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IGFBP-5 enhances epithelial cell adhesion and protects epithelial cells from TGFβ1-induced mesenchymal invasion☆

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ABSTRACT

TGFβ1 is a major fibrotic factor and its actions involve induction of epithelial cell death, together with the stimulation and transdifferentiation of fibroblasts into collagen- and fibronectin-secreting myofibroblasts. These actions of TGFβ1 are also consistent with a pro-metastatic role, by aiding epithelial cell escape through mesenchymal tissues. Recently IGFBP-5 has been described as a pro-fibrotic (pro-metastatic?) agent and the aim of this study was to compare and contrast the actions of IGFBP-5 with TGFβ1. We used NMuMG cells and cloned stable epithelial and mesenchymal lines from the parent cells. TGFβ1 induced apoptosis and/or EMT in the epithelial cells, whereas it enhanced mesenchymal cell survival and migration. IGFBP-5, in contrast, enhanced both cell–cell and cell–ECM adhesion and also improved wound closure in epithelial cells whereas, in mesenchymal cells, IGFBP-5 decreased adhesion and migration. Furthermore, IGFBP-5 was able to antagonise the actions of TGFβ1. In a co-culture model simulating epithelial–mesenchymal boundaries, IGFBP-5 was able to antagonise the disruptive transgressions induced by TGFβ1. Overall, these findings suggest that IGFBP-5 is important in maintaining epithelial–mesenchymal boundaries and thus may limit metastasis and fibrosis by inducing an orderly repair mechanism, very distinct from the fibrotic disruption induced by TGFβ1. A role for IGFBP-5 in the inhibition of metastasis is supported by immunohistochemical studies of breast cancer microarrays, where we show that elevated IGFBP-5 expression is associated with increased disease-free survival.

1. Introduction

Tissue boundaries must be formed and maintained, despite a range of traumatic insults, such as the wound healing process, which can ultimately manifest itself in a variety of chronic fibrotic disorders, where healing remains unresolved (Wynn and Ramalingam, 2012) or metastasis, where epithelial cells cross mesenchymal tissues and endothelial barriers.

The principal agent driving EMT is TGF-β1 (Margadant and Sonnenberg, 2010). TGFβ1 induces apoptosis in epithelial cells but can also induce EMT in the surviving cells. Thus, although TGFβ1 is thought to be protective in the early stages of tumour formation (pro-apoptotic) it is a poor prognostic factor during metastatic disease (pro-EMT) (Roberts and Wakefield, 2003; Siegel and Massague, 2003). By increasing collagen and fibronectin production from fibroblasts and inducing their trans-differentiation into myofibroblasts, TGF-β1 acts to disrupt the epithelial–mesenchymal boundary, generating a fibrotic response which impairs wound healing (Nakerakanti and Trojanowska, 2012). Insulin-like growth factor binding protein-5 (IGFBP-5) is increased in fibrotic disorders (Feghali and Wright, 1999; Zuo et al., 2002) and induces fibrotic responses similar to TGF-β1 (Yasuoka et al., 2006a, 2006b, 2008). However, we believe that IGFBP-5 and TGF-β1 serve very different functions. For example, we have demonstrated that IGFBP-5 increases epithelial cell adhesion to the ECM, whilst simultaneously inhibiting migration by maintaining E-cadherin expression (Sureshbabu et al., 2012). These responses to IGFBP-5 would be anticipated to reduce, rather than increase, metastatic potential and to limit fibrotic responses to the mesenchymal compartment by maintaining an effective epithelial barrier. Paradoxically, increased IGFBP-5 expression has been associated with poor prognosis

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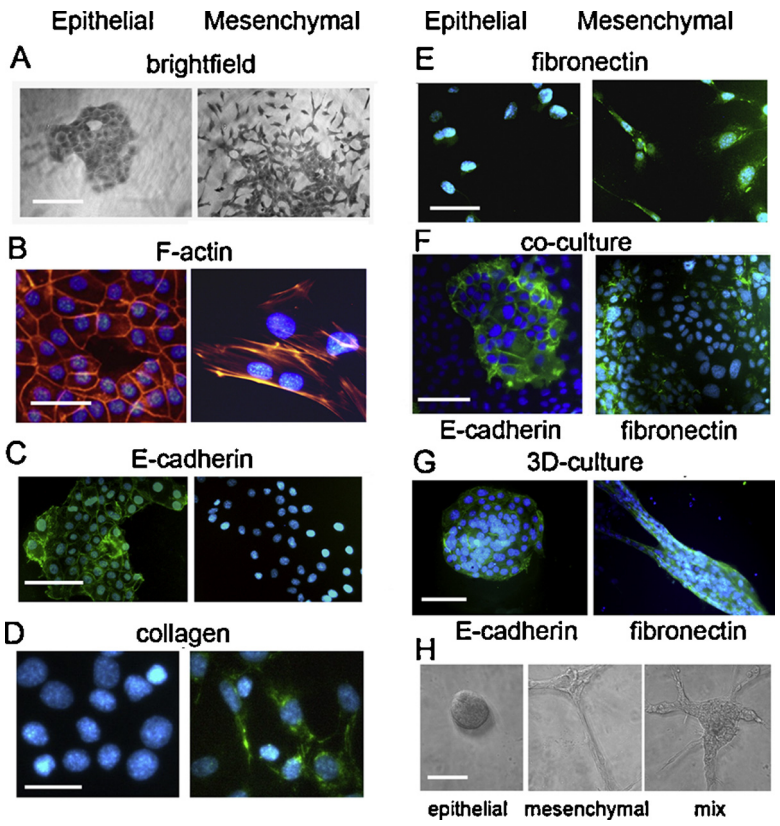

Fig. 1. Characterisation of NMuMG epithelial and mesenchymal clones. Panel A: Bright field images, demonstrating the difference in phenotype of the cells. Bar = 100 μm. Panel B: expression of F-actin, at the plasma membrane in epithelial clones and as stress fibres in the mesenchymal clones. Bar represents 50 μm. Panel C: Epithelial cells exclusively express E-cadherin whereas mesenchymal cells do not. Bar represents 100 μm. Panel D: Mesenchymal cells express collagen whereas epithelial cells do not. Bar represents 25 μm. Panel E: Expression of fibronectin is greatly increased in mesenchymal cells. Bar represents 20 μm. Panel F: When co-cultured, the cells organise into epithelial colonies surrounded by mesenchymal cells. Bar represents 100 μm. Panel G: In 3D culture, epithelial cells form regular spheroids (visualised via e-cadherin expression) whereas mesenchymal cells form elongated structures expressing fibronectin. Finally, when co-cultured in 3D, spheroids are connected to each other by duct-like structures. Bar represents 100 μm.

during metastasis (Hou et al., 2009; Huynh, 1998; McGuire et al., 1994; Mita et al., 2007; Pekonen et al., 1992). Rather than inducing metastasis however, we believe that IGFBP-5 secretion might actually reflect a host response to limit tumour escape.

To test this hypothesis, we compared the actions of TGFβ1 and IGFBP-5, exploring their individual roles in the maintenance of the epithelial–mesenchymal boundary. We took advantage of a normal mouse mammary cell line (NMuMG), where epithelial cells exhibit both apoptosis and EMT in response to TGFβ1. Furthermore, this cell line has previously been used to generate both epithelial and mesenchymal clones (Maeda et al., 2005) which allowed the actions of these growth factors to be examined in different phenotypic states of the same cell line. We describe actions of IGFBP-5 which suggest a role as a natural antagonist of TGF-β1 in the epithelial compartment, which would be anticipated to improve wound healing responses and to limit metastatic escape of epithelial cells. We also investigated if this was clinically relevant by assessing the prognostic significance of IGFBP-5 in a cohort of human breast cancer specimens.

