Insulin-Like Growth Factor-Binding Protein 7 Regulates Keratinocyte Proliferation, Differentiation and Apoptosis

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Insulin-like growth factor (IGF)-binding protein 7 (IGFBP7) belongs to the IGFBP superfamily, which is involved in the regulation of IGF and insulin signaling. Recently, a global gene expression study revealed that IGFBP7 is downregulated in the psoriatic epidermis, with UVB phototherapy restoring its expression to normal. In the present study, we confirmed that IGFBP7 expression is decreased in psoriatic lesions. Given the previous data suggesting a role for IGFBP7 in the control of cancer cell growth, we investigated its involvement in the regulation of keratinocyte (KC) proliferation and differentiation, which are abnormal in psoriasis. To model IGFBP7 downregulation *in vitro*, we used IGFBP7-specific small interfering RNA or small hairpin RNA-expressing lentiviral vectors in HaCaT cells or primary human KCs. Downregulation of IGFBP7 was found to markedly enhance KC proliferation in both systems, was associated with a significant decrease in KC susceptibility to tumor necrosis factor-α-induced apoptosis, but did not affect senescence. Downregulation of IGFBP7 was also shown to block expression of genes associated with calcium-induced differentiation of human KCs. Finally, recombinant IGFBP7 was found to inhibit KC proliferation and enhanced their apoptosis. These data position IGFBP7 as a regulator of KC proliferation and differentiation, suggesting a potential role for this protein in the pathophysiology and treatment of hyperproliferative dermatoses such as psoriasis.

Journal of Investigative Dermatology (2010) **130,** 378–387; doi:10.1038/jid.2009.265; published online 27 August 2009

INTRODUCTION

Psoriasis is a chronic inflammatory skin disease affecting ~2% of the general population worldwide (Lowes *et al.*, 2007). The etiology of psoriasis is multifactorial and thought to relate to interactions between environmental and genetic factors. Typical histopathological features in psoriasis include both epidermal (hyperparakeratosis) and immunological (neutrophil microabscesses, dermal mononuclear infiltrate, and blood vessel proliferation) abnormalities (Schon and Boehncke, 2005; Griffiths and Barker, 2007; Lowes *et al.*, 2007). Accordingly, both immunological dysfunction and epidermal defects are seemingly involved in the pathogenesis of the disease, as exemplified by the nature of the candidate genes found to be associated with genetic predisposition to develop the disease (Nair *et al.*, 2006, 2009; Zenz and Wagner, 2006; Griffiths and Barker, 2007; Lowes *et al.*, 2007; Yang *et al.*, 2008; Zenz *et al.*, 2008; Zhang *et al.*, 2009).

Recently, genomic-scale analysis of psoriatic skin revealed marked upregulation of *IGFBP7*, encoding the insulin-like growth factor (IGF)-binding protein 7 (IGFBP7), after phototherapy. IGFBP7 was found by immunohistochemistry to be downregulated in psoriatic skin as compared with normal skin, while UVB phototherapy was found to restore IGFBP7 expression in the epidermis to normal in parallel with clearing of the lesions (Hochberg *et al.*, 2007). These data suggested that IGFBP7 upregulation may either be directly affecting epidermal homeostasis or represent an epiphenomenon of no direct relevance to psoriasis pathogenesis.

Insulin-like growth factor-binding protein 7 belongs to the IGFBP superfamily, a large group of secreted proteins. A total of 16 IGFBP family members have been identified, 6 of which bind IGFs with a high affinity (IGFBP1–6), the other 10 members bind to IGFs with a low affinity (Burger *et al.*, 2005). IGFBP7 (also called IGFBP-rP1 or MAC25) binds IGFs with a low affinity, but in contrast, recognizes insulin with a high affinity, and thereby modifies its metabolism, distribution, and ability to bind to the insulin receptor (Yamanaka *et al.*,

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Abbreviations: Ab, antibody; ERK 1/2, extracellular signal-regulated kinase 1/2; IGF, insulin-like growth factor; IGFBP7, insulin-like growth factor-binding protein 7; Kc, keratinocyte; MTT, 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; rIGFBP7, recombinant IGFBP7; siRNA, small interfering RNA; shRNA, small hairpin RNA

Received 27 March 2009; revised 29 June 2009; accepted 10 July 2009; published online 27 August 2009

1997). IGFBP7 has also IGF/insulin-independent actions. For example, IGFBP7 contains a ''follistatin module'', which enables it to bind to activin (Kato, 2000), a member of the TGF-β (tumor growth factor β) superfamily of growth factors (Tamura *et al.*, 2007). IGFBP7 has been shown to regulate cell proliferation, cell adhesion, cellular senescence, and angiogenesis in different cancer cell lines, and it is therefore considered to function as tumor suppressor gene in numerous cancers (Akaogi *et al.*, 1996; Wilson *et al.*, 2002; Burger *et al.*, 2005; Ruan *et al.*, 2007; Sato *et al.*, 2007). More recently, IGFBP7 has been shown to mediate senescence and apoptosis in melanocytes, and to suppress melanoma growth *in vivo* (Wajapeyee *et al.*, 2008).

Insulin-like growth factor-binding protein 7 is expressed in an ubiquitous fashion (Degeorges *et al.*, 2000; Lopez-Bermejo *et al.*, 2003) and is inactivated by proteolytic processing (Ahmed *et al.*, 2003); in addition, hypermethylation has been reported to also affect its expression in neoplastic tissues (Kanemitsu *et al.*, 2000; Ahmed *et al.*, 2003; Lin *et al.*, 2007). IGFBP7 expression is induced by TGF-β (Burger *et al.*, 2005), glucocorticoids (Pereira *et al.*, 1999), and retinoic acid (Swisshelm *et al.*, 1995). IGFBP7 has been found to be one of several keratinocyte (KC)-specific genes differentially expressed in KCs compared with non-KC cell types (Gazel *et al.*, 2003).

