with 20 mM Na_2HPO_4, 8 M Urea, pH 7.4 before batch mode adsorption onto 10 mL of Heparin-sepharose resin equilibrated with the same buffer. Following binding, the resin was packed into a column and washed with 20 mM Na_2HPO_4, 8 M Urea, 150 mM NaCl, pH 7.4 and bound protein was eluted across a 0.15-1.5 M NaCl gradient at a flow rate of 1 mL/min. Fractions containing VN-immunoreactive protein were pooled and diluted in 100 mM NaH_2PO_4, 10 mM Tris.Cl, pH 8.0 before loading onto a second Ni-NTA Superflow Agarose column. After processing as above, fractions containing Chimera A were pooled and dialysed against 100 mM NaH_2PO_4, pH 7.4 containing 1.5 M NaCl. Total protein was precipitated by the addition of 40% (w/v) ammonium sulphate and the centrifuged protein pellet was resuspended in Dulbecco's Phosphate Buffered Saline (Invitrogen).

Chimeras B and C were purified using a similar method, however, Q-sepharose resin (GE Healthcare) was used in place of Heparin-sepharose. Purification of Chimera D was achieved through the exclusive use of 2 passes on Ni-NTA Superflow Agarose as describe above. The chimeric preparations were quantified via UV spectrophotometry using calculated extinction co-efficients at A_{280nm} (Wilkins et al. 1999). Purity of the preparations was assessed by silver stained SDS PAGE and was shown to be >90% (data not shown).

Transwell™ migration assays. As the MCF-7 cell line migrates poorly in the absence of serum and attachment factors, it is an ideal cell line to examine the effects of candidate factors as stimulators of migration (Noble et al. 2003). Migration assays were performed as previously described by our laboratory (Noble et al. 2003; Hyde et al. 2004; Hollier et al. 2005). Briefly, experimental treatments were added to the lower chamber of the Transwell™ plate immediately prior to cell harvesting and seeding into Tranwells™ (see **Table II** for

treatment schedule). Cells which has been serum-starved for 4 hours prior to trypsinisation were seeded at a density of 2 x 10^5 cells/well in SFM + 0.05% BSA into the upper chamber of Transwell™ inserts (12 µm), while the treatments were dispensed into the lower Transwell™ chambers. In some experiments the cells were pre-incubated with the indicated concentrations of inhibitory antibodies for 30 minutes at room temperature prior to seeding into the Transwell™. Plates containing the Transwell™ inserts were then incubated for 5 hours at 37°C, 5% CO_2. Cells which had migrated to the lower surface of the membrane were fixed in 10% para-formaldehyde and stained with 0.023% Crystal Violet in phosphate buffered saline. The number of cells which had migrated to the lower surface of the porous membrane was then quantified by extracting the crystal violet stain in 10% acetic acid and determining the optical density of these extracts at 595 nm (Leavesley et al. 1993).

[Insert Table II about here]

N-terminal sequencing and Mass-spectroscopy. Pure preparations of chimera B and chimera D were reduced and alkylated with 10 mM dithiothreitol (DTT) and 50 mM iodoacetamide (IAA). Samples were then electrophoresed on a 15% separating SDS-PAGE and transferred onto a sequence grade PVDF membrane (Bjerrum and Schafer-Nielsen 1986). The transferred proteins were stained with Ponceau S and the bands corresponding to the pure chimeras were excised and transferred to microcentrifuge tubes. The samples were then processed to determine the N-terminal 8 amino acids by the Australian Proteome Analysis Facility (APAF) (Sydney, NSW, Australia) using the Edman degredation method (Edman 1950). Peptide Mass Fingerprinting was also performed to confirm amino acid sequence fidelity. In brief, the chimeric proteins were run on a 4-12% gradient SDS-PAGE gel, the bands were excised and the proteins were reduced with 10 mM DTT and alkylated with 55 mM IAA. Sequence grade trypsin (Promega) was then added and the proteins were digested for 16 hr at 37^{o}C. The eluted peptides were spotted onto a target plate in a 1:1 mixture with 5 mg/mL α-cyano-4-hydroxycinnamic acid in 5 mM ammonium phosphate:60% acetonitrile

and allowed to air dry. The plate was then analysed using a Voyager DE-STR MALDI-TOF (Applied Biosystems, Foster City, CA, USA) mass spectrometer and the data were processed using the FindPep program available from the expasy website (www.expasy.org).

Assessment of signal transduction pathways. For ERK 1/2 and AKT activation, the experimental treatments were added to the wells of a 6-well plate immediately prior to cell harvesting (see **Table III** for treatment schedule). Serum-starved cells were then harvested and resuspended in SFM + 0.05% BSA and allowed to recover for 30 minutes at 37°C, 5% CO_2. Cells were then seeded into the wells of 6-well plates containing protein treatments and incubated at 37°C, 5% CO_2 for the times indicated. Cells were lysed in Radio Immunoprecipitation Buffer (RIPA) containing 2 mM Na_3VO_4, 10 mM NaF and were subsequently interrogated via immunoblotting using antibodies specific for the activated forms of the signaling molecules. Protein species were then visualized using enhanced chemiluminescence following the manufacturer's instructions (GE Healthcare). The same membranes were subsequently stripped and total levels of ERK 1/2 and AKT were detected to validate equal loading.

[Insert Table III about here]

Cell proliferation assays. The ability of select chimeric proteins to promote MCF-7 cell proliferation was assessed in cell growth assays utilising a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS)-based assay system (Cory et al. 1991). The treatments were diluted in SFM to concentrations equivalent to those used in the migration assays in a total volume of 50 μL (see **Table IV** for treatment schedule). Subsequently 5,000 cells in SFM (50 μL) were added to each well and incubated at 37^{o}C, 5% CO_2 for 96 hours. MTS reagent was then added and the cells were incubated for a further 2 hours at 37^{o}C, 5% CO_2 in order to allow the tetrazolium salt colour to develop.

Absorbance was read at 490 nm in a Beckman Coulter microplate reader (Beckman Coulter, Fullerton, CA, USA) and the results were expressed as percentage above SFM.

[Insert Table IV about here]

Statistical Analysis. The data is expressed as a percentage of the response observed in either wells containing SFM or VN alone, unless otherwise stated in the Figure Legends. The data was pooled from replicate experiments with each treatment tested separately in each experiment in at least duplicate wells (refer to Figure Legends). Data analysis was performed using ANOVA with Dunnets and Tukeys post hoc tests performed where appropriate. Statistical confidence was determined at 95% level ($p<0.05$).

Results

Production of four VN:IGF-I chimeric proteins. As detailed above, we sought to develop chimeric proteins able to recapitulate the action of the VN:IGFBP:IGF-I multiprotein complex within a single molecular species. We have designed, expressed and purified 4 recombinant chimeric proteins incorporating specific domains of VN linked to mature IGF-I (a schematic representation and amino acid sequences are depicted in **Fig. 1**). A series of chimeric expression constructs were designed in which various lengths of the VN protein were linked to the full-length mature IGF-I protein. Modification of the IGF-I protein was not pursued due to the discontiguous nature of the multiple sites in IGF-I that have been reported to interact with the IGF-1R (Cascieri et al. 1988; Bayne et al. 1989; Bayne et al. 1990; Denley et al. 2005; Keyhanfar et al. 2007). The first chimeric protein, chimera A, was designed to contain the complete mature VN sequence (amino acids 1-459) linked to IGF-I. Chimera B contains VN amino acids 1-311, creating a C-terminally-truncated VN without the heparin-binding domain, while chimera C, encoding amino acids 1-125 of VN, contains the somatomedin-B (SMB) domain as well as the connecting region of VN. This region is suggested to play a role in binding the thrombin-antithrombin complex as well as collagen (Schvartz et al. 1999). The shortest construct design, chimera D, contains amino acids 1-64 of VN and therefore includes the SMB domain, RGD integrin binding site and the acidic region as its basic functional elements. The acidic region was retained as it is thought to be pertinent to the function of VN whilst also allowing the binding of additional growth factors (Schvartz et al. 1999). In each case, the VN segments were linked to the mature IGF-I sequence via a $(Gly_4Ser)_4$Asn linker.

[Insert Figure 1 about here]

The four VN:IGF-I chimeric molecules were successfully expressed in Sf9 insect cells and were then purified from the conditioned media using heparin-sepharose, Q-sepharose and Ni^{2+}-NTA agarose under native and denaturing conditions as described in the *Materials and Methods* section. Western-blot analysis revealed that all four recombinant

proteins cross-react with a poly-clonal anti-VN antibody, while silver-stained SDS-PAGE analysis demonstrated purities >90% (**Fig. 2**).