2. Results

2.1. Generation of epithelial and mesenchymal clones of NMuMG cells

Using a limiting-dilution technique, we were able to clone stable lines which exhibited either epithelial or mesenchymal characteristics (Fig. 1). Epithelial clones were easily identifiable by the tight colonies formed, whereas there was an absence of such interaction in the mesenchymal lines (Fig. 1A). In the epithelial clones, F-actin was arranged around the periphery of the cell, whereas it was evident as stress fibres in the mesenchymal clones (Fig. 1B). The differences in phenotype were confirmed by demonstrating E-cadherin expression in the epithelial clones but not the mesenchymal clones (Fig. 1C), whilst the mesenchymal clones exhibited staining for collagen (Fig. 1D) and fibronectin (Fig. 1E), which the epithelial clones did not. When the cells were co-cultured by seeding as a mixture, the cells arranged themselves with epithelial colonies surrounded by mesenchymal cells (Fig. 1F). When cultured on matrigel, to encourage 3D growth, the epithelial clones formed characteristic spheroids, whereas the mesenchymal clones developed structures more closely resembling ductal or tubular structures (Fig. 1G). When mixed, the cells organised themselves into spheroids, linked by mesenchymal duct-like structures (Fig. 1H). Because the mesenchymal cells were apparently derived from the original parent cell and the possibility existed of a mesenchymal-epithelial transition (MET), which could make interpretation difficult in some of our co-culture experiments, some studies were also undertaken with a classic mesenchymal cell, the 3T3 fibroblast, which does not undergo MET. 3T3 cells also exhibited abundant collagen expression (see Fig. 6), which could be used as a reliable marker for their identification in culture.

2.2. Responses of NMuMG cells to TGFβ1, IGF-I and insulin

We undertook some initial studies to characterise the response of both epithelial and mesenchymal clones to TGFβ1 as well

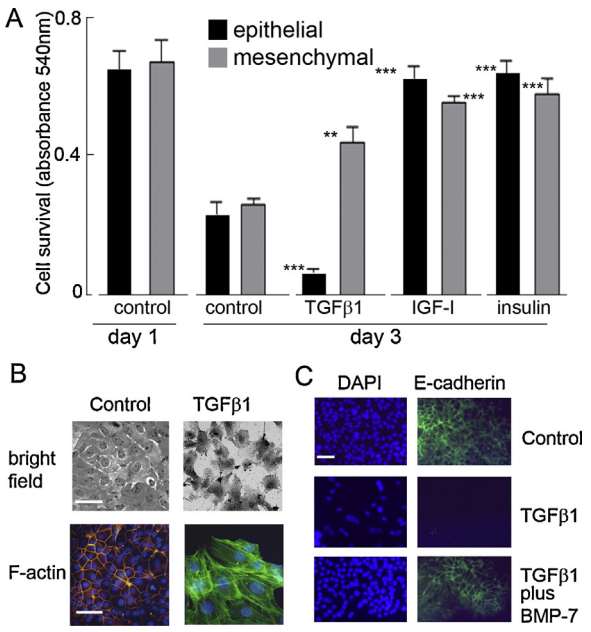

Fig. 2. TGFβ1 induces apoptosis in epithelial cells but survival in mesenchymal cells. IGF-I and insulin support survival in both cell types. Panel A: Cell survival response to growth factors in epithelial and mesenchymal clones of NMuMG cells. Cells were seeded in the presence of 10% serum overnight to allow attachment, after which the cells were washed to remove the serum and cultured in the presence of various growth factors in 2% FCS DMEM for 2 d. Cells were then fixed, stained with crystal violet and absorbance values determined at 540 nm. Values are means ± SEM of 6 observations. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control cells (ANOVA followed by Bonferroni's test for multiple comparisons). Panel B: TGFβ1 induces apoptosis of NMuMG epithelial clones (dying cells evident as dark, shrunken spheroids but EMT is evident in the surviving cells which show little cell–cell interaction). Acting re-arrangement is evident after TGFβ1 treatment with stress fibre formation, which contrasted with the expression of F-actin in the plasma membrane of control cells. Bar represents 25 μm. Panel C: TGFβ1 induces cell death and down-regulation of E-cadherin expression. Epithelial cells were cultured with TGFβ1 with or without BMP-7 for 3 d and then fixed and stained for E-cadherin (green) and DAPI (blue).The effects of TGFβ1 were completely abolished by concurrent treatment with BMP-7, a TGFβ1 inhibitor. Bar represents 50 μm.(For interpretation of the references to color in figure legend, the reader is referred to the web version of the article.)

as to IGF-I, which acts as a major survival factor for these cells. We also used insulin as a surrogate IGF-I because it is incapable of interaction with IGFBP-5 and can thus be used to help distinguish IGF-dependent and IGF-independent effects of IGFBP-5.

In order to examine the effects of growth factors, the cells were cultured in low (2%) serum, sufficient to allow the cells to adhere but limiting their survival and proliferative potential. By day 3, cell numbers were decreased by approximately 50% in control wells of both epithelial and mesenchymal cells. IGF-I and insulin prevented the decrease in cell numbers of epithelial and mesenchymal clones (Fig. 2A). In contrast, although TGFβ1 also inhibited the decrease in cell numbers of mesenchymal clones, it led to an even greater decrease in epithelial cells than in control wells, resulting in an 80% decrease in epithelial cell numbers.

When we examined the actions of TGFβ1 on epithelial clones, it was evident that the surviving cells had undergone a phenotypic epithelial–mesenchymal transition (EMT) (Fig. 2B) which involved down-regulation of E-cadherin expression (Fig. 2C), an effect which could be prevented by the TGFβ1 antagonist, BMP-7.

These results provided us with confidence that these clones would be suitable to examine the actions of IGFBP-5, in order to compare and contrast them with TGFβ1 in epithelial and mesenchymal cell lines.

2.3. Effects of IGFBP-5 on adhesion of epithelial clones

Based upon our previous studies which had demonstrated that IGFBP-5 could enhance adhesion of MCF-7 epithelial cells to both collagen and fibronectin, as well as other provisional matrices (Sureshbabu et al., 2012), we conducted short-term (30 min) experiments in serum-free conditions. IGFBP-5 potently enhanced the adhesion of epithelial clones to the substratum and this was particularly pronounced for collagen and fibronectin, when compared with laminin (Fig. 3A and B).

Epithelial clones survived poorly in serum-free conditions during prolonged (overnight) culture and this was evident from the fact that untreated cells showed little or no interaction with each other or adhesion to the substratum. Metabolic activity, judged by WST-1 activity was also very low (results not shown). In contrast both insulin and IGF-I stimulated considerable cell–cell adhesion, generating metabolically-active spheroids (Fig. 3C). To our surprise, IGFBP-5, despite having initially induced adhesion to the substratum, also induced the formation of spheroids. Quantification of spheroid number and size revealed that both IGF-I and insulin increased spheroid size, whereas IGFBP-5 increased spheroid numbers (Fig. 3D). Intriguingly, when cells were treated with both IGF-I and IGFBP-5 (in approximately equimolar concentrations), neither the number or size of spheroids was increased compared with controls, suggesting that they neutralised each other. This begs the question "Which factor inhibits which?" Support for independent actions of IGF-I and IGFBP-5 on NMuMG epithelial clones came from co-incubation of IGFBP-5 and insulin (which do not interact with each other). This resulted in the formation of even larger spheroids. TGFβ1 completely inhibited the formation of spheroids. Thus, in stark contrast to TGFβ1, IGFBP-5 supported epithelial cell survival, apparently by driving both cell adhesion and cell–cell contact. Furthermore, when epithelial cells were cultured in the presence of serum to allow attachment and survival, IGFBP-5, expressed from an adenoviral construct, was able to partially inhibit the apoptotic effects of TGFβ1 (Fig. 3E). IGFBP-5 could also limit the epithelial–mesenchymal transition induced by TGFβ1. IGFBP-5 had no effect on actin arrangement on its own, with cells showing the characteristic epithelial expression of actin around the plasma membrane (Fig. 3F upper panel). In contrast, TGFβ1 induced re-alignment of actin into stress fibres and led to dramatic elongation and alignment of cells. Although actin re-arrangement was also evident with the combined treatment of TGFβ1 and IGFBP-5, cell alignment and elongation were considerably reduced. However, IGFBP-5 was unable to prevent the down-regulation of E-cadherin induced by TGFβ1 (Fig. 3F lower panel) indicating that it was, at best, a partial antagonist of this action of TGFβ1.