Given the deranged expression of IGFBP7 in psoriasis and its role in cancer cell growth, we studied its involvement in the regulation of KC proliferation and differentiation, which are typically abnormal in psoriasis. Our data suggest that IGFBP7 may indeed regulate these processes in epidermal cells, and as such may constitute an attractive target for therapeutic interventions in common dermatoses.

RESULTS

IGFBP7 expression is decreased in psoriatic skin compared with normal skin

Previous data showed that psoriasis is associated with decreased expression of IGFBP7 (Hochberg *et al.*, 2007). To confirm these data in an independent set of patients, we examined by immunohistochemistry the expression of IGFBP7 protein in a series of psoriatic ($n=13$) and control ($n=13$) biopsies (Figure 1). IGFBP7 was found to be expressed strongly throughout the normal epidermis, whereas its expression was either absent or very weak in psoriatic epidermis (Figure 1). These and earlier data (Hochberg *et al.*, 2007) suggested that IGFBP7 may be involved in the pathogenesis of psoriasis, a disorder characterized by abnormal proliferation and differentiation of epidermal KCs.

IGFBP7 downregulation induces KC proliferation

As psoriasis has been shown to be associated with increased cell proliferation and IGFBP7 has been shown to regulate cell proliferation in cancer cells (Akaogi *et al.*, 1996; Wilson *et al.*, 2002; Burger *et al.*, 2005; Ruan *et al.*, 2007; Sato *et al.*, 2007), we assessed the role for IGFBP7 expression in the regulation of epidermal KC proliferation. We used small interfering RNA (siRNA) and small hairpin RNA (shRNA) to transiently and stably decrease IGFBP7 expression in HaCaT cells, respectively, and used shRNA to transiently decrease IGFBP7 expression in human primary KCs. Downregulation of IGFBP7 was confirmed by quantitative reverse transcriptase PCR (Figure 2a) and immunoblotting of the conditioned media (Figure 2b). To exclude off-target effects of the siRNA and shRNA used, we tested the effect of IGFBP7 downregulation on the levels of expression of other members of the IGFBP family and found no significant changes in their mRNA levels, suggesting that our siRNA and shRNA specifically target IGFBP7 (Supplementary Figure 1S).

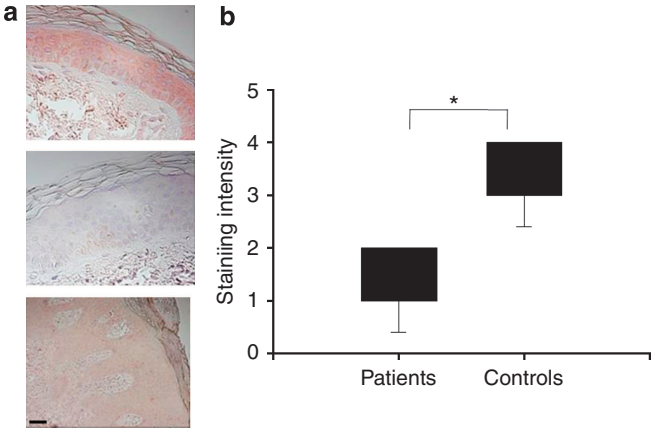

Figure 1. Insulin-like growth factor-binding protein 7 (IGFBP7) protein expression in psoriasis. (**a**) Tissue sections obtained from patients affected with plaque psoriasis ($n=13$; lower panel) or from healthy controls ($n=13$; upper panel) were stained with mouse anti-IGFBP7 and counterstained with hematoxylin. Bar = 40 μm. A control slide stained with non-immune serum is shown in the middle panel; (**b**) Staining intensity was graded from 1–4 by two independent observers. Data are presented as mean staining intensity grade. Bars indicate the group means ± SD (*$P<0.01$).

Downregulation of IGFBP7 in HaCaT cells increased cell viability, as assessed by the MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay (Figure 3a), and cell proliferation rates, as determined by BrDU incorporation (Figure 3b). Concomitant with an increase in cell proliferation, the expression of KRT6, a marker of epidermal proliferation, was upregulated in HaCaT cells (Figure 3c) and primary KCs (Supplementary Figure 3S) downregulated for IGFBP7. We confirmed these data in primary KCs using both the MTT (Figure 3d) and the BrDU incorporation assays (Figure 3e).

Decreased IGFBP7 expression is associated with decreased apoptosis in KCs

Insulin-like growth factor-binding protein 7 has been shown to induce cell apoptosis and senescence in a number of cancer cell lines (Akaogi *et al.*, 1996; Wilson *et al.*, 2002; Burger *et al.*, 2005; Ruan *et al.*, 2007; Sato *et al.*, 2007; Wajapeyee *et al.*, 2008), two phenomena of potential relevance to the pathogenesis of psoriasis (Wrone-Smith *et al.*, 1997). We assessed the effect of IGFBP7 downregulation on apoptotic activity in KCs. HaCaT cells were stably or transiently transfected with either IGFBP7-specific or control shRNA or siRNA, and apoptosis was estimated using the TUNEL and annexin V assays. We found out that

