

ORIGINAL PAPERS

The fibroblast growth factor binding protein is a novel interaction partner of FGF-7, FGF-10 and FGF-22 and regulates FGF activity: implications for epithelial repairHans-Dietmar Beer¹, Michaela Bittner², Gisela Niklaus¹, Christine Munding¹, Nicole Max², Andreas Goppelt² and Sabine Werner^{*1}¹Department of Biology, Institute of Cell Biology, ETH Zürich, Hönggerberg, CH-8093 Zürich, Switzerland; ²Switch Biotech AG, Floriansbogen 2-4, D-82061 Neuried, Germany

The fibroblast growth factor-binding protein (FGF-BP) binds and activates FGF-1 and FGF-2, thereby contributing to tumor angiogenesis. In this study, we identified novel binding partners of FGF-BP, and we provide evidence for a role of this protein in epithelial repair processes. We show that expression of FGF-BP increases after injury to murine and human skin, in particular in keratinocytes. This upregulation is most likely achieved by major keratinocyte mitogens present at the wound site. Most importantly, we demonstrate that FGF-BP interacts with FGF-7, FGF-10, and with the recently identified FGF-22, and enhances the activity of low concentrations of ligand. Due to the important functions of FGF-7 and FGF-10 for repair of injured epithelia, our findings suggest that upregulation of FGF-BP expression after injury stimulates FGF activity at the wound site, thus enhancing the process of epithelial repair.

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Introduction

Fibroblast growth factors (FGFs) comprise a group of structurally related polypeptide mitogens, which, in vertebrates, consists of 22 members (Ornitz, 2000; Powers *et al.*, 2000). They have a high affinity to heparin and heparan sulfate proteoglycans and require heparan sulfate to activate one of the four transmembrane tyrosine kinase receptors, which mediate their biological effects (Ornitz and Itoh, 2001). These receptors (FGFR1–FGFR4) are characterized by the presence of two to three immunoglobulin-like domains (Ig-domains), a stretch of acidic amino acids between Ig-domains I and II in the extracellular region, and a cytoplasmic tyrosine kinase domain (Johnson and

Williams, 1993). Additional diversity is generated by alternative splicing in the extracellular domains of FGFR1–FGFR3. This splicing event dramatically affects their ligand-binding specificities (Ornitz, 2000). For example, two isoforms of FGFR2 have been identified, FGFR2-IIIb and FGFR2-IIIc, which differ in the second half of the third Ig-like domain (Miki *et al.*, 1992). The IIIb variant is restricted to epithelial cell lineages and binds FGF-1, FGF-3, FGF-7, FGF-10 (Orr-Urtreger *et al.*, 1993; Ornitz *et al.*, 1996; Igarashi *et al.*, 1998), and possibly the highly homologous FGF-22, while the IIIc isoform is expressed by mesenchymal cells and by other nonepithelial cells and is a receptor for FGF-1, FGF-2, FGF-4, FGF-6, and FGF-9 (Orr-Urtreger *et al.*, 1993; Ornitz *et al.*, 1996).

In addition to the transmembrane signaling receptors, a secreted protein has been identified, which binds FGF-1 and FGF-2 (Wu *et al.*, 1991). This protein was designated heparin-binding protein 17 (Hbp17) due to its molecular weight of 17 kDa and its affinity to heparin (Wu *et al.*, 1991). It results from proteolytic processing in the carboxy-terminal half of a larger protein (Wu *et al.*, 1991), designated FGF-binding protein (FGF-BP) (Czubayko *et al.*, 1994). In follow-up studies, FGF-BP was found to mobilize FGF-2 from the extracellular matrix and to enhance the biological activities of FGF-1 and FGF-2 (Aigner *et al.*, 2001; Tassi *et al.*, 2001). This appears to be of particular importance in epithelial tumor tissues, since FGF-BP is highly expressed in different carcinoma cells (Okamoto *et al.*, 1996; Kurtz *et al.*, 1997; Sauter *et al.*, 2001; Aigner *et al.*, 2002a). Most interestingly, FGF-BP was shown to stimulate both tumor and endothelial cell proliferation through FGF-2 release (Aigner *et al.*, 2001) and to serve as the angiogenic switch in human cancer (Czubayko *et al.*, 1997). However, it is as yet unclear if FGF-BP is also important in normal tissues and under benign hyperproliferative conditions such as tissue repair, and if it binds other FGFs in addition to FGF-1 and FGF-2. Here we show that FGF-BP is expressed in normal skin and strongly upregulated after skin injury. The hyperproliferative wound epidermis was identified as the major site of expression of FGF-BP in the wound. We demonstrate that FGF-BP interacts with FGF-7 and