2.4. Actions of IGFBP-5 on adhesion in mesenchymal cells

When IGFBP-5 was used to treat mesenchymal clones of NMuMG cells or 3T3 cells, there was a remarkable decrease in cell adhesion to laminin, fibronectin or collagen (Fig. 3G and H) which was not evident in response to TGFβ1. The initial action of IGFBP-5, however, was to induce mesenchymal cells to migrate towards each other, again forming small spheroids (Fig. 3I – arrowheads). These structures did not, however, survive fixation and washing procedures. Once again, this action was distinct from that of TGFβ1, which induced mesenchymal cell survival, characterised by increased cell numbers, cell elongation and swirling patterns, characteristic of myofibroblast trans-differentiation (Fig. 3I). When TGFβ1 and IGFBP-5 were added together, the effect of IGFBP-5 pre-dominated over the actions of TGFβ1 (Fig. 3J).

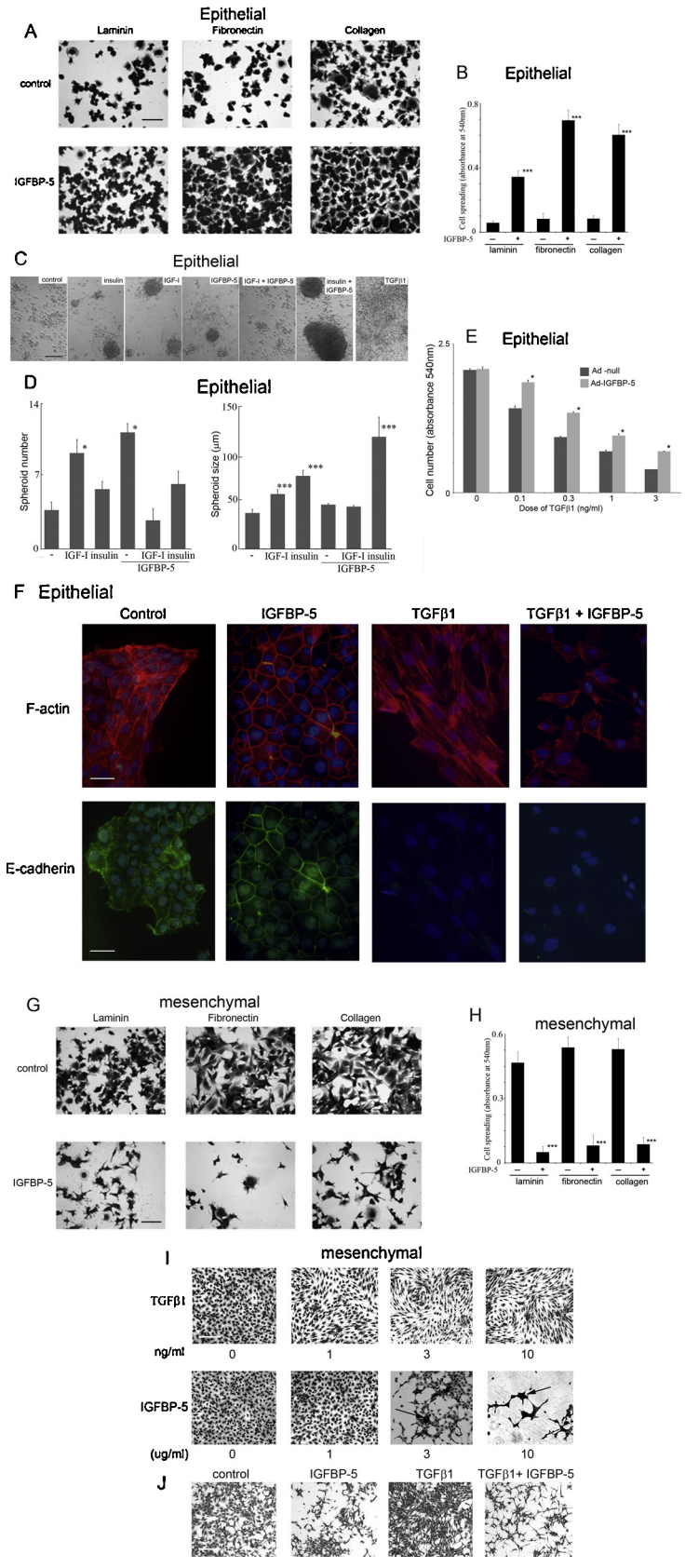

Fig. 3. IGFBP-5 enhances adhesion and survival of epithelial cells but decreases adhesion of mesenchymal cells. Panel A: Treatment with IGFBP-5 increases adhesion of NMuMG epithelial cells. NMuMG cells (in 0.1% BSA DMEM) were seeded into 96-well plates coated with laminin, fibronectin or collagen. After 30 min culture at 37 °C, the plates were inverted, washed and fixed in 4% paraformaldehyde. They were then stained with crystal violet, photographed and absorbances were determined at 540 nm. Bar represents 100 μm. Panel B: Quantification of the results of the study shown in Panel A. Values are means ± SEM of 6 observations. $^{***}p < 0.001$ compared with control cells (ANOVA followed by Bonferroni's test for multiple comparisons). Panel C: NMuMG cells were trypsinised and seeded into 96-well plates at 4×10^5 cells/ml in 0.1% BSA DMEM along with insulin (10 μg/ml), IGF-I (100 ng/ml), IGFBP-5 (10 μg/ml), TGFβ1 (10 ng/ml) or combinations of these. After 24 h culture at 37 °C, cells were photographed under

2.5. Mechanism of action of IGFBP-5

In order to assess the role of intracellular signalling in the actions of IGFBP-5, we utilised a variety of kinase inhibitors. The actions of IGFBP-5 on epithelial spheroid formation were unaffected by any of the classical kinase inhibitors tested (Fig. 4A) . In contrast, the action of TGFβ1 on epithelial cells was, as expected, blocked by the inhibitors of the type I receptors ALK4/5/7, A.8301 and SB431542 (Fig. 4B). TGFβ1 increased phosphorylation of Smad 2/3, whereas IGFBP-5 had no effect, either alone or in combination with TGFβ1 (Fig. 4C) The de-adhesive action of IGFBP-5 on mesenchymal clones was also unaffected by any of the kinase inhibitors tested although the PI3-kinase inhibitor LY294002 also inhibited cell adhesion alone, so no definitive conclusion could be made about PI3-kinase and IGFBP-5 (Fig. 4D and E).

2.6. Effects of IGFBP-5 and TGFβ1 on wound closure

Effects of IGFBP-5 and TGFβ1 on wound closure were examined either alone, or in combination, using ibidi 2-chamber migration inserts. TGFβ1 inhibited epithelial wound closure (Fig. 5A and C), whereas it enhanced wound closure in mesenchymal cells (Fig. 5B–D). In contrast, IGFBP-5 enhanced wound closure of epithelial cells, either alone or in combination with TGFβ1 (Fig. 5A–C), but significantly antagonised the effect of TGFβ1 in mesenchymal clones, although it had no effect alone.

2.7. Effects of IGFBP-5 and TGFβ1 on maintenance of epithelial–mesenchymal boundaries

We next examined the ability of IGFBP-5 and TGFβ1 to influence boundary formation between epithelial NMuMG clones and 3T3 fibroblasts. 3T3 fibroblasts were used to avoid potential MET transformations that might be induced in the mesenchymal NMuMG clones, which would make boundary determination difficult using the markers we had chosen (E-cadherin and collagen). After removal of the inserts in which the 2 cell types were allowed to attach overnight, they were cultured for a further 3 d. Treatment with TGFβ1 induced a clear transgression of 3T3 cells into the epithelial zone following boundary formation (Fig. 6). The activation of fibroblasts was also evident in the mesenchymal compartment, where cellular elongation and increased collagen expression were evident. In contrast, IGFBP-5 induced a more rounded phenotype to the mesenchymal cells and, although they were making abundant collagen, the boundary remained intact. When administered with TGFβ1, IGFBP-5 was again able to inhibit the activation of fibroblasts induced by TGFβ1 and thereby prevent disruption of the boundary between the epithelial and mesenchymal cells.