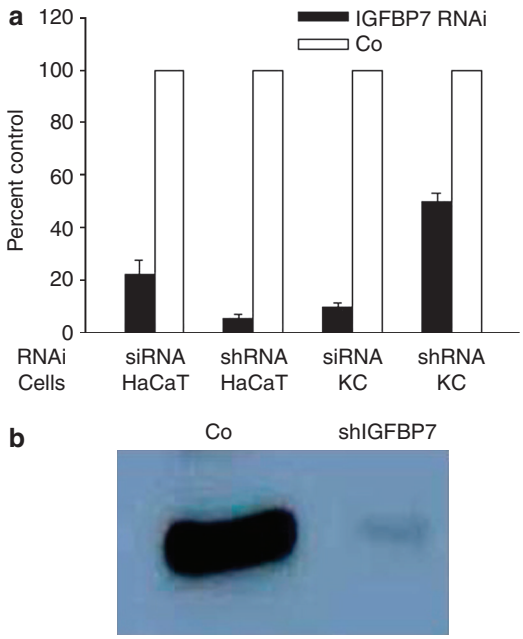

Figure 2. Downregulation of insulin-like growth factor-binding protein 7 (IGFBP7) expression in HaCaT cells and primary human keratinocytes (KCs). (**a**) HaCaT cell lines and primary human KCs were either transfected with small interfering RNA (siRNA) or infected with a lentiviral vector expressing an IGFBP7-specific small hairpin RNA (shRNA). A non-specific siRNA or shRNA served as controls. RNA was extracted after 48 hours of culture. mRNA expression was normalized to *ACTB* or *GAPDH* (not shown). Results are expressed as percent of control. Data shown represent mean values ± SD of three independent experiments performed in duplicates. (**b**) Protein extracts from conditioned media obtained from HaCaT cells stably expressing an IGFBP7-specific shRNA (shIGFBP7) or a non-specific shRNA (Co) were analyzed by immunoblotting and probed using an anti-IGFBP7. Note the significant decrease in IGFBP7 expression in the cells infected with IGFBP7-specific shRNA.

decreased IGFBP7 expression in HaCaT cells lead to a concomitant decrease in apoptotic activity, even in the presence of 10 ng μl^{-1} of recombinant tumor necrosis factor (TNF)-α (Figure 4a and b). Similarly, downregulation of IGFBP7 by IGFBP7-specific siRNA in primary KCs prevented TNF-α-induced cell apoptosis (Figure 4c).

In contrast, we did not observe any effect of IGFBP7 downregulation on senescence rates in human KCs (data not shown).

IGFBP7 is required for calcium-induced expression of genes associated with KC differentiation

To further investigate the role of IGFBP7 in epidermal homeostasis, we induced differentiation of HaCaT cells expressing either IGFBP7-specific or control shRNA in the presence of 1.4 mM Ca^{2+} medium, as previously described (Boukamp *et al.*, 1988). Downregulation of IGFBP7 was found to block the induction of three markers of KC differentiation, KRT10, involucrin (Figure 5a), and loricrin (Supplementary Figure 4S). In parallel, cells downregulated for IGFBP7 failed to show morphological changes, characteristic of calcium-induced differentiation (Supplementary Figure 2S). Similar results were obtained with primary KCs (Figure 5b), suggesting that IGFBP7 may also be involved in the regulation of KC differentiation. To further investigate this possibility at a broader level, we performed a global gene expression analysis to assess the effect of IGFBP7 downregulation on the expression of genes differentially expressed in HaCaT cells cultured under low and high extracellular calcium concentrations (Supplementary Table 2S). We found that IGFBP7 downregulation significantly attenuated the expression of 99.6 and 76.2% of genes displaying more than 2.5-fold change in expression in response to an increase in extracellular calcium concentration in HaCaT cells and primary KCs, respectively. A global pathway gene ontology term analysis of the two data sets (*P*-value <0.01) show that several of the process terms found to be significantly enriched in the analysis were relevant to regulation of proliferation and differentiation (Figure 5c). Taken together, these data suggest that IGFBP7 regulates the expression of genes associated with calcium-induced KC differentiation.

Recombinant IGFBP7 inhibits proliferation and induces apoptosis in human KCs

To further confirm the involvement of IGFBP7 in the regulation of KC proliferation and differentiation, we examined the effect of recombinant IGFBP7 (rIGFBP7) on primary KC cells. Addition of rIGFBP7 resulted in a decrease in viable cell counts in primary human KC cultures, as determined by the MTT assay (Figure 6a). This observation was most probably accounted for by a decrease in cell proliferation, as determined by the BrDU assay (Figure 6a), as well as by an increase in KC apoptosis, as shown in Figure 6b. rIGFBP7 lacked significant effect on cell differentiation (not shown).

Effect of IGFBP7 downregulation on TGF-β and insulin signaling

To investigate the signaling pathway(s) affected by IGFBP7 downregulation, we used HaCaT cells stably downregulated for IGFBP7. IGFBP7 downregulation was found to induce the phosphorylation of insulin receptor-associated IRS-1 (insulin receptor substrate 1) and of the tyrosine kinase, extracellular signal-regulated kinase 1/2 (ERK 1/2), suggesting interference with signaling through the insulin receptor (Figure 7). In contrast, IGFBP7 did not influence SMAD 2/3 phosphorylation status (not shown).

DISCUSSION

Our results as well as that of a previous study (Hochberg *et al.*, 2007) indicate that the expression of IGFBP7 is downregulated in the psoriatic epidermis. Although this mere observation cannot imply in itself a direct causal role for IGFBP7 in the pathogenesis of psoriasis, the fact that IGFBP7 has been shown to function as a tumor suppressor gene in non-cutaneous systems (Swisshelm *et al.*, 1995; Akaogi *et al.*, 1996; Wilson *et al.*, 2002; Mutaguchi *et al.*, 2003; Burger *et al.*, 2005; Ruan *et al.*, 2007; Wajapeyee *et al.*, 2008) suggests that its low expression in psoriasis may contribute to the pathogenesis of the disease. We therefore studied the effect of IGFBP7 on four parameters of potential relevance to