[Insert Figure 2 about here]

Cell migration. The four chimeric molecules were assessed compared side-by-side with the VN:IGFBP-3:IGF-I complex in MCF-7 cell migration assays (**Fig. 3**). The treatments were added in molar ratio according to the VN portion of the protein; hence 1x VN equals 1 µg of VN per mL. The data are expressed as % of the 1x VN alone treatment (1 µg/mL) (100%) and indicates that the VN:IGFBP-3:IGF-I complex treatment stimulated migration to approximately 280%. Chimera A was unable to stimulate cell migration as effectively as the VN:IGFBP-3:IGF-I complex, enhancing migration only 37% above the 1x VN only control at its highest dose (10x). Although chimera B was no more active than chimera A at low concentrations (1x and 3x), the migration response observed with chimera B at 10x (300%) was equivalent to that found with VN:IGFBP-3:IGF-I complexes. The third chimera, C, did not significantly increase cell migration above the negative control (67% and 31% respectively). With respect to chimera D, the 1x, 3x and 10x treatments were not dissimilar to the VN alone treatments. However, 30x chimera D was able to promote cell migration to similar levels as found with the 1x VN:IGFBP-3:IGF-I complex, that is 240%. Further, both chimeras B and D stimulated MCF-7 cell migration in a dose-responsive manner.

[Insert Figure 3 about here]

N-terminal sequencing and Mass-Spectrum analysis of chimeric proteins. The chimeras of interest after assessment in the migration assay described above, namely chimeras B and D, were interrogated to determine whether the N-terminal signal peptide had been correctly cleaved. Hence, samples of chimeras B and D were processed as described in the *Materials and Methods* section and their N-terminal 8 amino acids deduced via Edman degradation. Both chimeras B and D returned a sequence complementary to that expected for native VN with the secretion peptide (amino acids 1 – 19) removed (see **Table V**). A blank at position 5

indicates a Cysteine residue or another modified amino acid. In the expected sequence for mature VN a Cysteine (C) is positioned at residue 5, therefore the sequence results confirm that the constructs have been successfully produced as designed. N-terminal sequencing was only performed on chimeras B and D but the same result can be extrapolated to chimeras A and C as there are no amino acid differences between all 4 chimeras at the N-terminus, nor were the expression conditions different.

[Insert Table V about here]

However, to provide a broader picture of post-translational protein modifications, all 4 chimeras were also assessed in Mass-Spectroscopy experiments along with human VN (Promega) as a control (**Table VI**). Upon analysis, the VN control returned 9 matches for theoretical peptides generated after trypsin digestion. Interestingly, no matches were observed N-terminal to amino acid 187. Chimera A retrieved 8 positive specific matches to the expected sequence. Of these 8, 5 peptides concurred with the VN portion, with the most upstream thereof being at amino acid 177, whilst 3 peptides pertained to sequences within the IGF-I portion of the chimeric protein. Chimera B on the other hand had 4 positive matches, with 2 in each of the VN and IGF-I regions. Here the most N-terminal fragment started at amino acid 198. Chimeras C and D both returned 3 positive matches. However, all 3 peptides corresponded to the IGF-I portion of the chimeras and there were no matches to any part of their VN segments.

[Insert Table VI about here]

Receptor blocking cell migration. As described above, Chimeras B and D, at 10x and 30x respectively, were found to induce cell migration to levels similar to that found with the 1x VN:IGFBP-3:IGF-I complex; hence these chimeras were selected for further evaluation in migration assays employing function blocking antibodies. Assays of each treatment without antibody were defined as 100% and the relative inhibitory effect of the function-specific blocking antibodies determined (**Fig. 4**). The anti IGF-1R antibody reduced cell migration of VN alone by 30% while the anti-αv-integrin antibodies significantly decreased migration by

70%. Similarly, treatments of the VN:IGFBP-3:IGF-I complex showed reduced migration by 30% and 56% in the presence of anti-IGF-1R and anti-αv-integrin antibodies, respectively. Interestingly, incubation of the cells with function blocking antibodies was more effective in assays of chimeras B and D. Thus, when anti-IGF-1R antibodies were included with chimera B, cell migration was reduced by 64% while the addition of anti-αv-integrin antibodies inhibited migration by 83%. Similar results were observed with chimera D, where cell migration was decreased by more than 60% and 80% when the cells were pre-incubated with anti-IGF-1R and anti-αv-integrin antibodies, respectively.

[Insert Figure 4 about here]

Cell signalling activation. To determine if the chimeric proteins could activate intracellular signalling in a similar fashion to the VN:IGFBP-3:IGF-I complexes (Hollier et al. 2007), phosphorylation of ERK 1/2 and AKT was examined (**Fig. 5**). Cells were incubated with concentrations of the controls (VN, VN:IGFBP-3:IGF-I and VN:IGF-I) and chimeric proteins (chimeras B and D) equivalent to those found to be the most effective in stimulating MCF-7 migration (that is 1x for the controls, 10x for chimera B and 30x for chimera D) for time periods of 10 and 60 minutes. At both time points the VN alone control stimulated phosphorylation of ERK 1/2 while no activation of AKT was observed. Both VN:IGF-I and the VN:IGFBP-3:IGF-I complex, however, were effective in stimulating phosphorylation of both ERK 1/2 and AKT. Chimera D was able to stimulate ERK 1/2 and AKT phosphorylation to levels comparable to the multiprotein VN:IGFBP-3:IGF-I complex, at both time points tested. Chimera B on the other hand did not stimulate activation of ERK 1/2 to the same extent as chimera D or the VN:IGFBP-3:IGF-I complex, nor did it stimulate phosphorylation of AKT whatsoever. The activation stimulated by chimera B was instead similar to that induced by VN alone.

[Insert Figure 5 about here]

Cell proliferation. To ascertain whether the IGF-I domain of chimeras B and D retained biological function, we assessed the ability of these chimeras to stimulate cell proliferation in the MCF-7 cells. We have previously found that unlike the situation in cell migration assays VN alone, or added to IGF-I ± IGFBP treatments, has little effect on MCF-7 proliferative responses leading us to deduce that the proliferative responses induced by the trimeric VN:IGFBP-3:IGF-I complexes is primarily due to the presence of IGF-I (Hollier et al. 2007). Hence, the ability of chimeras B and D to promote cell proliferation was assessed in 96 hr cell growth assays (**Fig. 6**). The treatments assessed were of the same molar ratios as used in the cell migration assays. All concentrations of VN alone were ineffective at inducing cell growth, while VN:IGF-I and the VN:IGFBP:IGF-I complex were able to significantly enhance cell proliferation above the control. Cells treated with 10x Chimera B, 30x Chimera D and 1x VN:IGFBP:IGF-I complex showed equivalent stimulation of cell proliferation.

[Insert Figure 6 about here]

Discussion

We have previously reported that insulin-like growth factor-I (IGF-I) binds to the extra-cellular matrix protein vitronectin (VN) via the involvement of select IGF-binding proteins (IGFBPs) (Kricker et al. 2003). Further, we have comprehensively demonstrated that these multiprotein complexes significantly enhance cell growth and migration in a range of cells, including continuous cell lines (Kricker et al. 2003; Noble et al. 2003; Hyde et al. 2004; Hollier et al. 2005), cells derived from primary tissues and used for the production of clinical grafts, and more recently, cells involved in the re-epithelialization of wounds *in vivo* (Upton et al. 2007). Furthermore, the activity of the multiprotein complex has been shown to depend on the co-activation of IGF-I and VN-binding cell surface receptors (Hollier et al. 2007; Upton et al. 2007). Currently, translation of the VN:IGFBP:IGF-I complex technology into approved cell culture reagents and clinical treatments is limited to some extent by the fact that each of the components needs to be produced recombinantly, along with the cost and complex methodology required for the incorporation of three separate components into one effective treatment. In order to address these limitations, we hypothesised that the functional constituents of the complexes could be reproduced in a single protein that could emulate the enhanced cellular responses previously observed with the multiprotein complex (Kricker et al. 2003; Hyde et al. 2004; Hollier et al. 2005; Schleicher et al. 2005; Ainscough et al. 2006; Dawson et al. 2006). The aim of this investigation therefore was to produce unique protein species that incorporated the critical functional elements of the VN:IGFBP:IGF-I complex in one bio-active protein and in doing so, facilitate the translation of this technology into clinical and industrial therapies through improved production efficiencies and reduced development costs.

As described herein, 4 recombinant chimeric proteins incorporating specific domains of VN linked to mature IGF-I were designed, expressed and purified (**Fig. 1 and 2**). Critically, functional studies indicate that 2 of these novel recombinant chimeric proteins, namely chimeras B and D, can indeed mimic the action of the VN:IGFBP:IGF-I multiprotein

complex in cell migration experiments (**Fig. 3**). However, higher molar ratios of the chimeras were required (10x Chimera B and 30x Chimera D) to obtain responses equivalent to the VN:IGFBP:IGF-I complex. Despite this need for higher molar amounts of the chimeras, chimeric protein treatments able to induce responses equivalent to the VN:IGFBP:IGF-I complexes would in fact contain no more than 3 times the total protein, due to the removal of the need for the IGFBP and the overall reduced molecular weight of the chimeric proteins.