In order to begin to assess the relevance of these findings to metastasis, we undertook immunohistochemical studies to determine IGFBP-5 expression in breast cancer biopsy tissue microarrays. IGFBP-5 expression was examined in the nucleus, cytoplasm and plasma membrane individually. Whilst no relationships were evident for IGFBP-5 expression in the plasma membrane or cytoplasm, high IGFBP-5 expression in the nucleus was associated with increased disease-free intervals when compared to those with low IGFBP-5 nuclear expression ($p = 0.004$, 93 vs 83 months respectively). In particular, high expression of IGFBP-5 was associated with significant increases in disease-free periods in patients who subsequently received chemotherapy ($p = 0.031$, 87 vs 77 months), but not in those who did not (Fig. 7). Additionally, this relationship between disease-free survival and expression of IGFBP-5 was evident in patients with high levels of apoptosis ($p = 0.002$, 84 vs 100 months in low and high IGFBP-5 expressers respectively) but not in those with low levels of apoptosis.

3. Discussion

NMuMG cells are able to form luminal, polarised structures similar to the normal mammary alveolar structure, (Swamydas et al., 2010). Our studies confirmed previous findings (Maeda et al., 2005) that NMuMG cells can be separated into stable clones with either epithelial or mesenchymal characteristics. We now demonstrate, for the first time, the responses of the mesenchymal clones to various growth factors. We generated three independent clones for both epithelial and mesenchymal cells with almost identical characteristics for clones of the same cell type (although there are subtle differences, for example in the number of epithelial cells killed by TGFβ1). Both epithelial and mesenchymal cells are stable in passage for more than 12 months and we believe that they represent a valuable and novel tool for comparing both EMT and MET in the same cell line.

3.1. IGFBP-5 and epithelial cell adhesion and survival

We recently identified a novel role for IGFBP-5 in the induction of epithelial cell adhesion and spreading which could play an important part in the prevention of metastasis and/or limit fibrosis (Sureshbabu et al., 2012). In this study, we now also demonstrate this effect in a normal mouse mammary gland epithelial cell, as well as describing the ability of IGFBP-5 to antagonise the disruptive actions of TGFβ1. The adhesion of epithelial cells induced by IGFBP-5 was particularly evident in the presence of collagen or fibronectin, components of the mesenchymal matrix but was smaller in magnitude in the presence of laminin, the major component of the epithelial basement membrane. Thus, this action of IGFBP-5 would be exaggerated during exposure of epithelial cells

bright field conditions. Bar represents 100 μm. Panel D: Quantification of spheroid numbers and size from experiment described in Panel C. Spheroids were classified as having a minimum diameter of 25 μm (typically 8–10 cells). Values are means ± SEM of 4 observations. $^{*}p < 0.05$, $^{***}p < 0.001$ compared with control. Data for TGFβ1 are not shown as no spheroids were evident. Panel E: IGFBP-5 antagonises the apoptotic effect of TGFβ1 on epithelial cells. Epithelial cells were infected with either adenovirus expressing IGFBP-5 or the null vector and cultured for 24 h to allow expression of IGFBP-5 to be established. Cells were then trypsinised and 4×10^5 epithelial cells were seeded into 96-well plates in DMEM containing 10% serum. After overnight culture to allow attachment, and to permit IGFBP-5 secretion to become established, TGFβ1 (0.1–3 ng/ml) was added to the wells. After 3 d the cells were fixed and stained with crystal violet and absorbances determined at 540 nm. Values are means ± SEM. $^{*}p < 0.01$ compared with TGFβ1 alone. Panel F: IGFBP-5 reduces the epithelial–mesenchymal rearrangement induced by TGFβ1 in epithelial cells. Epithelial cells were infected with either adenovirus expressing IGFBP-5 or the null vector and cultured for 24 h to allow expression of IGFBP-5 to be established. Cells were then trypsinised and 4×10^5 epithelial cells were seeded into chamber slides in DMEM containing 10% serum. After overnight culture to allow attachment, and to permit IGFBP-5 secretion to become established, TGFβ1 (0.1–3 ng/ml) was added to the wells. After 3 d the cells were fixed and stained with phalloidin to reveal actin filaments in red (upper panel) or with anti-E-cadherin in green (lower panel). Nuclei were stained blue with DAPI. Bar represents 25 μm. Panel G: Treatment with IGFBP-5 decreases adhesion of NMuMG mesenchymal cells. Mesenchymal cells (in 0.1% BSA DMEM) were seeded into 96-well plates coated with laminin, fibronectin or collagen. After 30 min culture at 37 °C, the plates were inverted rinsed and fixed in 4% paraformaldehyde. They were then stained with crystal violet, photographed and absorbances determined at 540 nm. Panel H: Quantification of the results of the study shown in Panel G. Values are means ± SEM of 6 observations. $^{***}p < 0.001$ compared with control cells (ANOVA followed by Bonferroni's test for multiple comparisons). Panel I: TGFβ1 induces activation of mesenchymal cells whereas IGFBP-5 induces de-adhesion, an action which overrides the effect of TGFβ1. TGFβ1 induces a dose-dependent activation of mesenchymal cells, seen as cell elongation and swirling patterns. IGFBP-5 induces a dose-dependent de-adhesion and formation of cell clumps (arrows). Panel J: When TGFβ1 and IGFBP-5 were administered together, the effect of IGFBP-5 predominated. (For interpretation of the references to color in figure legend, the reader is referred to the web version of the article.)