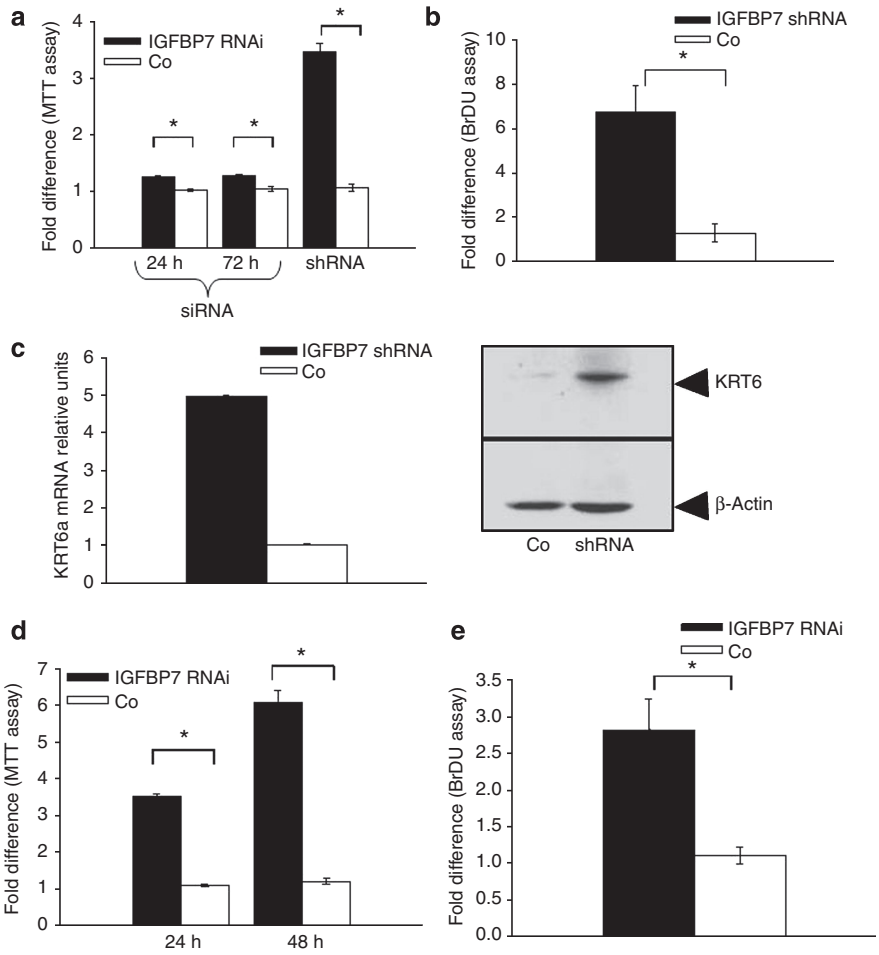

Figure 3. Antiproliferative effect of insulin-like growth factor-binding protein 7 (IGFBP7) on keratinocytes (KCs). (**a**) Cell viability was assessed using the MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay in HaCaT cells downregulated for IGFBP7 using small interfering RNA (siRNA) and cultured for 24 and 72 hours, as well as in HaCaT Cells stably expressing an IGFBP7-specific small hairpin RNA (shRNA) or a control shRNA (Co). Data represent mean values ± SD of three independent experiments. *$P<0.01$ compared with control cells. (**b**) HaCaT Cells stably expressing an IGFBP7-specific shRNA or a control shRNA (Co) were assessed using the BrDu assay (stable transfection). Data represent mean values ± SD of three independent experiments. *$P<0.01$ compared with control cells. (**c**) KRT6a mRNA and protein levels were assessed by quantitative reverse transcriptase PCR (left panel) and immunoblotting (right panel) in HaCaT cells stably expressing an IGFBP7-specific shRNA or a control shRNA (Co). Primary KCs were transiently transfected with IGFBP7-specific siRNA (IGFBP7) or with control siRNA (Co) and assessed using (**d**) the MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay (24 and 48-hours posttransfection) and (**e**) the BrDu assay (24 hours posttransfection). Data represent mean values ± SD of three independent experiments (*$P<0.01$ compared with control cells).

the disease cardinal features: cell proliferation, differentiation, apoptosis, and senescence. We found that low IGFBP7 expression triggered cell proliferation, blocked differentiation, decreased apoptosis, and had no effect on senescence rates both in HaCaT cells and in primary human KCs. Conversely, rIGFBP7 was found to induce cell apoptosis and to decrease cell proliferation.

These data may bear direct relevance to the pathogenesis of psoriasis. Indeed, excessive KC proliferation is characteristic of psoriasis (Krueger and Bowcock, 2005; Lowes *et al.*, 2007); in addition, psoriatic KCs display a number of markers of proliferation, such as KRT6a (McKay and Leigh, 1995), which was also found to be induced by IGFBP7 downregulation in cultured KCs. Conversely, KC differentiation is altered in the psoriatic epidermis (McKay and Leigh, 1995; Krueger and Bowcock, 2005; Lowes *et al.*, 2007), and in KCs expressing decreased IGFBP7 levels, calcium-induced differentiation was similarly blocked. More specifically, IGFBP7 downregulation was associated with attenuation of calcium-induced KRT10 upregulation; similarly, KRT10 expression is low in psoriasis (Bernerd *et al.*, 1992; Bovenschen *et al.*, 2005), whereas various treatment modalities restores KRT10 expression to normal in the psoriatic epidermis (Vissers *et al.*, 2008). Of note, despite the fact IGFBP7 downregulation brought about a decrease in involucrin upregulation during differentiation, involucrin expression has been shown to be upregulated in psoriasis (Haider *et al.*, 2006). IGFBP7 was also found to regulate apoptotic activity in cultured KCs: IGFBP7 downregulation almost completely abrogated TNF-α-induced KC apoptosis, whereas rIGFBP7 significantly