We elected to produce these chimeric proteins in an insect cell expression system because VN has been successfully produced in insect cells. In addition, we believe post-translational modifications such as glycosylation may be critical to protein function. Therefore, as post-translational modifications and protein processing are suggested to be similar in insect cells compared to mammalian systems (Geisse et al. 1996; Hu 2005; Harrison and Jarvis 2006), the insect cells were chosen as the most appropriate expression system. In order to verify compliant processing of the N-terminus which contained the native human VN secretion signal peptide, chimeras B and D were sequenced. The N-terminal sequencing results revealed that the first 8 amino acids of each chimera were identical to those of mature human VN from which the signal peptide had been cleaved, confirming equivalent processing in this instance (**Table V**).

To gain further insights into the structure and processing of the recombinant proteins produced, the chimeras were digested with trypsin and interrogated by mass-spectroscopy (**Table VI**). Peptides corresponding to VN were only observed in tryptic-digested preparations of human VN and chimeras A and B, and of those fragments, none were more N-terminal than amino acid 177 of VN. These findings correlate with those of Josic and co-workers who described the presence of VN in 'pure' factor IX preparations (Josic et al. 2001). Analysis of their data reveals that no peptides more N-terminal than amino acid 187 were detected by MALDI-TOF MS. A possible explanation for these results may be that the protein sequence up to amino acid 177, thus the entire VN portion of chimeras C and D, has been modified to incorporate factors such as phosphates and carbohydrates. In addition, previous studies have demonstrated that stretches of acidic amino acids, such as amino acids

52 to 64 of VN, have a low ionization efficiency altering the time of flight of the peptides. The presence of such entities has been reported to interfere with the tryptic cleavage reaction and/or alters the mass of fragments containing such modifications or characteristics, thus making them impossible to match with theoretical tryptic peptide masses (Ewing and Cassady 2001). Each chimera, however, gave consistent results with 2 to 3 positive fragments homologous to the IGF-I portion, indicating homology to this aspect of their original designs.

The data indicating that chimeras B and D are functionally equivalent to the VN:IGFBP:IGF-I complex in cell migration assays, whereas chimeras A and C are not is perplexing. Presumably this lack of activity is due to unfavourable folding resulting in a conformation that may inhibit activation of one or both of the αv-integrins and IGF-1R. However, there are many examples of the compatibility of insect and mammalian systems in the literature (Geisse et al. 1996), and given that two of the four chimeras were able to promote cellular responses equivalent to the VN:IGFBP:IGF-I complex, it is unlikely that post-translational modifications performed by the insect cells caused chimeras A and C to function poorly. Another aspect that may impact the biological activity of the final chimeric protein, however, is the linker sequence itself. The linker sequence used in the chimeras was a derivation of those used in previously reported studies in which fragments of the variable region of antibodies were linked together, or to other secondary effector molecules, to provide therapeutic and diagnostic reagents for a wide variety of uses (Huston et al. 1988; Kortt et al. 1997; Turner et al. 1997; Hudson 1998). These previous studies demonstrated that the linker was able to act as a connecting region with negligible impact on the function of the linked entities, thus making the Gly_4Ser linker a prime candidate for our chimeric proteins. In addition, a recent study by Lynn and co-workers described a detailed structural model of VN (Lynn et al. 2005) and on this representative VN structure, the maximum separation of the C-terminus from the charged regions of the SMB domain is ~75Å. We therefore sought a linker sequence that allowed the C-terminal IGF-I domain to interact with all of the VN domains. Hence, the VN:IGF-I chimeras we generated incorporated a linker with 4 repeats of the Gly_4Ser motif since such a linker will span approximately 70Å (assuming an average amino

acid length of 3.25Å) and would be quite flexible. Nevertheless, in terms of chimeras A and C, further investigations directed at modifying the linker sequence may well result in an optimal linker sequence that enables both αv-integrin and IGF-1R receptor activation in these chimeras.

It has been suggested by many that the enhanced cellular responses to IGFs in the presence of VN may be a result of "cross-talk" between the IGF-1R and αv-integrins (Zheng and Clemmons 1998; Clemmons et al. 1999; Maile et al. 2001). While direct co-operation between the IGF-1R and αv-integrins has yet to be determined, the downstream signalling pathways are clearly interconnected (Vuori and Ruoslahti 1994; Eliceiri 2001; Clemmons and Maile 2005; Maile et al. 2006). Additional investigations also demonstrate that a number of growth factor receptors interact with ECM components to modulate cellular functions, suggesting that interactions between growth factor receptors and the ECM is important (Majack et al. 1986; Nurcombe et al. 2000; Eliceiri 2001; Rahman et al. 2005). In order to understand the mechanism by which the multiprotein complex and the VN:IGF-I chimeras were acting to induce cell migration, additional migration experiments were performed that utilised function blocking antibodies directed against the IGF-1R and αv-integrins (**Fig. 4**). In treatments of VN alone, the VN:IGFBP:IGF-I complex and chimeras B and D, function-blocking antibodies to the IGF-1R and the αv-integrins abrogate cell migration responses. This confirms that co-activation of the VN-binding αv-integrins and the IGF-1R is required to confer a migration response. Furthermore, these results corroborate previous studies by others in which inhibition of the IGF-1R and αvβ5 integrin reduced IGF-I-stimulated migration on VN in MCF-7 cells (Doerr and Jones 1996).

One mechanism by which growth factor receptors and integrins can collaborate is via synergistic responses in their respective down-stream signalling pathways (Plopper et al. 1995; Miyamoto et al. 1996; Eliceiri 2001). Binding of ligands to both αv-integrins and the IGF-1R induces a number of cascades, including the ERK 1/2/MAPK and AKT/PI-3K pathways (Klemke et al. 1994; Fincham et al. 2000; Yamboliev et al. 2001; Yamboliev and Gerthoffer 2001; Tai et al. 2003; Huang et al. 2004). In addition, activation of both these

pathways has been implicated in cell motility (Klemke et al. 1997; Fincham et al. 2000; Yamboliev et al. 2001; Tai et al. 2003; Huang et al. 2004). Moreover, blocking VN binding to αvβ3 via disintegrin antagonists has been demonstrated to abolish IGF-I-stimulated responses such as cell migration, IGF-1R autophosphorylation and PI3-K activation (Zheng and Clemmons 1998; Clemmons et al. 1999; Ling et al. 2003; Maile et al. 2006). Therefore, we investigated the ability of the VN:IGFBP:IGF-I complex and chimeras B and D to induce activation of these pathways. We document here that VN:IGFBP:IGF-I complexes, along with chimeric proteins B and D, can stimulate transient and synergistic activation of the ERK 1/2/MAPK signalling pathway. Coincidently, both the multiprotein complex and chimera D produced a sustained activation of the AKT/PI3-K pathway (**Fig. 5**). When compared to the VN:IGFBP:IGF-I treatment, only chimera D was equivalent in the level of phosphorylated ERK 1/2 and AKT present, while chimera B was only able to activate the signalling molecules to levels found with VN alone. These results demonstrate that chimera D and the VN:IGFBP:IGF-I complex are similar functionally. Thus the synergistic increases in cell signalling observed in response to the VN:IGFBP:IGF-I complex and chimera D appear to arise from co-activation of both the IGF-1R and αv-integrins through the concomitant delivery of both IGF-I and VN (or fragments there of) to the cell surface, since substantial activation of the AKT/PI3-K pathway required both components to be present (**Fig. 5**).

In addition to cell migration, the proliferation of cells is another key response critical for successful cell growth and repair processes. Furthermore, a significant number of growth factor chimera studies have used cell proliferation experiments to characterise the novel proteins (Congote and Li 1994; Congote 1995; Difalco and Congote 1997; Michieli et al. 2002; Sandoval et al. 2002; Sandoval et al. 2003; Brewster et al. 2004; Brewster et al. 2006; Ishikawa et al. 2006). Therefore, the chimeric proteins that mimicked responses to VN:IGFBP-3:IGF-I complexes in cell migration assays, namely chimeras B and D, were assessed for their ability to induce cell growth using MCF-7 breast carcinoma cells (**Fig. 6**). As per the cell migration assays, both chimeric proteins were able to significantly increase cell number equivalent to the VN:IGFBP-3:IGF-I multiprotein complex. These results

indicate that the IGF-I portion of the chimeras confer function in terms of cell proliferation. Moreover, these results further demonstrate that chimeras B and D and the VN:IGFBP:IGF-I complexes are equivalent functionally in terms of cell proliferation.