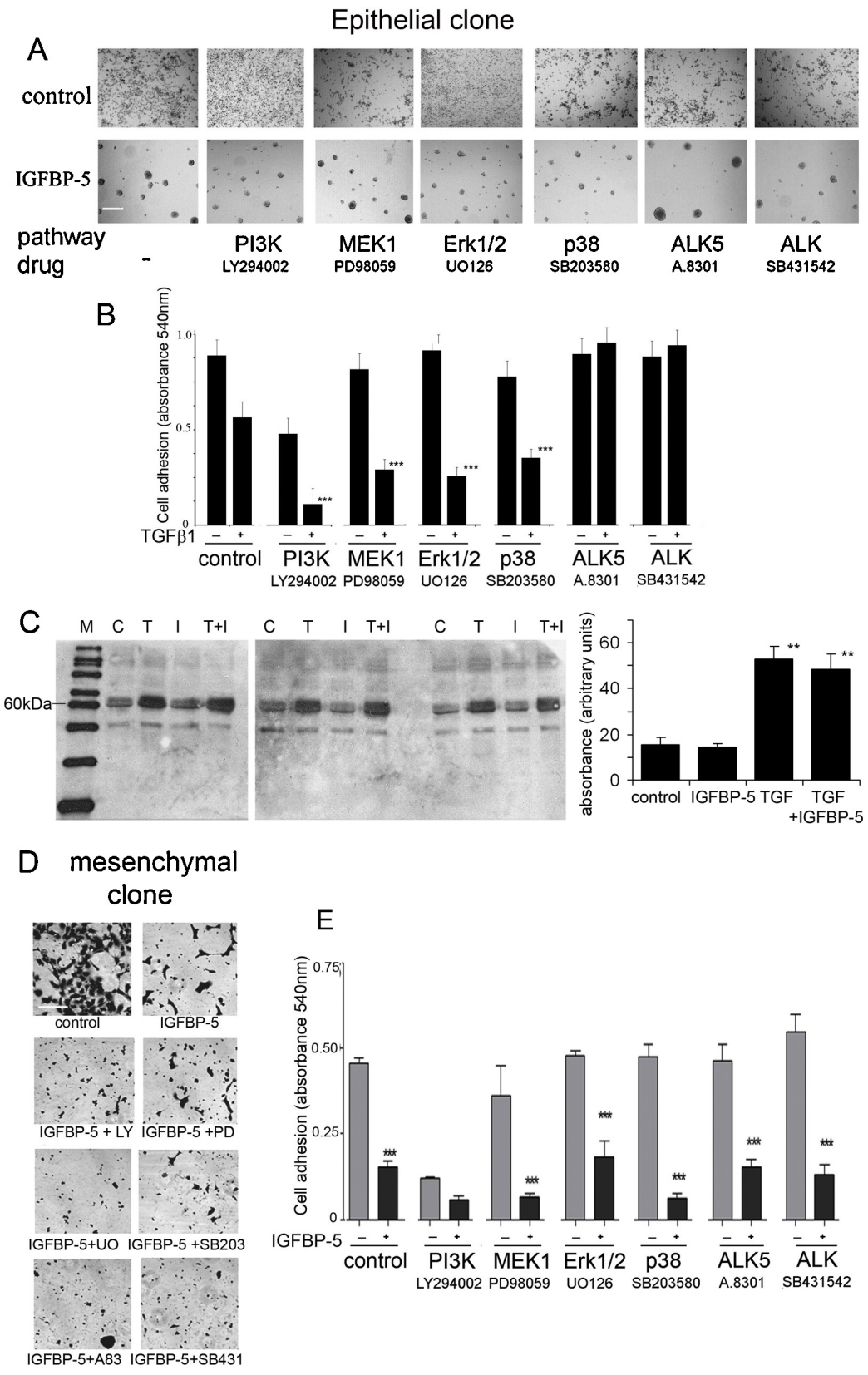

Fig. 4. Panel A: IGFBP-5 induces the formation of epithelial spheroids and its actions are unaffected by various kinase inhibitors. NMuMG epithelial cells were trypsinised and seeded into 96-well plates at 4×10^5 cells/ml in 0.1% BSA DMEM along with various kinase inhibitors (see Section 4 for concentrations) with or without IGFBP-5 (10 μg/ml). Control wells received equivalent concentrations of DMSO. After 24 h culture at 37 °C, cells were photographed under bright-field conditions. Panel B: TGFβ1 induces epithelial cell death and its actions are prevented by inhibitors of the Smad pathway. NMuMG epithelial cells were trypsinised and seeded into 96-well plates at 4×10^5 cells/ml in DMEM containing 10% serum along with various kinase inhibitors (see Section 4 for concentrations) with or without TGFβ1 (10 ng/ml). Control wells received equivalent concentrations of DMSO. After 24 h culture at 37 °C, cells were fixed, stained with crystal violet and absorbances determined at 540 nm. Values are means ± SEM of 6 observations. $^{***}p < 0.001$ compared with respective control wells (ANOVA followed by Bonferroni's test for multiple comparisons). Panel C: TGFβ1 increases phosphorylation of Smad 2/3 in epithelial cells, whereas IGFBP-5 has no effect. NMuMG epithelial cells were seeded into 12-well plates overnight. They were then washed and treated with

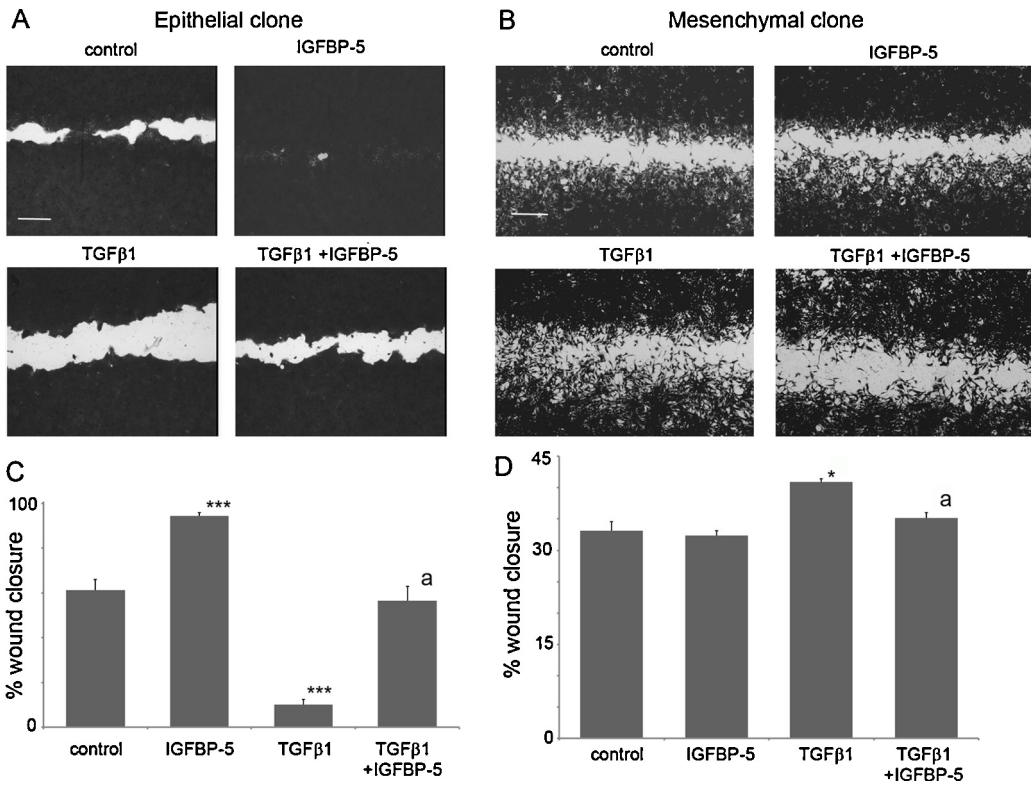

Fig. 5. IGFBP-5 enhances wound closure in NMuMG epithelial cells but inhibits it in NMuMG mesenchymal cells, thereby antagonising the actions of TGFβ1. Panel A: Epithelial cells were seeded into ibidi 2-chamber inserts to produce confluent monolayers overnight. The inserts were then removed and the cells cultured in DMEM containing 10% serum with or without TGFβ1 (2 ng/ml) or IGFBP-5 (10 μg/ml) or a combination of both. Migration was allowed to proceed for 24 h and the cells were then fixed, stained with crystal violet and photographed. Bar represents 250 μm. Panel B: Mesenchymal cells were seeded into ibidi 2-chamber inserts to produce confluent monolayers overnight. The inserts were then removed and the cells cultured in DMEM containing 10% serum with or without TGFβ1 (2 ng/ml) or IGFBP-5 (10 μg/ml) or a combination of both. Migration was allowed to proceed for 24 h and the cells were then fixed, stained with crystal violet and photographed. Bar represents 250 μm. Panels C and D: Quantitative analysis of the studies described in panels A and B, respectively. Values are means ± SEM of 6–8 observations. $^{*}p < 0.05$, $^{***}p < 0.001$ compared with control. $^{a}p < 0.01$ compared with TGFβ1 alone.

to a mesenchymal environment, such as occurs during the tissue disruption evident in wound-healing or metastasis. Similar alterations in the response of both IGFBP-3 and IGFBP-5 in the presence of laminin or fibronectin have been described previously (McCaig et al., 2002; Sureshbabu et al., 2012).

Although IGFBP-5 significantly extended epithelial cell survival, initially by driving cell adhesion to the substratum, it ultimately induced the formation of spheroids where, presumably, the forces involved in cell–cell contact were stronger than the cell-substratum interaction and thus became the major determinant of phenotype. This response of the epithelial cells was similar to the effect of IGFBP-5 in MCF-7 breast cancer cells, where IGFBP-5 induced adhesion to the substratum. and maintained E-cadherin expression and strong cell–cell contact (Sureshbabu et al., 2012). What role might this response play? IGFBP-5 expression is increased in the brain during hypoxia (O'Donnell et al., 2002) in Crohn's disease (Zimmermann et al., 1997) in atherosclerotic plaques and in senescing cells (Kim et al., 2007). Survival in nutrient-deprived/oxygen-depleted conditions is advantageous for tissue repair after injury, when vasculature is compromised and hypoxia is common and thus IGFBP-5 may play an important role in aiding epithelial cell survival and repair at these times (Johnson and Haun, 2009; Sureshbabu et al., 2012).

TGFβ1, in complete contrast to IGFBP-5, induced apoptosis of >80% of cells and induced EMT in the remaining cells, as evidenced by the complete loss of E-cadherin expression and the re-organisation of actin into stress fibres, confirming previous findings (Maeda et al., 2005). Furthermore, when epithelial clones were treated with TGFβ1 in the presence of IGFBP-5 both the apoptotic and EMT effects of TGFβ1 were considerably reduced, though not ablated. Such antagonism of TGFβ1 actions would again support a pro-survival action of IGFBP-5 in the epithelium.