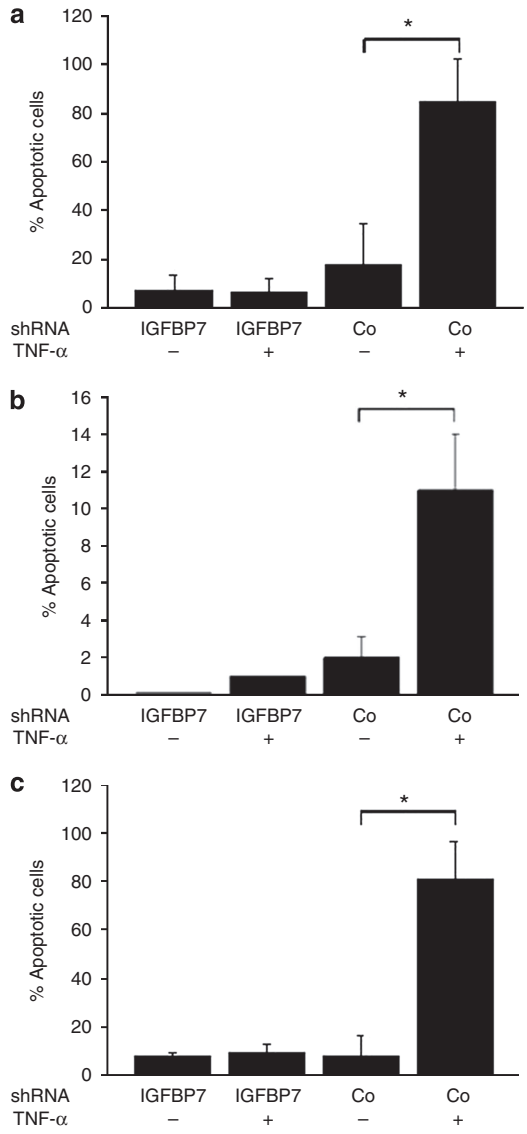

Figure 4. Decreased insulin-like growth factor-binding protein 7 (IGFBP7) expression is associated with decreased apoptosis in human keratinocytes. (**a**) HaCaT cells stably expressing IGFBP7-specific (IGFBP7) or control small hairpin RNA (shRNA) (Co), were exposed to 10 ng μl^{-1} of recombinant TNF-α (+) or vehicle (−) 24 hours after transfection. Apoptosis was measured using the TUNEL assay. (**b**) HaCaT cells stably expressing IGFBP7-specific (IGFBP7) or control shRNA (Co), were exposed to 10 ng μl^{-1} of recombinant TNF-α (+) or vehicle (−) 24 hours after transfection. Apoptosis was measured using the Annexin V assay. (**c**) Primary KCs were transiently transfected with IGFBP7-specific small interfering RNA (siRNA) (IGFBP7) or with scrambled control siRNA (Co) and exposed to 10 ng μl^{-1} of recombinant TNF-α (+) or vehicle (−). Apoptotic activity was assessed by the TUNEL assay. All experiments were repeated thrice. Results are provided as mean values ± SD. Results were considered significant for $*P<0.01$.

augmented the apoptotic response of the cells. Conflicting data on the role of apoptosis in the pathogenesis of psoriasis (Bowen *et al.*, 2004; Gunduz *et al.*, 2006; Yang *et al.*, 2009) may reflect the fact that apoptotic activity significantly changes depending on the stage of evolution of the psoriatic plaque, with markedly decreased apoptotic indices being found in the established lesions and increased apoptosis correlating with regression (Laporte *et al.*, 2000). In fact, most studies lend support to a role for decreased apoptosis in the pathogenesis of psoriasis (Raj *et al.*, 2006). Of note, serum stimulation was found to downregulate IGFBP7 expression, suggesting a possible role for growth factor (for example, EGFR) signaling in the regulation of IGFBP7 expression (Supplementary Figure 5S). In contrast we did not observe any effect of calcium on IGFBP7 expression (not shown).

Insulin-like growth factor-binding protein 7 has been shown to bind and block the activity of activins, members of the TGF-β superfamily of growth factors (Kato, 2000). Activins are thought to have a complex role in skin homeostasis, promoting KC differentiation (Seishima *et al.*, 1996) or epidermal thickening (Munz *et al.*, 1999), and stimulating fibroblast proliferation (Mukhopadhyay *et al.*, 2007), especially in the context of wound healing, a process considered by many to be in some aspects reminiscent of psoriasis (Nickoloff *et al.*, 2006). We did not detect a significant effect of IGFBP7 downregulation on SMAD phosphorylation (not shown). IGFBP7 has also been shown to bind, and thereby modulate, the activity of IGFs and insulin. Interestingly, insulin and IGF-1 signaling pathways have been shown to enhance KC proliferation (Neely *et al.*, 1991; Wertheimer *et al.*, 2001; Sadagurski *et al.*, 2007) and to be involved in skin differentiation (Wertheimer *et al.*, 2000, 2001; Sadagurski *et al.*, 2007). The fact that IGFBP7 is able to block insulin (and to a lesser degree IGF-1) binding to its receptors (Yamanaka *et al.*, 1997) suggests a possible pathway through which IGFBP7 may affect KC differentiation and growth. Accordingly, IGFBP7 downregulation was found to be associated with increased levels of phosphorylated IRS-1 and ERK 1/2, which attests to the activation of the insulin signaling pathway (Genua *et al.*, 2009). Interestingly, increased levels of phosphorylated ERK 1/2 were also observed in lesional psoriatic skin (Yu *et al.*, 2007).

In conclusion, independent of their possible relevance to the pathogenesis of psoriasis (which initially motivated this study), our data position IGFBP7 as a key regulator of KC differentiation and proliferation, and therefore suggest that this protein may represent an attractive target for the treatment of dermatoses associated with KC abnormal proliferation and differentiation.

MATERIALS AND METHODS

Cell cultures

HaCaT cells, a spontaneously immortalized human KC line, were kindly provided by Dr Dina Ron (Technion, Haifa, Israel). The cells were maintained in high-glucose DMEM medium containing 0.075 or 1.4 mM $CaCl_2$ supplemented with 10% fetal calf serum, 1% L-glutamine, and 1% penicillin/streptomycin (Biological Industries, Beit-Haemek, Israel).

Primary human KCs were purchased from CELLnTEC Advanced Cell Systems (Bern, Switzerland). Cells were grown in KC growth medium containing 0.15 mM $CaCl_2$ supplemented with growth factor bullet kit (Lonza, Walkersville, MD). Medium was changed every 2–3 days. Cells were used at passage 3. For differentiation, cells were cultured in KC growth media and 1.4 mM $CaCl_2$.