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FGF-10, which are crucial mediators of epithelial cell growth in normal, wounded and diseased tissues and organs (Steiling and Werner, 2003). Furthermore, it was shown to bind the recently identified FGF-22, which is also expressed and upregulated in wounded skin (Beyer *et al.*, 2003). Finally, FGF-BP stimulated the activity of low concentrations of FGF-7, indicating that over-expression of FGF-BP after injury may enhance FGF activity at the wound site.

Results

Enhanced expression of FGF-BP in wounded skin

Recent studies demonstrated a strong expression of FGF-BP in normal skin and in particular in skin cancer (Kurtz *et al.*, 1997; Sauter *et al.*, 2001; Aigner *et al.*, 2000, 2002b). This finding suggested that FGF-BP might also play a role in wound repair. To address this question, we isolated RNA from control skin and from full-thickness excisional wounds of female Balb/c mice at different time points after injury and analysed the samples for the presence of FGF-BP mRNA by RNase protection assay. Highest levels were observed between 12 h and 3 days after wounding (Figure 1a). This result was confirmed for human wound healing as determined by real-time reverse transcriptase–polymerase chain reaction (RT–PCR) with RNAs from healthy adult volunteers (Figure 1b). In this case, an ~3-fold increase was found between 1 h and 24 h post-wounding. The levels subsequently declined and returned to the levels seen in normal skin at day 15 after injury when the wound was fully healed.

To localize FGF-BP expression in the skin, sections from nonwounded human skin (Figure 1c, d) and from

5-day full-thickness excisional skin wounds (Figure 1g, h) were stained with an antibody against FGF-BP. This protein was exclusively detected in keratinocytes throughout the normal epidermis (Figure 1c). A similar staining pattern was previously observed in human skin using a monoclonal antibody against FGF-BP (Mongiati *et al.*, 2001). Most interestingly, our FGF-BP antibody also stained predominantly the epidermis of wounded skin (Figure 1g). Particularly high levels were found in cells of the cornified layer. In addition, patches of strongly positive cells were seen in several areas of the