3.2. *Effects of IGFBP-5 on adhesion and survival of mesenchymal cells*

When the actions of IGFBP-5 were examined on mesenchymal NMuMG cells or classical 3T3 fibroblasts, IGFBP-5 induced a striking inhibition of their adhesion to the substratum, in complete contrast to its effects upon epithelial cells. However, this lack of adhesion was somewhat artefactual, since the cells began to form small spheroids, apparently demonstrating a preference for

TGFβ1 with or without IGFBP-5 for 30 min. Cells were harvested and subjected to Western blotting for phospho- Smad 2/3. Representative blots are shown on the left and quantitative analysis using Image J is shown on the right. M, molecular weight markers; C, control; T, TGFβ1; I, IGFBP-5. Values are means ± SEM of 5 observations. $^{**}p < 0.01$ compared with control cells. Panel D: IGFBP-5 induces de-adhesion of NMuMG mesenchymal cells and is unaffected by various kinase inhibitors. NMuMG mesenchymal cells were trypsinised and seeded into 96-well plates at 4×10^5 cells/ml in 0.1% BSA DMEM along with various kinase inhibitors (see Section 4 for concentrations) with or without IGFBP-5 (10 μg/ml). Control wells received equivalent concentrations of DMSO. After 24 h culture at 37 °C, cells were fixed, stained with crystal violet, photographed and absorbances determined at 540 nm. Panel E: Quantitative analysis of data derived from the study shown in Panel D. Values are means ± SEM of 6 observations. $^{***}p < 0.001$ compared with respective control wells (ANOVA followed by Bonferroni's test for multiple comparisons).

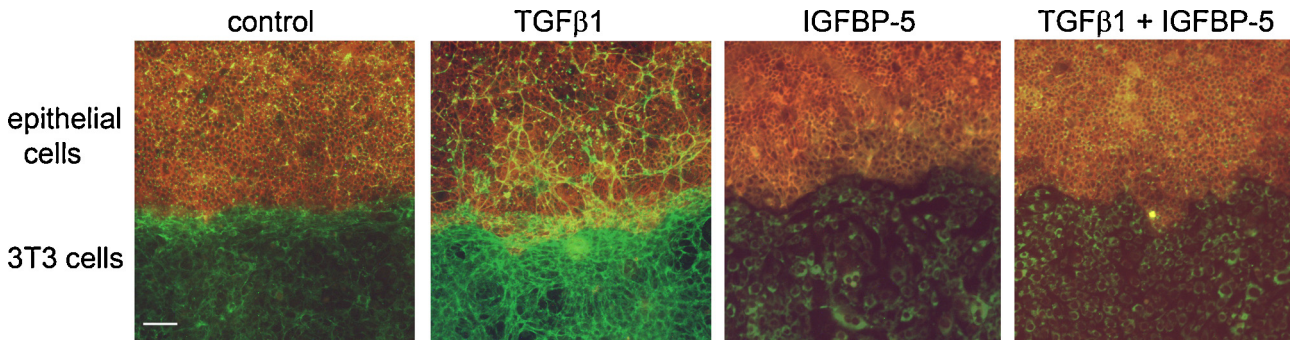

Fig. 6. TGFβ1 induced mesenchymal cell invasion of the epithelial cell layer: inhibition by IGFBP-5. NMuMG epithelial cells were infected with IGFBP-5 adenovirus or null adenovirus and after overnight culture, the cells were trypsinised and 3×10^4 cells were seeded into one chamber of an ibidi 2 chamber insert in a 24-well tissue culture plate. 2×10^4 3T3 cells were seeded into the opposite chamber. After overnight culture, to allow adhesion, the inserts were removed and the cells cultured for a further 3 d in the presence or absence of TGFβ1 (2 ng/ml). Cells were then fixed and stained for E-cadherin (red) or collagen (green). Bar represents 100 μm. (For interpretation of the references to color in figure legend, the reader is referred to the web version of the article.)

cell–cell contact rather than cell substratum interactions (similar to that observed in the epithelial clones). This could be interpreted as a more epithelial characteristic, suggestive of an induction of MET. Whilst we would have liked to investigate this possibility by, for example, examining E-cadherin expression in these mesenchymal clones this proved impossible because the poor adhesion of the spheroids to the substratum meant that any immunohistochemical approach (involving multiple washes) resulted in the loss of all of the spheroids. Once again however, these actions of IGFBP-5 were in complete contrast to those of TGFβ1 and, importantly, IGFBP-5 was able to completely antagonise the actions of TGFβ1 on mesenchymal cells when they were administered together.

3.3. Effects of IGFBP-5 on wound closure

IGFBP-5 enhanced epithelial cell wound closure, whereas TGFβ1 induced the opposite effect and, when administered in combination, IGFBP-5 was able to partially inhibit this action of TGFβ1. In mesenchymal cells (both NMuMG cells and 3T3 cells)

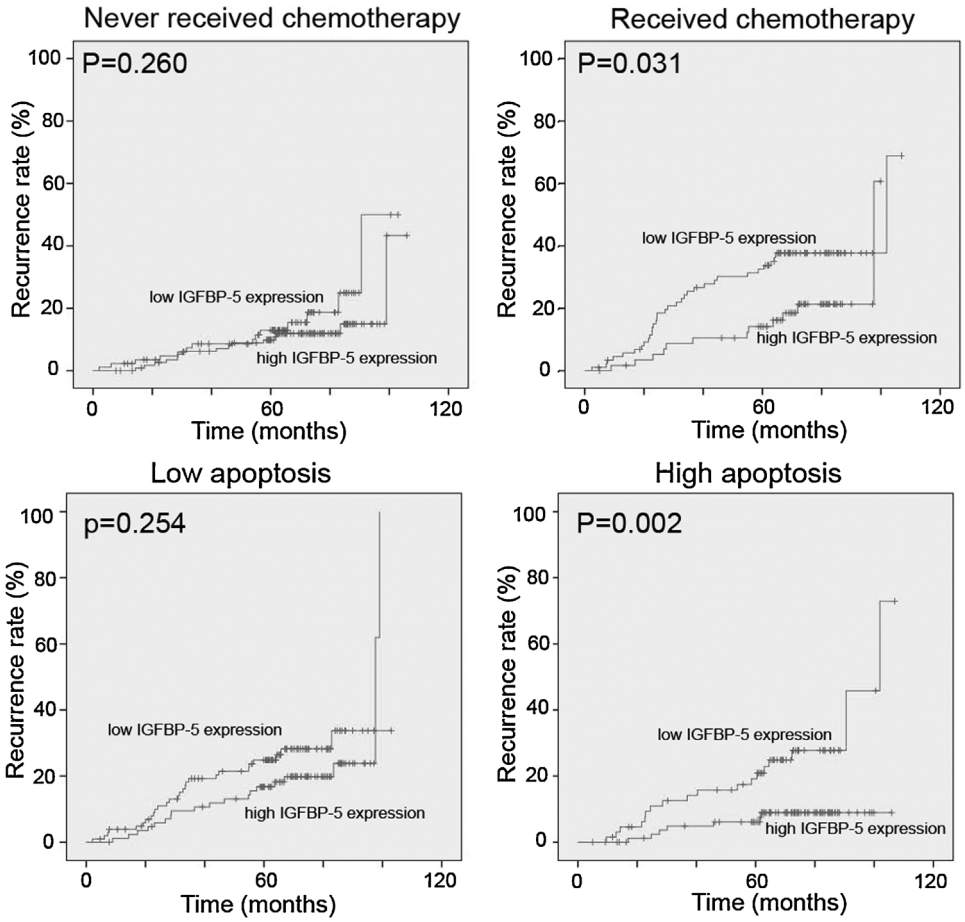

Fig. 7. High expression of IGFBP-5 increase disease-free intervals in patients receiving chemotherapy or with high TUNEL expression. Kaplan–Meier survival plots showing the effect of expression of nuclear IGFBP-5 on recurrence of breast cancer in patients who did or did not receive chemotherapy and in patients with high or low TUNEL expression.

the opposite was evident, where TGFβ1 increased wound closure, whereas IGFBP-5 antagonised this effect.