Recent studies in other laboratories have also drawn attention to the potential of combining proteins with functionally disparate properties into one molecule to generate novel proteins with activity surpassing that of the individual components alone. For example, basic fibroblast growth factor (bFGF) was linked to the ECM heparin-binding growth-associated molecule and this novel protein has been demonstrated to enhance re-endothelialisation in injured arteries above that obtained with bFGF alone (Brewster et al. 2006). Another recent report describes the linkage of the anti-viral protein interferon-α (IFN-α) to human serum albumin (Balan et al. 2006). The resultant chimera sustained the activity of the IFN-α portion whilst the stabilizing proteolytic antagonist properties of albumin were retained. Furthermore, Ishikawa *et al*. (2006) utilised the collagen binding domain of FN to locate a non-heparin binding mutant of vascular endothelial growth factor (VEGF), VEGF121, to the ECM (Ishikawa et al. 2006). In this situation the focal concentration of VEGF121 was sustained in a collagen matrix to promote enhanced growth and differentiation of endothelial cells (Ishikawa et al. 2006).

These recent reports, taken together with the novel data we present herein, highlight the importance of interactions between ECM proteins and growth factors. In addition, these data confirm that co-ordinate activation and "cross-talk" between the IGF-I receptor and the VN-binding αv-integrins underlie the enhanced responses observed in cells exposed to the VN:IGFBP:IGF-I multiprotein complexes (Hollier et al. 2007; Upton et al. 2007). Furthermore, we demonstrate that these responses can be recapitulated in a single recombinant chimeric protein. Critically, these experimental data have important implications in terms of reducing manufacturing costs and addressing regulatory issues in translating these ECM protein:growth factor complexes into industrial-scale cell culture applications, as well as cell-based regenerative therapies and wound healing applications.

REFERENCES

Ainscough, S. L., Z. Barnard, et al. (2006). "Vitronectin supports migratory responses of corneal epithelial cells to substrate bound IGF-I and HGF, and facilitates serum-free cultivation." Exp Eye Res 83(6): 1505-14.

Balan, V., D. R. Nelson, et al. (2006). "Modulation of interferon-specific gene expression by albumin-interferon-alpha in interferon-alpha-experienced patients with chronic hepatitis C." Antivir Ther 11(7): 901-8.

Bayne, M. L., J. Applebaum, et al. (1990). "The roles of tyrosines 24, 31, and 60 in the high affinity binding of insulin-like growth factor-I to the type 1 insulin-like growth factor receptor." J Biol Chem 265(26): 15648-52.

Bayne, M. L., J. Applebaum, et al. (1989). "The C region of human insulin-like growth factor (IGF) I is required for high affinity binding to the type 1 IGF receptor." J Biol Chem 264(19): 11004-8.

Bjerrum, O. J. and C. Schafer-Nielsen (1986). "Buffer systems and transfer parameters for semidry electroblotting with a horizontal apparatus." In M. J. Dunn (ed.), Electrophoresis ’86 VCH Publishers, Deerfield Beach, Fla.: 315-327.

Brewster, L., E. M. Brey, et al. (2006). "Improving endothelial healing with novel chimeric mitogens." Am J Surg 192(5): 589-93.

Brewster, L. P., E. M. Brey, et al. (2004). "Heparin-independent mitogenicity in an endothelial and smooth muscle cell chimeric growth factor (S130K-HBGAM)." Am J Surg 188(5): 575-9.

Cascieri, M. A., G. G. Chicchi, et al. (1988). "Mutants of human insulin-like growth factor I with reduced affinity for the type 1 insulin-like growth factor receptor." Biochemistry 27(9): 3229-33.

Clemmons, D. R., G. Horvitz, et al. (1999). "Synthetic alphaVbeta3 antagonists inhibit insulin-like growth factor-I-stimulated smooth muscle cell migration and replication." Endocrinology 140(10): 4616-21.

Clemmons, D. R. and L. A. Maile (2005). "Interaction between insulin-like growth factor-I receptor and alphaVbeta3 integrin linked signaling pathways: cellular responses to changes in multiple signaling inputs." Mol Endocrinol 19(1): 1-11.

Congote, L. F. (1995). "Increased heparin binding by site directed mutagenesis of a recombinant chimera of bombyxin and insulin-like growth factor II." Biochim Biophys Acta 1243(3): 538-42.

Congote, L. F. and Q. Li (1994). "Accurate processing and secretion in the baculovirus expression system of an erythroid-cell-stimulating factor consisting of a chimaera of insulin-like growth factor II and an insect insulin-like peptide." Biochem J 299 (Pt 1): 101-7.

Cory, A. H., T. C. Owen, et al. (1991). "Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture." Cancer Commun 3(7): 207-12.

Dawson, R. A., Z. Upton, et al. (2006). "Preparation of cultured skin for transplantation using insulin-like growth factor I in conjunction with insulin-like growth factor binding protein 5, epidermal growth factor, and vitronectin." Transplantation 81(12): 1668-76.

Denley, A., L. J. Cosgrove, et al. (2005). "Molecular interactions of the IGF system." Cytokine Growth Factor Rev 16(4-5): 421-39.

Difalco, M. R. and L. F. Congote (1997). "Preparation of a recombinant chimaera of insulin-like growth factor II and interleukin 3 with high proliferative potency for haemopoietic cells." Biochem J 326 (Pt 2): 407-13.

Doerr, M. E. and J. I. Jones (1996). "The roles of integrins and extracellular matrix proteins in the insulin-like growth factor I-stimulated chemotaxis of human breast cancer cells." J Biol Chem 271(5): 2443-7.

Edman, P. (1950). "Method for determination of the amino acid sequence in peptides." Acta Chemica Scandinavica 4: 283.
Eliceiri, B. P. (2001). "Integrin and growth factor receptor crosstalk." Circ Res 89(12): 1104-10.
Ewing, N. P. and C. J. Cassady (2001). "Dissociation of multiply charged negative ions for hirudin (54-65), fibrinopeptide B, and insulin A (oxidized)." J Am Soc Mass Spectrom 12(1): 105-16.
Fincham, V. J., M. James, et al. (2000). "Active ERK/MAP kinase is targeted to newly forming cell-matrix adhesions by integrin engagement and v-Src." Embo J 19(12): 2911-23.
Fryklund, L. and H. Sievertsson (1978). "Primary structure of somatomedin B: a growth hormone-dependent serum factor with protease inhibiting activity." FEBS Lett 87(1): 55-60.
Geisse, S., H. Gram, et al. (1996). "Eukaryotic expression systems: a comparison." Protein Expr Purif 8(3): 271-82.
Giancotti, F. G. and E. Ruoslahti (1999). "Integrin signaling." Science 285(5430): 1028-32.
Harrison, R. L. and D. L. Jarvis (2006). "Protein N-glycosylation in the baculovirus-insect cell expression system and engineering of insect cells to produce "mammalianized" recombinant glycoproteins." Adv Virus Res 68: 159-91.
Hollier, B., D. G. Harkin, et al. (2005). "Responses of keratinocytes to substrate-bound vitronectin: growth factor complexes." Exp Cell Res 305(1): 221-32.
Hollier, B. G., J. A. Kricker, et al. (2007). "The PI3-K/AKT pathway mediates substrate-bound IGF-I:IGFBP:Vitronectin complex stimulated breast cell migration." Endocrinology Submitted.
Hu, Y. C. (2005). "Baculovirus as a highly efficient expression vector in insect and mammalian cells." Acta Pharmacol Sin 26(4): 405-16.
Huang, C., K. Jacobson, et al. (2004). "MAP kinases and cell migration." J Cell Sci 117(Pt 20): 4619-28.
Hudson, P. J. (1998). "Recombinant antibody fragments." Curr Opin Biotechnol 9(4): 395-402.
Huston, J. S., D. Levinson, et al. (1988). "Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in Escherichia coli." Proc Natl Acad Sci U S A 85(16): 5879-83.
Hyde, C., B. Hollier, et al. (2004). "Insulin-like growth factors (IGF) and IGF-binding proteins bound to vitronectin enhance keratinocyte protein synthesis and migration." J Invest Dermatol 122(5): 1198-206.
Ishikawa, T., M. Eguchi, et al. (2006). "Establishment of a functionally active collagen-binding vascular endothelial growth factor fusion protein in situ." Arterioscler Thromb Vasc Biol 26(9): 1998-2004.
Josic, D., C. Kannicht, et al. (2001). "Vitronectin in clotting factor IX concentrates." Haemophilia 7(3): 250-7.
Keyhanfar, M., G. W. Booker, et al. (2007). "Precise mapping of an IGF-I-binding site on the IGF-1R." Biochem J 401(1): 269-77.
Klemke, R. L., S. Cai, et al. (1997). "Regulation of cell motility by mitogen-activated protein kinase." J Cell Biol 137(2): 481-92.
Klemke, R. L., M. Yebra, et al. (1994). "Receptor tyrosine kinase signaling required for integrin alpha v beta 5-directed cell motility but not adhesion on vitronectin." J Cell Biol 127(3): 859-66.
Kortt, A. A., M. Lah, et al. (1997). "Single-chain Fv fragments of anti-neuraminidase antibody NC10 containing five- and ten-residue linkers form dimers and with zero-residue linker a trimer." Protein Eng 10(4): 423-33.
Kricker, J. A., C. L. Towne, et al. (2003). "Structural and functional evidence for the interaction of insulin-like growth factors (IGFs) and IGF binding proteins with vitronectin." Endocrinology 144(7): 2807-15.

Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." Nature 227(5259): 680-5.
Leavesley, D. I., M. A. Schwartz, et al. (1993). "Integrin beta 1- and beta 3-mediated endothelial cell migration is triggered through distinct signaling mechanisms." J Cell Biol 121(1): 163-70.
Ling, Y., L. A. Maile, et al. (2003). "Tyrosine phosphorylation of the beta3-subunit of the alphaVbeta3 integrin is required for embrane association of the tyrosine phosphatase SHP-2 and its further recruitment to the insulin-like growth factor I receptor." Mol Endocrinol 17(9): 1824-33.
Lynn, G. W., W. T. Heller, et al. (2005). "A model for the three-dimensional structure of human plasma vitronectin from small-angle scattering measurements." Biochemistry 44(2): 565-74.
Maile, L. A., J. Badley-Clarke, et al. (2001). "Structural analysis of the role of the beta 3 subunit of the alpha V beta 3 integrin in IGF-I signaling." J Cell Sci 114(Pt 7): 1417-25.
Maile, L. A., W. H. Busby, et al. (2006). "Insulin-like growth factor-I signaling in smooth muscle cells is regulated by ligand binding to the 177CYDMKTTC184 sequence of the beta3-subunit of alphaVbeta3." Mol Endocrinol 20(2): 405-13.
Majack, R. A., S. C. Cook, et al. (1986). "Control of smooth muscle cell growth by components of the extracellular matrix: autocrine role for thrombospondin." Proc Natl Acad Sci U S A 83(23): 9050-4.
Michieli, P., S. Cavassa, et al. (2002). "An HGF-MSP chimera disassociates the trophic properties of scatter factors from their pro-invasive activity." Nat Biotechnol 20(5): 488-95.
Miyamoto, S., H. Teramoto, et al. (1996). "Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: roles of integrin aggregation and occupancy of receptors." J Cell Biol 135(6 Pt 1): 1633-42.
Morris, C. A., P. A. Underwood, et al. (1994). "Relative topography of biologically active domains of human vitronectin. Evidence from monoclonal antibody epitope and denaturation studies." J Biol Chem 269(38): 23845-52.
Noble, A., C. Towne, et al. (2003). "Insulin-like growth factor-II bound to vitronectin enhances MCF-7 breast cancer cell migration." Endocrinology 144(6): 2417-24.
Nurcombe, V., C. E. Smart, et al. (2000). "The proliferative and migratory activities of breast cancer cells can be differentially regulated by heparan sulfates." J Biol Chem 275(39): 30009-18.
Plopper, G. E., H. P. McNamee, et al. (1995). "Convergence of integrin and growth factor receptor signaling pathways within the focal adhesion complex." Mol Biol Cell 6(10): 1349-65.
Rahman, S., Y. Patel, et al. (2005). "Novel hepatocyte growth factor (HGF) binding domains on fibronectin and vitronectin coordinate a distinct and amplified Met-integrin induced signalling pathway in endothelial cells." BMC Cell Biol 6(1): 8.
Sandoval, C., H. Curtis, et al. (2002). "Enhanced proliferative effects of a baculovirus-produced fusion protein of insulin-like growth factor and alpha(1)-proteinase inhibitor and improved anti-elastase activity of the inhibitor with glutamate at position 351." Protein Eng 15(5): 413-8.
Sandoval, C., A. Stojanova, et al. (2003). "The fusion of IGF I with stromal cell-derived factor I or alpha1 proteinase inhibitor alters their mitogenic or chemotactic activities while keeping their ability to inhibit HIV-1-gp120 binding." Biochem Pharmacol 65(12): 2055-63.
Schleicher, I., A. Parker, et al. (2005). "Surface modification by complexes of vitronectin and growth factors for serum-free culture of human osteoblasts." Tissue Eng 11(11-12): 1688-98.
Schvartz, I., D. Seger, et al. (1999). "Vitronectin." Int J Biochem Cell Biol 31(5): 539-44.

Sunstrom, N. A., R. D. Gay, et al. (2000). "Insulin-like growth factor-I and transferrin mediate growth and survival of Chinese hamster ovary cells." Biotechnol Prog 16(5): 698-702.

Tai, Y. T., K. Podar, et al. (2003). "Insulin-like growth factor-1 induces adhesion and migration in human multiple myeloma cells via activation of beta1-integrin and phosphatidylinositol 3'-kinase/AKT signaling." Cancer Res 63(18): 5850-8.

Tai, Y. T., K. Podar, et al. (2003). "CD40 induces human multiple myeloma cell migration via phosphatidylinositol 3-kinase/AKT/NF-kappa B signaling." Blood 101(7): 2762-9.

Turner, D. J., M. A. Ritter, et al. (1997). "Importance of the linker in expression of single-chain Fv antibody fragments: optimisation of peptide sequence using phage display technology." J Immunol Methods 205(1): 43-54.

Upton, Z., L. Cuttle, et al. (2007). "Topical application of nanogram amounts of a novel vitronectin:growth factor complex significantly enhances wound healing *in vivo*." Journal of Investigative Dermatology Submitted.

Upton, Z., H. Webb, et al. (1999). "Identification of vitronectin as a novel insulin-like growth factor-II binding protein." Endocrinology 140(6): 2928-31.

Vuori, K. and E. Ruoslahti (1994). "Association of insulin receptor substrate-1 with integrins." Science 266(5190): 1576-8.

Wilkins, M. R., E. Gasteiger, et al. (1999). "Protein identification and analysis tools in the ExPASy server." Methods Mol Biol 112: 531-52.

Yamboliev, I. A., J. Chen, et al. (2001). "PI 3-kinases and Src kinases regulate spreading and migration of cultured VSMCs." Am J Physiol Cell Physiol 281(2): C709-18.

Yamboliev, I. A. and W. T. Gerthoffer (2001). "Modulatory role of ERK MAPK-caldesmon pathway in PDGF-stimulated migration of cultured pulmonary artery SMCs." Am J Physiol Cell Physiol 280(6): C1680-8.

Zheng, B. and D. R. Clemmons (1998). "Blocking ligand occupancy of the alphaVbeta3 integrin inhibits insulin-like growth factor I signaling in vascular smooth muscle cells." Proc Natl Acad Sci U S A 95(19): 11217-22.

FOOTNOTES

We thank: Gemma Topping (Tissue Repair and Regeneration Program, Institute of Health and Biomedical Innovation, Queensland University of Technology, Kelvin Grove, QLD, Australia) for her contributions in the design and formatting of all figures in the manuscript; and Christopher Towne (currently at EPFL, Lausanne, Switzerland) and Dr. Mark Egerton (Fisher Adams Kelly, Brisbane, QLD, Australia) for assistance in the design of the chimeric proteins.

[a]Abbreviations used: IGF: insulin-like growth factor; ECM: extracellular matrix; VN: vitronectin; IGFBP: IGF binding protein; IGF-1R: IGF type-1 receptor; ERK: extracellular signal-regulated kinase; MAPK: mitogen activated protein kinase; AKT: protein kinase-B; SMB: somatomedin-B domain of vitronectin.

TABLE I

Primer sequences for site-directed mutagenesis PCRs.

Construct / Modification	Sense Primer	Anti-sense Primer
Chimera A / linker insertion	5′-p-GGTGGTGGTGGTTCCGGTGGTGGTGGTTCC GGTGGTGGTGGTTCCGGTGGTGGTGGTTCCAAC GGTCCTGAGACCCTGTGCGGT-3′	5′-CAGGTGGCCAG GAGCA-3′
Chimera B / linker insertion	5′-p-GGTGGTGGTGGTTCCGGTGGTGGTGGTTCC GGTGGTGGTGGTTCCGGTGGTGGTGGTTCCAAC GGTCCTGAGACCCTGTGCGGT-3′	5′-ACGAGTACCAG CGGAGGTACG-3′
Chimera C / linker insertion	5′-p-GGTGGTGGTGGTTCCGGTGGTGGTGGTTCC GGTGGTGGTGGTTCCGGTGGTGGTGGTTCCAAC GGTCCTGAGACCCTGTGCGGT-3′	5′-ACCAGGGTGCA GAGTCTCAGG-3′
Chimera D / linker insertion	5′-p-GGTGGTGGTGGTTCCGGTGGTGGTGGTTCC GGTGGTGGTGGTTCCGGTGGTGGTGGTTCCAAC GGTCCTGAGACCCTGTGCGGT-3′	5′-CTCCTCGCCGTCGT CGTACAC-3′
All chimeras / TEV insertion	5′-p-GACAACCTGTACTTTCAGGGTAAGCCTAT CCCTAA-3′	5′-AGCGGACTTAG CAGGC-3′

Sense primers bound downstream on the coding sequence whilst anti-sense primers bound upstream. Amplification occurred in opposite directions and continued along the complete template length of the synthetic genes.