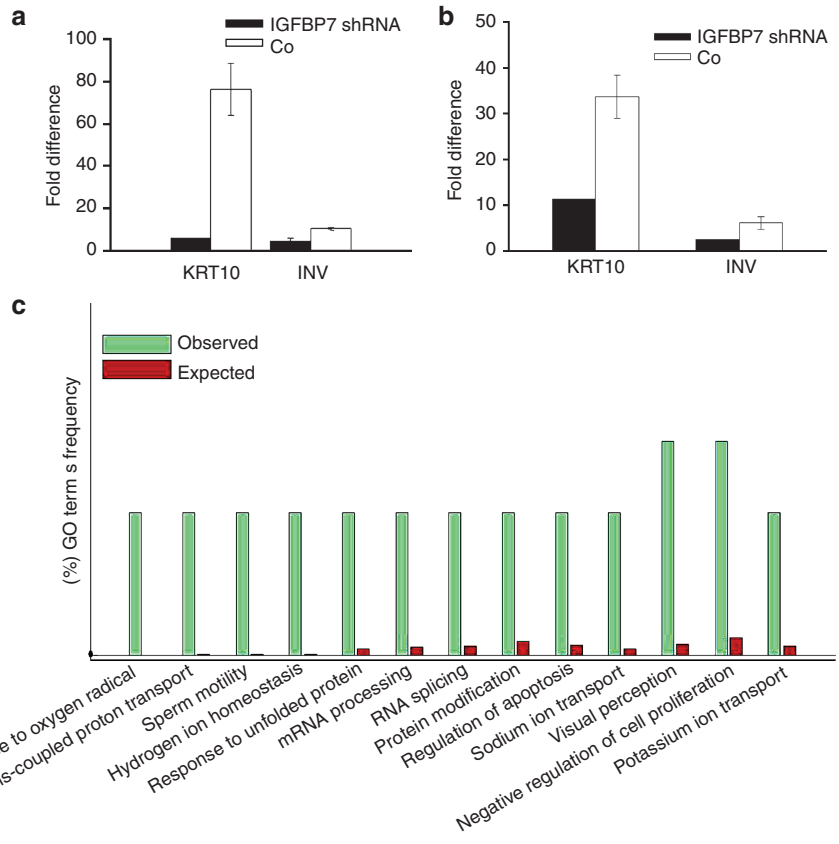

Figure 5. Insulin-like growth factor-binding protein 7 (IGFBP7) is required for calcium-induced keratinocyte (KC) differentiation. (**a**) HaCaT cells stably expressing an IGFBP7-specific or a control small hairpin RNA (shRNA) were induced to differentiate by increasing the extracellular calcium concentration. KRT10 and involucrin (INV) gene expression was assessed as a measure of differentiation-associated events. Data represent mean values ± SD of three independent experiments performed in duplicate. (**b**) Primary human KCs stably expressing an IGFBP7-specific or a control shRNA were induced to differentiate by increasing the extracellular calcium concentration. KRT10 and involucrin (INV) gene expression was assessed as a measure of early and late differentiation-associated events, respectively. Data represent mean values ± SD of three independent experiments performed in duplicate; (**c**) A global process gene ontology (GO) term analysis was performed using the top 100 genes that showed a maximal response (in data sets obtained in primary KCs and in HaCaT cells) to IGFBP7 silencing, after calcium induction. Significant terms (*P*-value <0.01) encompass processes of direct relevance to cell proliferation and differentiation. The green bars correspond to the observed frequency of the GO terms identified as compared with their expected frequency (brown bars).

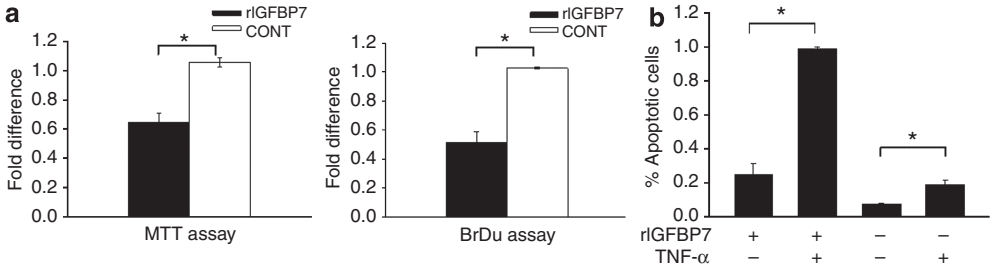

Figure 6. Recombinant insulin-like growth factor-binding protein 7 (IGFBP7) inhibits keratinocyte (KC) proliferation and induces apoptosis in KCs. Primary KCs were treated with 1.8 μg μl^{-1} rIGFBP7 for 72 hours, and then cultured in medium lacking rIGFBP7 for 12 hours. (**a**) Cell viability and proliferation were assessed using the MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (left panel) and BrDU (right panel) assays. (**b**) Apoptosis was quantitated by TUNEL assay. All experiments were repeated thrice. Results are provided as mean values ± SD. Results were considered significant for $*P<0.01$.

Immunohistochemistry

Formaldehyde-fixed 5-μm paraffin-embedded sections were treated with 3% H_2O_2 in methanol for 15 minutes at room temperature, warmed in a microwave oven in citrate buffer for 15 minutes at 90 °C, and stained with mouse monoclonal anti-IGFBP7 antibodies (Abs) (R&D Systems, Minneapolis, MN), anti-keratin 14 Abs (BioGenex, San Ramon, CA), or preimmune rabbit antiserum for 1 hour at room temperature. After extensive washings in phosphate-buffered saline (PBS), the Abs were revealed using the ABC technique (Zymed Laboratories, South San Francisco, CA) and the slides were counterstained with hematoxylin.