Taken together, these results suggest that IGFBP-5 can inhibit the pro-fibrotic/pro-metastatic actions of TGFβ1, both in terms of the effects of TGFβ1 on epithelial cells (pro-apoptotic, induction of EMT) and mesenchymal cells (pro-adhesion, pro-migratory). However, there is undoubtedly a pro-fibrotic response to IGFBP-5, based upon a number of published studies (Pilewski et al., 2005; Yasuoka et al., 2006a, 2006b). So, how might these apparently contradictory findings be reconciled? We believe our studies on epithelial–mesenchymal boundaries shed some light on this. IGFBP-5 clearly inhibited the disruptive effects of TGFβ1 on the maintenance of epithelial–mesenchymal boundaries. IGFBP-5 has also been shown to increase the production of laminin-1 a major constituent of the epithelial basement membrane which aids in re-enforcement of the boundary (Abrass and Hansen, 2010). If we take into account the fact that IGFBP-5 is expressed in a number of tissues during injury or remodelling such as hypoxia in the brain, atherosclerotic plaques and involution of the mammary gland (Tonner et al., 2002), then it is conceivable that IGFBP-5 produced by injured/dying epithelial cells, induces spreading/adhesion of neighbouring cells to provisional matrices or mesenchymal environments (to which they would not normally be exposed). This would allow more effective maintenance/repair of the epithelial barrier. At the same time, by promoting collagen and fibronectin production in the mesenchymal compartment, IGFBP-5 would support a "back-up" scar tissue response which would be present until epithelial repair was achieved. IGFBP-5 would, therefore, be able to support a controlled fibrotic response, rather than the massive and typically unresolved fibrotic scarring evident when TGFβ1 is active. Thus, although IGFBP-5 is up-regulated in fibrosis (Feghali and Wright, 1999), this may reflect a positive repair strategy by epithelial tissues.

Precisely how IGFBP-5 acts remains uncertain. Our studies were almost exclusively done in the absence of exogenous IGF-I, although we cannot rule out endogenous production of IGFs by the cells. However, our studies in MCF-7 cells included the use of a mutant form of IGFBP-5 which could not bind to IGFs (Allan et al., 2006) and this was fully active in inducing adhesion and inhibiting migration (Sureshbabu et al., 2012) suggesting that the actions of IGFBP-5 are indeed IGF-independent. We found no evidence that IGFBP-5 could inhibit the actions of TGFβ1 on its receptor or immediate downstream signal via Smad2/3. These results are consistent with a previous study which showed that IGFBP-3 but not IGFBP-5 could influence Smad signalling (Fanayan et al., 2002). Our previous study identified a novel interaction of IGFBP-5 with α2β1 integrins which was required for adhesion and an Akt-induced survival pathway (via integrin- rather than via PI-3 kinase). The fact that none of the classical signalling pathways appeared to have a major role in the adhesive action of IGFBP-5 is consistent with our previous findings, although it is at odds with the activation of MAPK by IGFBP-5 along with stimulation of collagen and fibronectin production in another study (Yasuoka et al., 2009). However, the induction of fibronectin and collagen production by IGFBP-5 supports, rather than inhibits, the actions of TGFβ1 and this suggests that IGFBP-5 functions in at least 2 ways in mesenchymal cells. This mechanism of action resembles that of members of the CCNs, a family of proteins structurally-related to IGFBP-5, which involves interactions of integrins with the extracellular matrix (Leask and Abraham, 2006).

3.4. IGFBP-5 and cancer

So what of the associations of IGFBP-5 with cancer? Early studies identified increased IGFBP-5 expression in tumours and led to the proposal that IGFBP-5 was pro-metastatic (Hou et al., 2009; Huynh, 1998; McGuire et al., 1994; Mita et al., 2007; Pekonen et al., 1992). However, our data, along with other recent reports, suggest that IGFBP-5 is protective. For example, IGFBP-5 expression has recently been shown to be associated with increased survival times in breast cancer patients as well as being responsible for maintaining sensitivity to tamoxifen (Ahn et al., 2010). In addition, overexpression of IGFBP-5 in MCF-7 xenografts inhibited tumour development in mice (Ahn et al., 2010) whilst recent studies have demonstrated a tumour suppressor role for IGFBP-5 in ovarian cancer cells (Rho et al., 2008) in osteosarcoma (Su et al., 2011) and in breast cancer after foetal alcohol exposure (Polanco et al., 2010). An intriguing relationship between IGFBP-5 and tumour dormancy has also been described. Genome-wide transcriptional analysis identified a small cohort of genes, of which IGFBP-5 was one, which were up-regulated in dormant tumours of breast carcinoma, glioblastoma, osteosarcoma, and liposarcoma (Almog et al., 2009; Satchi-Fainaro et al., 2012). Our own observations of increased time to recurrence of breast cancer in individuals with high expression of IGFBP-5 add further weight to an anti-metastatic role for IGFBP-5. The fact that the relationship was evident for nuclear expression of IGFBP-5 is intriguing as IGFBP-5 has a nuclear localisation signal and nuclear actions of IGFBP-5 have been described. Although these remain the subject of debate (Jurgeit et al., 2007) this intra-nuclear role has been reported to be pro-apoptotic (Lee et al., 2004) and thus may be another mechanism by which IGFBP-5 increases survival of breast cancer patients. Why the role of IGFBP-5 should be most evident in those patients who undergo chemotherapy compared with those who do not, also requires explanation. For example, is the action of IGFBP-5 more effective in more aggressive tumours, those which typically require chemotherapy?

In summary, IGFBP-5 plays an important role in the epithelial cell response to injury/insult by inducing adhesion of epithelial cells to the provisional matrix and enhancing epithelial wound closure, in order to maximise the efficiency of barrier repair. At the same time, IGFBP-5 enhances deposition of scar tissue by mesenchymal cells but prevents their transgression into the epithelial compartment, partly by re-enforcement of the epithelial barrier and partly by inducing an MET-like response and decreasing migration potential of mesenchymal cells. As such IGFBP-5 may be an important regulator of the pro-fibrotic and pro-metastatic actions of TGFβ1 and could thus offer alternative routes to the generation of anti-fibrotic and anti-metastatic agents.

4. Materials and methods

4.1. Production of recombinant IGFBP-5

Wild type IGFBP-5 was produced as described previously (Allan et al., 2002; Shand et al., 2003).

4.2. Generation of adenovirus containing IGFBP-5

This was performed exactly as described previously (Sureshbabu et al., 2012)

4.3. Cell culture

NMuMG cells, a mouse mammary epithelial cell line, were obtained from ECACC (Cat No: 94081121). They were cultured in DMEM supplemented with 2 mM glutamine, penicillin (5.0 U/ml) streptomycin (5 mg/ml) insulin (10 μg/ml) and 10% foetal bovine serum. Insulin was omitted from the medium for all experiments in which a response to IGF-I was to be determined.

NIH 3T3 cells (ATCC no: CRL-1658) were cultured in DMEM with 2 mM glutamine, penicillin (5.0 U/ml), streptomycin (5 mg/ml), and 10% FBS.

4.4. Establishment of NMuMG clones

The NMuMG clones were established by limiting dilution of the original parent population of NMuMG cells. Generating two distinct cell types, one (epithelial) in which cell:cell adhesion was strong another (mesenchymal), where the cells showed little interaction.

4.5. Determination of optimum concentrations for adenoviral infection of cells

Replication-deficient adenovirus containing the IGFBP-5 gene was used to infect NMuMG cells with a null adenovirus serving as a negative control.

After trypsinisation, NMuMG cells were washed in serum-free DMEM. The cells were then infected with adenovirus in the wells of a 6-well tissue culture plate pre-coated with 1 μg/ml of collagen to support the adhesion of the NMuMG cells in the absence of serum. Plates were cultured for 3 h, followed by supplementation with 10% FCS (to aid survival), followed by overnight incubation. The following day the cells were trypsinised, washed, counted and added to 24-well plates and cultured at 37 C in 95% air/5% CO_2. Medium from the wells was collected every day, for the determination of the IGFBP-5 concentration by enzyme linked immunosorbent assay (ELISA) to determine appropriate infection levels.

4.6. Enzyme linked immunosorbent assay (ELISA)

ELISA (IGFBP-5 Mouse ELISA Kit, aβ100693,) was performed to determine the concentration of the IGFBP-5 produced from the adenoviral infected NMuMG cells exactly according to the manufacturer's instructions (Abcam, Cambridge UK).