TABLE II

Treatment schedule for Transwell™ migration assays

Treatments	Treatments			
	1x	3.3x	10x	30x
VN	1 µg/mL	3.3 µg/mL	10 µg/mL	
Trimeric Complex	1 µg/mL VN 1 µg/mL IGFBP3 0.3 µg/mL IGF-I	3.3 µg/mL VN 3.3 µg/mL IGFBP3 1 µg/mL IGF-I	10 µg/mL VN 10 µg/mL IGFBP3 3 µg/mL IGF-I	
Chimera A	1 µg/mL	3.3 µg/mL	10 µg/mL	
Chimera B	0.67 µg/mL	2.21 µg/mL	6.7 µg/mL	
Chimera C	0.26 µg/mL	0.86 µg/mL	2.6 µg/mL	
Chimera D	0.14 µg/mL	0.46 µg/mL	1.4 µg/mL	4.2 µg/mL

The composition and concentration of the treatments used in the cell migration assays assessing the chimeric proteins is indicated. The proteins were added in 1 mL of SFM to the lower chamber of the Transwell™ plates prior to seeding cells into the upper chamber inserts.

TABLE III

Treatment schedule for cell signalling assays

Treatments	Concentration
VN	2.50 µg/mL
Trimeric Complex	2.50 µg/mL VN 2.50 µg/mL IGFBP-3 0.75 µg/mL IGF-I
VN + IGF-I	2.50 µg/mL VN 0.75 µg/mL IGF-I
Chimera B	16.75 µg/mL
Chimera D	10.50 µg/mL

The composition of the treatments used in the cell signalling assays assessing the chimeric proteins is indicated. The proteins were added to the wells of a 6-well plate in 1 mL SFM. The final concentrations of the proteins assessed, after the addition of cells to the wells, are indicated above.

TABLE IV

Treatment schedule for proliferation assays

Treatments	Treatments					
	0.33x	1x	3x	10x	30x	90x
VN	0.28 µg/mL	0.83 µg/mL	2.50 µg/mL			
Trimeric Complex	0.28 µg/mL VN 0.28 µg/mL IGFBP3 0.08 µg/mL IGF-I	0.83 µg/mL VN 0.83 µg/mL IGFBP3 0.25 µg/mL IGF-I	2.50 µg/mL VN 2.50 µg/mL IGFBP3 0.75 µg/mL IGF-I			
VN + IGF-I	0.28 µg/mL VN 0.08 µg/mL IGF-I	0.83 µg/mL VN 0.25 µg/mL IGF-I	2.50 µg/mL VN 0.75 µg/mL IGF-I			
Chimera B			1.86 µg/mL (3.3x)	5.58 µg/mL	16.75 µg/mL	
Chimera D				1.16 µg/mL	3.50 µg/mL	10.50 µg/mL

The composition of the treatments used in the proliferation assays assessing the chimeric proteins is indicated. The proteins were added to the wells of a 96-well plate in 50 µL SFM. The final concentrations of the proteins assessed, after the addition of cells to the wells, are indicated above.

TABLE V

N-terminal sequencing results for Chimeras B and D.

Position	Chimera B	Chimera D	VN
1	D	D	D
2	Q	Q	Q
3	E	E	E
4	S	S	S
5	-	-	C
6	K	K	K
7	G	(G)	G
8	R	(R)	R

The first eight amino acids of the N-terminus of chimeras B and D were determined using the Edman degradation method. '-' = C or modified amino acid, '( )' = tentative assignment. The mature VN N-terminal sequence is detailed in the far right row (Fryklund and Sievertsson 1978). The single letter amino acid code is depicted.

TABLE VI

Mass-Spectrum results for the chimeric proteins.

Protein	Mass	ΔMass	Positive sequence	Position	VN	IGF-I
VN	875.561	-0.087	(R)/QPQFISR/(D)	331-337	✓	
	887.599	-0.087	(K)/AVRPGYPK/(L)	187-194	✓	
	988.623	-0.097	(R)/ERVYFFK/(G)	269-275	✓	
	1314.82	-0.142	(R)/RVDTVDPPYPR/(S)	453-463	✓	
	1422.817	-0.163	(R)/FEDGVLDPDYPR/(N)	230-241	✓	
	1448.928	-0.195	(K)/TYLFKGSQYWR/(F)	219-229	✓	
	1448.928	-0.167	(R)/TSAGTRQPQFISR/(D)	325-337	✓	
	1647.001	-0.183	(R)/DVWGIEGPIDAAFTR/(I)	198-212	✓	
	1666.962	-0.186	(R)/DWHGVPGQVDAAMAGR/(I)	338-353	✓	
A	1304.585	-0.038	(R)/GQYCYELDEK/(A)	177-186	✓	
	1314.730	-0.049	(R)/RVDTVDPPYPR/(S)	453-463	✓	
	1422.709	-0.055	(R)/FEDGVLDPDYPR/(N)	230-241	✓	
	1646.870	-0.052	(R)/DVWGIEGPIDAAFTR/(I)	198-212	✓	
	1666.793	-0.017	(R)/DWHGVPGQVDAAMAGR/(I)	338-353	✓	
	1667.827	-0.045	(R)/GFYFNKPTGYGSSSR/(R)	521-535		✓
	1708.842	-0.052	(R)/RAPQTGIVDECCFR/(S)	536-549		✓
			(K)/SAENLYFQGKPIPNPLLGLD			
	2530.387	-0.057	STR/(T)	568-590		✓
B	1422.750	-0.096	(R)/FEDGVLDPDYPR/(N)	230-241	✓	
	1646.913	-0.095	(R)/DVWGIEGPIDAAFTR/(I)	198-212	✓	
	1667.885	-0.103	(R)/GFYFNKPTGYGSSSR/(R)	373-387		✓
			(K)/SAENLYFQGKPIPNPLLGLD			
	2530.439	-0.109	STR/(T)	420-442		✓

C	1667.992	-0.210	(R)/GFYFNKPTGYGSSSR/(R)	188-202	✓
			(K)/SAENLYFQGKPIPNPLLGLD		
	2530.546	-0.216	STR/(T)	235-257	✓
D	1668.017	-0.235	(R)/GFYFNKPTGYGSSSR/(R)	126-140	✓
	1709.021	-0.231	(R)/RAPQTGIVDECCFR/(S)	141-154	✓
			(K)/SAENLYFQGKPIPNPLLGLD		
	2530.633	-0.303	STR/(T)	173-195	✓

Expected amino acid sequences and peptide masses of tryptic-digested samples of the chimeric proteins were entered into the FindPep program on the Expasy website (www.expasy.org). Positive matches were determined to pertain to regions of the VN or IGF-I portions of the chimeras. The “Mass” column indicates experimentally-derived masses, “ΔMass” indicates difference between experimentally-derived and theoretical masses. ’/’ indicate cleavage sites and bracketed ‘( )’ letters denote amino acids preceding and following the peptides N- and C-terminal cleavage sites.

FIGURE LEGENDS

Figure 1: ***Schematic representation of the chimeric proteins and amino acid sequences encoded by the chimeric expression constructs.*** i. All chimeric proteins contain the $(Gly_4Ser)_4$Asn linker motif (LINK) and the mature IGF-I molecule (IGF-I). Chimera A (A) contains the full-length VN molecule (amino acids 1 – 459) including the somatomedin-B domain (SMB), the integrin binding RGD domain (RGD), the connecting region (CON), the heamopexin-like 1 domain (HEM 1) and the heamopexin-like 2 domain (HEM 2). Chimera B (B), comprises amino acids 1 – 311 of VN and excludes the heparin binding domain. Chimera C (C), comprises amino acids 1-125 of VN, while Chimera D (D), comprises amino acids 1-64 of VN, and is truncated at the end of the polyanionic region (- - -). ii. All constructs contain DNA sequences encoding the secretion signal peptide of human VN (1-19) followed by DNA encoding: select amino acid sequences of the mature VN protein (purple highlighted); a $(Gly_4Ser)_4$Asn linker sequence (green highlighted); the mature IGF-I sequence (blue highlighted); and the TEV cleavage site, V5 epitope and the 6x His tag (underlined) are at the C-terminal end of the protein.

Figure 2: ***Purified chimeric VN:IGF-I proteins.*** Chimeric proteins A, B, C and D were purified using a variety of chromatographic methods as described in the *Materials and Methods*. Purity of the chimeras was assessed using Silver-Stained SDS-PAGE gels (left-hand lane in each pair) and Western blotting utilizing a polyclonal anti-VN antibody (right-hand lane in each pair). Molecular weight markers are indicated.