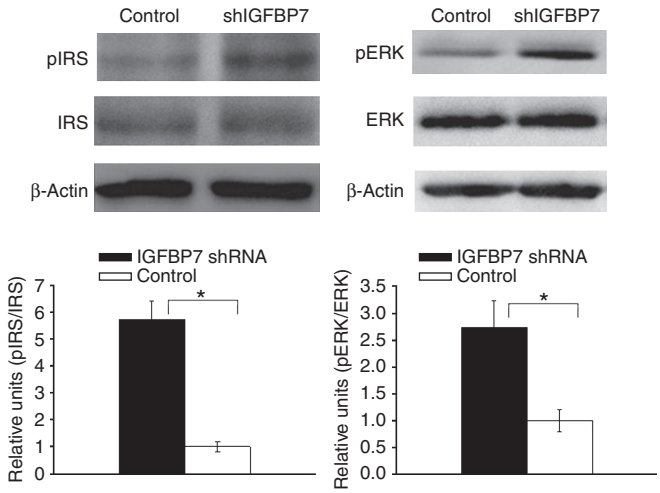

Figure 7. Effect of insulin-like growth factor-binding protein 7 (IGFBP7) down regulation on signaling through the insulin receptor. To assess the effect of IGFBP7 downregulation on signaling through the insulin receptor, protein was extracted from HaCaT cells stably expressing an IGFBP7-specific small hairpin RNA (shRNA) or a control shRNA. Protein extracts were analyzed using immunoblotting with antibodies directed against phosphorylated IRS-1 (p-IRS), IRS-1, phosphorylated ERK 1/2 (p-ERK), ERK2, and β-actin. Band intensity was assessed by densitometry. The experiments were repeated thrice and a graph depicting the mean ± SD is given below each corresponding gel. $*P<0.01$

siRNA transfection

Primary KC cells were cultured in six-well plates at a density of 8×10^4 cells per well before transfection with 66 nmol l^{-1} siRNA duplexes against IGFBP7 or negative control siRNA (Invitrogen, Carlsbad, CA) using Lipofectamine 2000 (Invitrogen). We tested five different siRNA species for downregulation of IGFBP7. The siRNA IGFBP7 duplex that we selected for further use in this study consisted of 5′-rGrCUrGrGUrAUrCUrCrCUrCUrArArGUTT-3′ and 5′-rArCUUrArGrArGrGrArGrGrAUrArCrCrArGrCTT-3′ (Sigma-Proligo, The Woodlands, TX). As a negative control, we used a standard scrambled siRNA purchased from Invitrogen.

shRNA lentiviral transduction

To achieve stable gene downregulation, we used a DNA shRNA-expressing lentiviral vector containing viral packaging signals, regulatory elements, and puromycin resistance gene to package the shRNA sequence into infectious virions (Sigma-Aldrich, St Louis, MO).

We transduced HaCaT cells with shRNA lentiviral particles according to the manufacturer's recommendations. Briefly, 24 hours before transduction, cells were grown in six-well plates up to 1.6×10^4 cells per well. 1–5 μl of viral stock and 2 μl of 4 mg ml^{-1} polybrene were added to the cells and incubated for 18–20 hours at 37 °C in 5% CO_2-humidified incubator. The amount of the viral stock was determined according to desired MOI (multiplicity of infection) (MOI = 5) and total transducing units (TU) per milliliter, supplied by Sigma. The formula for calculation is (total number of cells per well) × (desired MOI) = total TU needed; TU needed/(TU per ml supplied) = total milliliter of lentiviral particles for each well. At 24 hours after transduction, the cells were washed twice in 1 × PBS and maintained in complete growth medium. After expansion in culture for 48 hours, the cells were maintained in growth medium supplemented with puromycin at a final concentration of 4 μg ml^{-1}. Selection was performed in the presence of puromycin for 1 week. Selected clones were frozen in liquid nitrogen before further use.

Primary KCs were transduced according to the same protocol as described for HaCaT cells with slight modifications. At 24 hours after transduction, the cells were washed twice in 1 × PBS and maintained in KC growth medium containing 0.15 mM or 1.4 mM $CaCl_2$. After expansion in culture for 72 hours, the cells were used for *in vitro* assays.

Quantitative reverse transcription PCR

RNA was extracted from the cultured cells using an RNA extraction kit (Roche, Mannheim, Germany). cDNA was synthesized from 500 ng of total RNA using the Reverse-iT first strand synthesis kit (ABgene, Epson, UK) and random hexamers. cDNA PCR amplification was carried out using the SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) on a Mx3000p/5p multifilter system (Stratagene, Cedar Creek, TX) with gene-specific intron-crossing oligonucleotide pairs listed in Supplementary Table S1. To ensure the specificity of the reaction conditions, at the end of the individual runs, the melting temperature (Tm) of the amplified products was measured to confirm its homogeneity. Cycling conditions were as follows: 95 °C for 10 minutes, 95 °C for 10 seconds, 62 °C for 15 seconds, and 72 °C for 25 seconds for a total of 40 cycles. Each sample was analyzed in triplicate. For quantification, standard curves were obtained using serially diluted cDNA amplified in the same real-time PCR run. Results were normalized to *ACTB* and *GAPDH* mRNA levels. After the quantification procedure, the products were resolved by 2.5% agarose gel electrophoresis to confirm that the reaction had amplified DNA fragments of expected size.

Microarray hybridization and data analysis

Total RNA (200 ng) was reverse transcribed and cRNA prepared using TotalPrep RNA Amplification Kit (Applied Biosystems/Ambion, Austin, TX) according to the manufacturer's protocol. 1.5 μg of biotinylated cRNA was hybridized to Sentrix Human WG-6 v2 array (encompassing 48,701 transcript targets), washed, and scanned on a BeadArray Reader (Illumina, San Diego, CA). The scanning data were exported to MatLab sofware (MathWorks, Natick, MA), quantile normalized, and transcripts with detection *P*-value >0.01 were removed from the analysis (more than 13,000 transcripts had a *P*-value <0.01). In the global gene ontology term analysis, we tested all gene ontology terms that were present in our gene set more than once. The gene set was composed of the top 100 genes whose calcium-induced up- or downregulation was most markedly affected by IGFBP7 silencing. For each term, we randomly selected the same number of genes as in our gene set and calculated the number of times it appeared in this set. We repeated the process 100 times and built a histogram of this gene ontology term frequency. The results were analyzed using the one-sample Wilcoxon signed-ranks test to assess relative enrichment in our experimental gene set.