4.7. Cell adhesion

Various concentrations of IGFBP-5, TGFβ1, IGF-I and insulin were prepared in 0.1% BSA: DMEM. The treatments (as described in Section 2) were added to 96 well plates. 3×10^4 NMuMG epithelial clones, mesenchymal clones or 3T3 cells, also in 0.1% BSA: DMEM, were added to triplicate wells. Plates were then cultured for either 30 min or overnight at 37 °C in a 5% CO_2 atmosphere. The cells were then photographed, after which the medium was removed and cells were fixed with 100 μl of 4% (w/v) paraformaldehyde in phosphate buffer saline (PBS) for 20 min at room temperature, After staining with crystal violet, and washing, the absorbance was determined at 540 nm.

4.8. Cell signalling studies: Inhibition assays

To examine the possible intracellular signalling pathways of IGFBP-5 and TGFβ1, various kinase inhibitors were added to 96-well plates containing 3×10^4 cells in DMEM containing 10% serum, in the presence or absence of IGFBP-5 or TGFβ1 and cultured for 24 h, after which cells were fixed and stained with crystal violet. Final concentrations of the inhibitors used were LY-294,002 (5 μM), PD98,059 (25 μM), SB203,580 (1 μM) UO126 (10 μM) A83.01 (10 μM) and SB431542 (10 μM). Control wells contained an equivalent concentration of DMSO.

4.9. WST-1 assay

To determine metabolic activities, at the end of the culture period, WST-1 reagent (5 μl/well) was added to 96-well plates and culture continued for 1 h before the absorbance was read at 450 nm.

4.10. Western immunoblotting

NMuMG cells grown to 80% confluency on 12 well plates were treated with either TGF-β1 (10 ng/ml) alone, IGFBP-5 (10 μg/ml) alone or TGF-β1 plus IGFBP-5 for 30 min before harvesting. Cells were harvested in sample buffer containing (125 mM Tris-Base (pH 6.7), 0.5 mM $Na_4P_2O_7$, 1.25 mM EDTA, 1.25% (v/v) glycerol, 0.5% (w/v) SDS, 25 mM 1,4-dithiothreitol (DTT) and 0.02% (w/v) bromophenol blue) and were subjected to SDS-PAGE and Western blotting as previously described (Alderton et al., 2001). Resolved proteins were Western blotted with anti-phospho-smad 2 (Cell signalling, code 3108P)and visualised using the enhanced chemiluminescence method.

4.11. Immunofluorescence staining

Cells were cultured in 8-well chamber slides. At the end of the culture period, medium was removed from the wells by inverting the chamber slides and cells were fixed with 200 μl of 2% (w/v) paraformaldehyde in PBS for 10 min followed by permeabilization with 200 μl of 0.5% triton X-100 in PBS for 15 min at room temperature. Non-specific staining was diminished by incubating with 10% heat-inactivated serum, derived from the species in which the second antibody was produced. Cells were incubated with 200 μl of mouse anti-E-cadherin (5 μg/ml) (Invitrogen, Cat no: 334000) anti-collagen (1:500) (Abcam, Cat .No: ab88147) or anti-fibronectin (1:500) (Abcam, Cat No: ab23750). This was followed by 1 h incubation with secondary antibody labelled with either Texas red or FITC (1:200). F-actin was detected using either rhodamine- or FITC-phalloidin (Biotium, USA). Slides were covered with antifade DAPI nucleic acid mountant (Molecular Probes, Invitrogen, USA). The slides were visualised with a Nikon TE300 (Nikon, Kingston upon Thames, UK) inverted epifluorescence microscope using ×40 or ×100 objectives with oil immersion and a Hamamatsu CCD camera (Hamamatsu Photonics, Welwyn Garden City, UK) controlled by Metamorph software (Molecular Devices, Palo Alto, CA, USA) or a Leica DMIRB microscope (Leica Microsystems (UK) Ltd Milton Keynes, Bucks, UK) using a ×40 objective (Leica N PLAN 40×/0.55 NA CORR PH2 0–2/C) a ×10 objective (Leica N PLAN 10×/0.25 PH1 -/A 5.8) or a ×5 objective (Leica N PLAN 5×/0.12 PH0 -/A). Images were captured on a Leica DC200 (DMIRB) and analysed using a Leica Q550 Image Analysis Workstation (v2.2.1) combined with Leica Qwin Software for image acquisition.

The images were optimised using Adobe Photoshop. Composite pictures were taken and adjustments in brightness and contrast were applied equally across all images to prevent the introduction of any bias.

4.12. Migration assays

The cell migration assay was carried out in 24-well plates using ibidi 2-chamber inserts (Thistle Scientific Ltd, Glasgow). Chambers were added to the wells and then seeded with, typically 30,000–50,000 cells to achieve a confluent monolayer overnight. Treatments were also added at this time. After overnight culture the inserts were removed to reveal 2 patches of cells separated by a 500 μm gap. Fresh medium containing treatments was added and migration allowed to proceed until stopped by fixing in 4% PFA. Cells were stained with crystal violet and images taken for analysis using ImageJ software.

4.13. Boundary assays

Boundary assays utilised the same ibidi chambers but each chamber received different cells (epithelial in one and mesenchymal in the other). After overnight culture the inserts were removed

and the culture continued for a further 24 h to allow boundary formation to occur. Exogenous treatments were then added and cells cultured for a further 48 h, after which they were fixed and stained for E-cadherin (epithelial marker and collagen (mesenchymal marker).

4.14. Patients and tissue microarray (TMA) construction

A total of 371 patients were included in this study, all presenting with invasive breast cancer between 1995 and 1998 and recruited in the Greater Glasgow area. Available clinico-pathological data for each patient included age, tumour grade, tumour invasive grade, lymph node status, oestrogen receptor (ER) status, HER2 status and what therapy the patient had received. The data was retrieved from the NHS electronic patient notes by a member of the health care team as this was a retrospective study. Recurrence was determined when the patients returned to clinic with symptoms and recurrence was evident, or on a routine follow-up appointment when a recurrence was identified.

TMAs were already available for use in this study. These were constructed using 0.6 mm^2 cancer tissue cores taken from representative areas of tumour from each patient. All blocks were constructed in triplicate containing three individual tumour cores taken from the same embedded tissue sample.

4.15. Immunohistochemistry

Immunohistochemistry was used to assess protein expression in the tissue. TMAs were rehydrated in graded alcohols followed by heat induced antigen retrieval in EDTA buffer pH9 for 5 min in a pressure cooker. Endogenous peroxidase was quenched with 3% hydrogen peroxide for 20 min and non-specific binding of primary antibody was blocked by incubation of TMAs in 5% horse serum in 1× Tris-buffered saline (TBS) for 30 min. Tissue was then incubated in 5 μg/ml IGFBP5 mouse monoclonal antibody (MAB875, R&D systems, USA) overnight at 4 °C. Slides were incubated in Dako EnVision™ secondary antibody (Dako, Denmark) for 30 min and staining was detected using the chromogen 3,3′-diaminobenzidine (DAB) for 5 min. Tissue was then counterstained with haemotoxylin, dehydrated in graded alcohols and xylene and mounted with glass coverslips using DPX.

4.16. Scoring

Protein expression was assessed using the weighted histoscore method (Kirkegaard et al., 2006). This method first grades the intensity of staining from 0 (negative) to 3 (strong) and then multiplies the grade by the percentage of tumour cells within each category. The final histoscore varies between 0 (minimum) and 300 (maximum) and is averaged for the triplicate samples.

Statistical analysis was performed using SPSS version 19.0 for Windows. Univariate outcome analyses were conducted by Kaplan–Meier methods, using the log-rank test to compare outcome between patients grouped according to clinico-pathologic parameters and high/low protein expression. Disease-free survival rates were obtained using Kaplan–Meier method. The log-rank test was used to compare survival curves between subgroups.

4.17. Patient approval

Ethical approval was gained from the local ethics committee. Due to the retrospective nature of the study patient consent was not required.

4.18. Statistical analysis

In vitro study data were analysed using Analysis of Variance followed by Bonferroni's post hoc test.

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