Figure 3: ***Chimeric VN:IGF-I proteins stimulate cell migration in MCF-7 cells***. Transwell™ migration assays were performed with treatments of SFM (negative control), VN alone, the trimeric VN:IGFBP-3:IGF-I complex, as well as chimeric proteins A, B, C and D at the concentrations indicated in **Table 2**. Data is expressed as % of VN alone at 1x dose. Values shown are mean ± SEM of independent triplicate treatments present in 2 replicate

experiments. Asterisks (*) indicate treatments where migration was significantly different (p<0.01) compared to the 1x VN alone, while crosses (†) indicate treatments where migration was not significantly different (p>0.05) compared to the trimeric 1x VN:IGFBP-3:IGF-I complex.

Figure 4: ***Function blocking antibodies inhibit chimera induced cell migration***. Chimeras B and D showed similar activity to the VN:IGFBP-3:IGF-I complex in Transwell™ migration assays, thus these chimeras were further characterised in migration assays utilising function-blocking antibodies. Treatments of VN alone, the VN:IGFBP-3:IGF-I complex at 1x concentration, and chimeric proteins B, at 10x, and D, at 30x, were assessed in Transwell™ migration assays; cells for each treatment were pre-incubated with no antibody (white bars), IgG control (25 µg/mL, shaded bars), anti-IGF-IR (10 µg/mL, dark blue bars) and anti-αv-integrin (1:10, light blue bars) antibodies. Data is expressed for each treatment group as % of the migration observed for the treatment with no added antibody. Values shown are mean ± SEM of triplicate wells from from 2 replicate experiments with independent triplicate treatments in each experiment. Asterisks (*) indicate treatments where migration was significantly different (p<0.01) compared to the control treatment within each group (no added antibody).

Figure 5: ***Phosphorylation of ERK 1/2 and AKT in MCF-7 cells.*** Total protein was extracted from MCF-7 cells treated with VN alone, the trimeric VN:IGFBP-3:IGF-I complex, VN and IGF-I, chimera B and chimera D, at the concentrations indicated in Table 3.3, at 10 minutes (10') and 60 minutes (60'). Cell lysates were probed for phosphorylated (pERK 1/2 and pAKT) and total (ERK 1/2 and AKT) forms of ERK 1/2 and AKT in western blots.

Figure 6: ***Chimeric VN:IGF-I proteins stimulate MCF-7 cell proliferation.*** Treatments of SFM (negative control), VN alone, VN + IGF-I, the VN:IGFBP-3:IGF-I complex, as well as chimeras B and D, were assessed in cell proliferation assays. Data is expressed as % above

the SFM control. Values shown are mean ± SEM from 2 replicate experiments with independent triplicate treatments in each experiment. Asterisks (*) indicate treatments where migration was significantly different ($p<0.05$) compared to the control (SFM). Crosses (†) indicate treatments where proliferation was not significantly different ($p>0.05$) compared to the 1x VN:IGFBP-3:IGF-I complex.

FIGURE 1

i

Chimera A

```
MAPLRPLLILALLAWVALADQESCKGRCTEGFNVDKKCQCDELCSYYQSCCTDYTAECKP      60
QVTRGDVFTMPEDEYTVYDDGEEKNNATVHEQVGGPSLTSDLQAQSKGNPEQTPVLKPEE     120
EAPAPEVGASKPEGIDSRPETLHPGRPQPPAEEELCSGKPFDAFTDLKNGSLFAFRGQYC     180
YELDEKAVRPGYPKLIRDVWGIEGPIDAAFTRINCQGKTYLFKGSQYWRFEDGVLDPDYP     240
RNISDGFDGIPDNVDAALALPAHSYSGRERVYFFKGKQYWEYQFQHQPSQEECEGSSLSA     300
VFEHFAMMQRDSWEDIFELLFWGRTSAGTRQPQFISRDWHGVPGQVDAAMAGRIYISGMA     360
PRPSLAKKQRFRHRNRKGYRSQRGHSRGRNQNSRRPSRAMWLSLFSSEESNLGANNYDDY     420
RMDWLVPATCEPIQSVFFFSGDKYYRVNLRTRRVDTVDPPYPRSIAQYWLGCPAPGHLGG     480
GGSGGGGSGGGGSGGGGSNGPETLCGAELVDALQFVCGDRGFYFNKPTGYGSSSRRAPQT     540
GIVDECCFRSCDLRRLEMYCAPLKPAKSAENLYFQGKPIPNPLLGLDSTRTGHHHHHH-     598
```

Chimera B

```
MAPLRPLLILALLAWVALADQESCKGRCTEGFNVDKKCQCDELCSYYQSCCTDYTAECKP      60
QVTRGDVFTMPEDEYTVYDDGEEKNNATVHEQVGGPSLTSDLQAQSKGNPEQTPVLKPEE     120
EAPAPEVGASKPEGIDSRPETLHPGRPQPPAEEELCSGKPFDAFTDLKNGSLFAFRGQYC     180
YELDEKAVRPGYPKLIRDVWGIEGPIDAAFTRINCQGKTYLFKGSQYWRFEDGVLDPDYP     240
RNISDGFDGIPDNVDAALALPAHSYSGRERVYFFKGKQYWEYQFQHQPSQEECEGSSLSA     300
VFEHFAMMQRDSWEDIFELLFWGRTSAGTRGGGGSGGGGSGGGGSGGGGSNGPETLCGAE     360
LVDALQFVCGDRGFYFNKPTGYGSSSRRAPQTGIVDECCFRSCDLRRLEMYCAPLKPAKS     420
AENLYFQGKPIPNPLLGLDSTRTGHHHHHH-                                   450
```

Chimera C

```
MAPLRPLLILALLAWVALADQESCKGRCTEGFNVDKKCQCDELCSYYQSCCTDYTAECKP      60
QVTRGDVFTMPEDEYTVYDDGEEKNNATVHEQVGGPSLTSDLQAQSKGNPEQTPVLKPEE     120
EAPAPEVGASKPEGIDSRPETLHPGGGGGSGGGGSGGGGSGGGGSNGPETLCGAELVDAL     180
QFVCGDRGFYFNKPTGYGSSSRRAPQTGIVDECCFRSCDLRRLEMYCAPLKPAKSAENLY     240
FQGKPIPNPLLGLDSTRTGHHHHHH-                                        265
```

Chimera D

```
MAPLRPLLILALLAWVALADQESCKGRCTEGFNVDKKCQCDELCSYYQSCCTDYTAECKP      60
QVTRGDVFTMPEDEYTVYDDGEEGGGGSGGGGSGGGGSGGGGSNGPETLCGAELVDALQF     120
VCGDRGFYFNKPTGYGSSSRRAPQTGIVDECCFRSCDLRRLEMYCAPLKPAKSAENLYFQ     180
GKPIPNPLLGLDSTRTGHHHHHH-                                          203
```