Western blotting

Cells were homogenized in Cellytic MT lysis/extraction reagent (Sigma-Aldrich) and protease inhibitors mix, including 1 mM phenylmethanesulphonylfluoride, and 1 mg ml^{-1} aprotinin and

leupeptin (Sigma-Aldrich). Following centrifugation at 10,000 × *g* for 10 minutes at 4 °C, proteins were electrophoresed through a 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Trans-Blot, Bio-Rad, Hercules, CA). After blocking for 1 hour using 1 × Tris-buffered saline (20 mM Tris, 150 mM NaCl) with 3% BSA and 0.01% Tween 20, blots were incubated with primary Abs. The primary Abs included Abs to p-IRS-1, IRS-1, p-ERK 1/2, ERK 2, SMAD 2/3, p-SMAD 2/3 (Santa Cruz Biotechnology, Santa Cruz, CA); IGFBP7 (R&D Systems), and cytokeratin 6 (Abcam, Cambridge, MA). The blots were washed thrice with Tris-buffered saline–Tween 20 (20 mM Tris HCl, 4 mM Tris base, 140 mM NaCl, 1 mM EDTA, and 0.1% Tween 20). After incubation with secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit Ab (Sigma-Aldrich), and subsequent washings, proteins were detected using the EZ-ECL chemiluminescence detection kit (Biological Industries). To compare the amount of protein in different samples, the blots were re-probed using a mouse mAb to β-actin (Abcam, Cambridge, UK) and secondary horseradish peroxidase-conjugated anti-mouse Ab (Sigma-Aldrich).

MTT assay

The MTT test is based on the selective ability of living cells to reduce the yellow salt MTT (Sigma-Aldrich) to a purple-blue insoluble formazan precipitate. MTT was dissolved in PBS at 5 mg ml^{-1} and added to each well (10% of total volume) for 30-minute incubation at 37 °C. After incubation, the media was removed and the purple formazan product dissolved in DMSO. The supernatants were collected and then scanned with an ELISA reader Zenyth 200 (Anthos Labtec, Cambridge, UK) at 560 nm.

BrDu assay

The incorporation rate of BrdU was determined by Cell Proliferation ELISA BrdU colorimetric kit (Roche) according to the manufacturer's protocol. Absorbance at 450 nm was measured using an ELISA reader. Briefly, cells were cultured in six-well plates and incubated with BrdU for 6 hours at 37 °C. Then the cells were fixed and the DNA was denatured by adding FixDenat solution (Roche Applied Science, Penzberg, Germany). The anti-BrdU peroxidase conjugated Ab was added for 90 minutes at room temperature and the cells were rinsed. Immune complexes were detected by adding substrate solution at an absorbance of 450 nm using ELISA reader Zenyth 200 (Anthos Labtec).

TUNEL assay

Apoptosis was assessed using the TUNEL kit (Roche) according to the manufacturer's protocol. Briefly, cells were plated on cover slips with or without the addition of 10 ng ml^{-1} of TNF-α (PeproTech, Rocky Hill, NJ) for 12 hours, air-dried, and fixed with a freshly prepared fixation solution (4% paraformaldehyde in PBS) and then rinsed twice with PBS. Cells were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate solution. Cell samples were incubated in a humidified atmosphere for 1 hour at 37 °C in the dark and in the presence of the TUNEL reaction mixture, and counterstained with DAPI (4′,6-diamidino-2-phenylindole). More than 1,000 cells were counted for each slide and examined under a fluorescent microscope Zeiss Axioscope 2 (Carl Zeiss MicroImaging, Thornwood, NY). Image analysis was performed with Image-Pro Plus 5 software (Media Cybernetics, Bethesda, MD). Differences in apoptotic activity were considered significant at *P*-values <0.01 calculated using a standard student's *t*-test.

Annexin V assay

Annexin V assay was performed using ApoAlert Annexin V Apoptosis Kit (Clontech Laboratories, Mountain View, CA) according to the manufacturer's protocol. Briefly, cells were plated on cover slips with or without the addition of 10 ng ml^{-1} of TNF-α (PeproTech) for 12 hours, rinsed with the supplied binding buffer and incubated with Annexin V and propidium iodide at room temperature for 15 minutes in the dark. Hoechst stain was used to counterstain the cells. More than 1,000 cells were counted for each slide and examined under a fluorescent microscope (Carl Zeiss MicroImaging). Image analysis was performed with Image-Pro Plus 5 software. Differences in apoptotic activity were considered significant at *P*-values <0.01 calculated using a standard student's *t*-test.

Senescence-associated β-galactosidase assay

Cells were seeded 48 hours before staining at a concentration of 2 to 4×10^4 cells per well in six-well plates. This cell density ensures that the staining is performed before the cultures reach confluency. SA-β-Gal (senescence-associated β-Gal) staining was performed, as previously described, with minor modifications (Dimri *et al.*, 1995). Briefly, the cells were washed with cold PBS and fixed for 5 minutes with 0.5% glutaraldehyde diluted in cold PBS. After fixation, cells were washed in PBS and incubated for 8 hours at 37 °C in staining solution containing 1 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) (Roche) and the rest of the components as previously described (Dimri *et al.*, 1995). For staining at different pH values, 0.1 M citric acid and 0.2 M Na_2HPO_4 solutions were mixed at appropriate proportions. After the incubation period at 37 °C, cells were washed thrice with cold PBS and stored in PBS at 4 °C until images were collected.

Quantitative analysis of the images was performed using a Matlab application for cell marking (SegmentGui) and color analysis (http://md.technion.ac.il/pictures/storage/45/47.zip). For each measurement, a minimum of 250 randomly chosen cells were marked manually. Kolmogorov–Smirnov test was used for statistical analysis. Differences below *P*-value of 0.05 were considered significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We wish to thank Dr Karl Scorezki and Dr Liran Shlush for their help with the performance of the senescence assay and Dr Dina Ron for the gift of the HaCaT cell line. This study was supported, in part, by grants provided by the Wolfson Family Charitable Trust (UK); The Galil Center for Telemedicine, Medical Informatics & Personalized Medicine; the Rappaport Institute for Research in the Medical Sciences, the VPR fund of the Technion-Israel Institute of Technology, Haifa, Israel, and the Israel Science Foundation.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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