ii

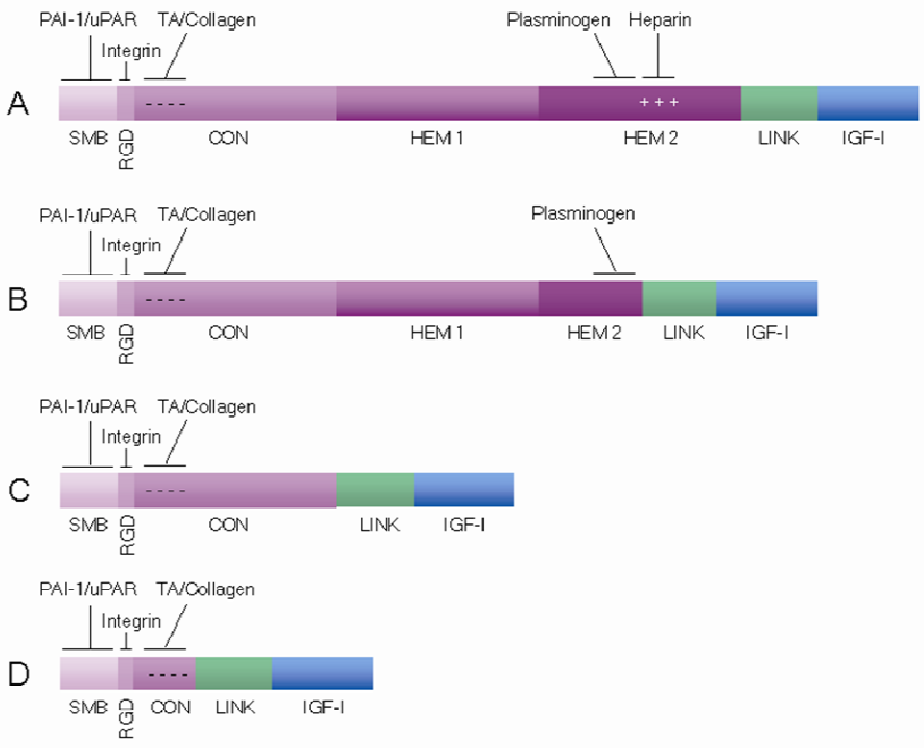


FIGURE 2

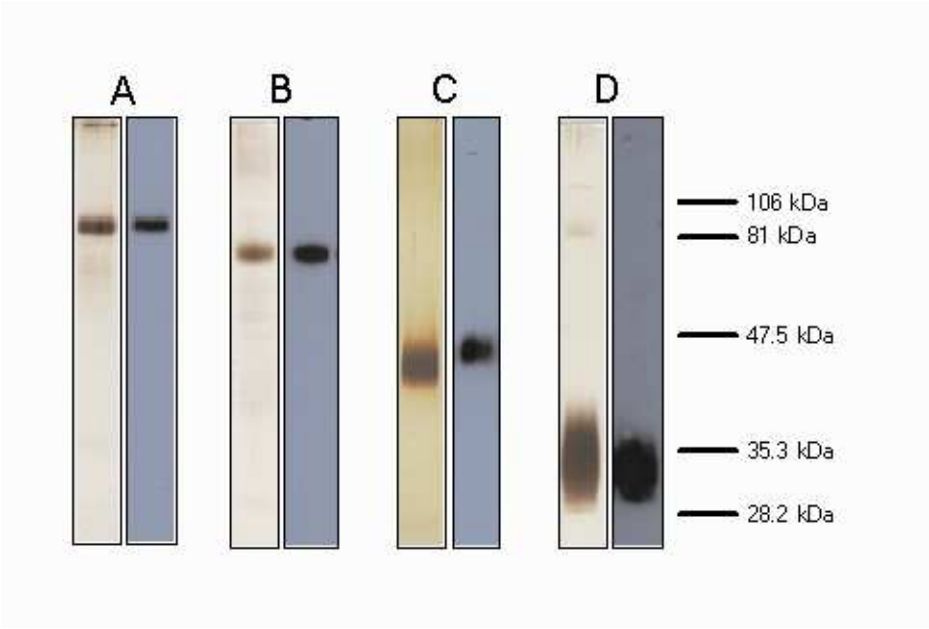


FIGURE 3

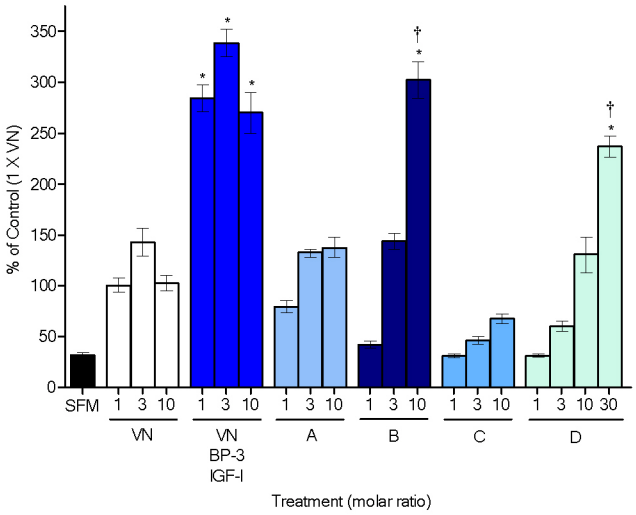

% of Control (1 X VN)
350
300
250
200
150
100
50
0
SFM
1 3 10
1 3 10
1 3 10
1 3 10
1 3 10
1 3 10 30
VN
VN
BP-3
IGF-I
A
B
C
D
Treatment (molar ratio)

FIGURE 4

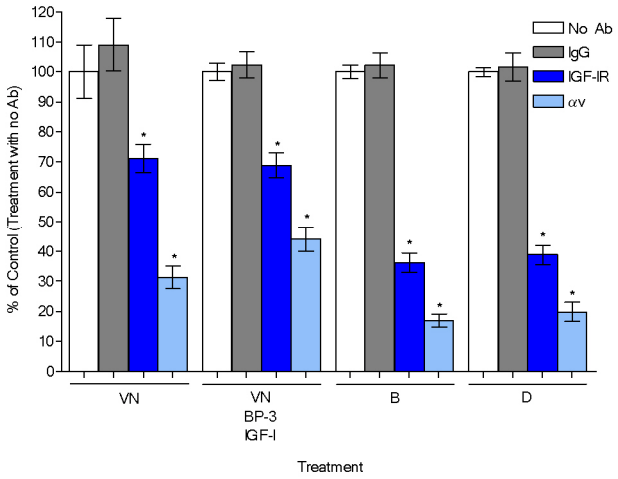

No Ab
IgG
IGF-IR
αv
% of Control (Treatment with no Ab)
120
110
100
90
80
70
60
50
40
30
20
10
0
VN
VN
BP-3
IGF-I
B
D
Treatment

FIGURE 5

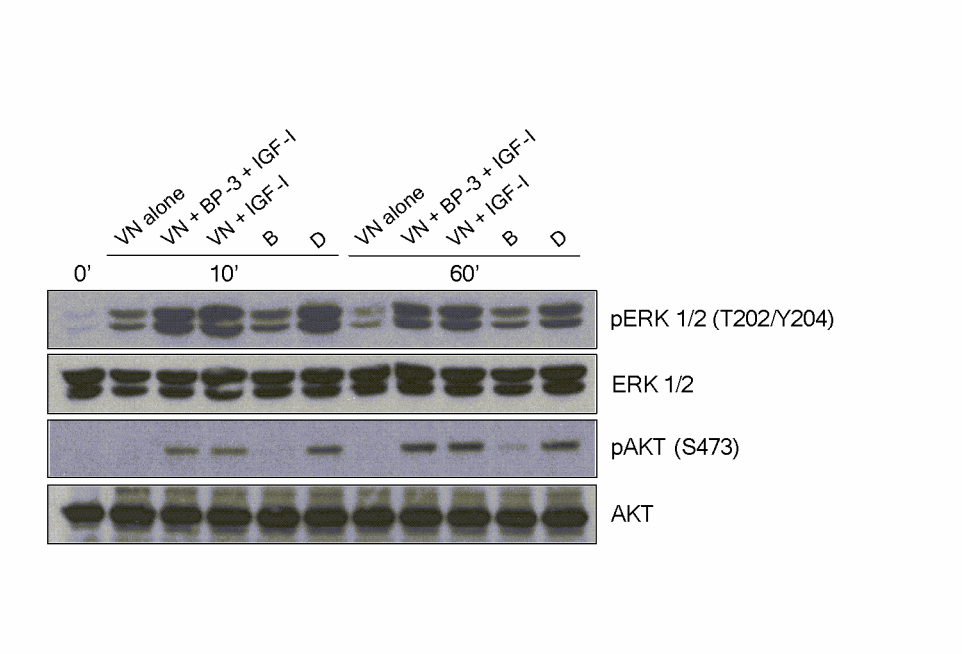
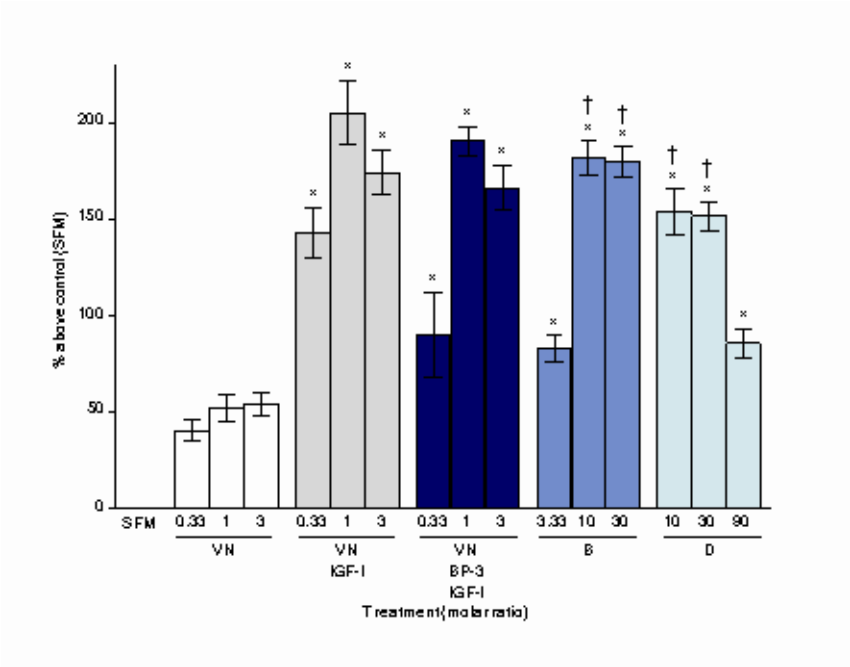

VN alone
VN + BP-3 + IGF-I
VN + IGF-I
B
D
VN alone
VN + BP-3 + IGF-I
VN + IGF-I
B
D
0'
10'
60'
pERK 1/2 (T202/Y204)
ERK 1/2
pAKT (S473)
AKT

FIGURE 6


% above control (SFM)
200
150
100
50
0
SFM
0.33
1
3
0.33
1
3
0.33
1
3
3.33
10
30
10
30
90
VN
VN
IGF-I
VN
BP-3
IGF-I
B
D
Treatment (molar ratio)