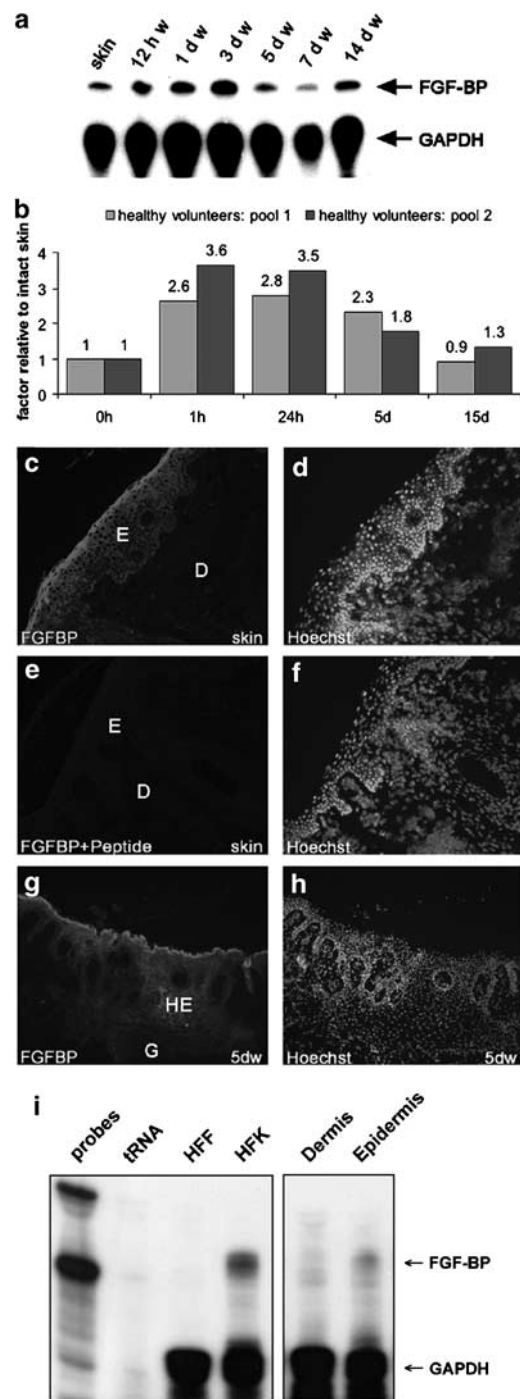


Figure 1 Expression of FGF-BP in murine and human wounds. (a) RNA was isolated from full-thickness excisional wounds of BALB/c mice at different time points after injury as indicated. 20 μ g total cellular RNA from nonwounded skin (skin) and from wounds at different stages were analysed by RNase protection assay for the expression of *fgf-bp*. Hybridization of the same samples with a *gapdh* riboprobe served as a loading control. (b) RNA was isolated from two pools of six biopsies each of normal skin and 1 h, 24 h, 5 days and 15 days full-thickness excisional wounds of healthy adult volunteers and analysed by real-time RT–PCR for the presence of FGF-BP mRNA. The levels expressed in normal skin (normalized to cyclophilin A) were arbitrarily set as 1. (c–h) Frozen sections from normal human skin (c–f) and from 5-day full-thickness excisional wounds (g, h) were stained with an antibody against FGF-BP (c, g) or with the same antibody that had been pretreated with the immunization peptide (e). Counterstaining with Hoechst was performed to visualize cell nuclei (d, f, h). Magnification $\times 100$. D: Dermis, E: Epidermis, G: Granulation tissue, HE: Hyperproliferative epithelium. (i) RNA (20 μ g) from human dermis and epidermis, human primary foreskin fibroblasts (HFF), and human primary foreskin keratinocytes (HFK) were analysed by RNase protection assay for the presence of *fgf-bp* mRNAs. Hybridization with a *gapdh* riboprobe served as a loading control. A longer exposure time was chosen for the assay with dermal and epidermal RNAs compared to RNAs from cultured fibroblasts and keratinocytes. In all, 1000 c.p.m. of the hybridization probe was used as a size marker. tRNA (20 μ g) was used as a negative control

wound epidermis. The specificity of the signal was confirmed by immunostaining with the FGF-BP antibody that had been preincubated with the immunization peptide. No signal was observed under these conditions (Figure 1e, f). Furthermore, no signal was observed with the second antibody alone (data not shown). Finally, we verified the epidermal expression of *fgf-bp* using RNase protection assay with RNA from human dermis and epidermis and from cultured primary fibroblasts and keratinocytes. Consistent with the immunofluorescence data, we found a much stronger expression in the epidermis compared to the dermis. In addition, *fgf-bp* mRNA was detected in keratinocytes but not in fibroblasts (Figure 1i). The lack of *fgf-bp* expression in fibroblasts is consistent with previous results from Wu *et al.* (1991).

Keratinocyte mitogens are potent inducers of fgf-bp expression in HaCaT keratinocytes

What are the factors that induce expression of the *fgf-bp* gene in wounded skin? To address this question, we treated HaCaT keratinocytes with different growth factors that are known to be upregulated in wounds, for example, FGF-7 and the ligands of the epidermal growth factor (EGF) receptor, EGF and transforming growth factor (TGF- α) (Werner and Grose, 2003). Since the culture of HaCaT cells in the absence of serum results in a downregulation of *fgf-bp* expression within the first few hours (Figure 2a), we also harvested cells incubated in the absence of any growth factor at each time point, in order to have the optimal control. EGF and TGF- α caused a strong induction of *fgf-bp* expression (Figure 2a, compare EGF and control lanes). The upregulation of *fgf-bp* expression by EGF does not require *de novo* protein synthesis, since it was also observed in the presence of the protein synthesis inhibitor cycloheximide (Figure 2b). A weaker, but significant induction was observed with 10% fetal calf serum (FCS) (Figure 2a), whereas the effect of FGF-7 was marginable. By contrast, TGF- β 1, a strong inhibitor of keratinocyte proliferation (Shipley *et al.*, 1986), suppressed the expression of *fgf-bp* in keratinocytes (Figure 2a). The regulation of *fgf-bp* expression by serum and growth factors was confirmed at the protein level. A protein with an apparent molecular weight of 34 kDa was detected in the lysate of HaCaT cells (Figure 2c) and to a lesser extent in the supernatant (data not shown). The levels of this protein increased in the lysates (Figure 2c) and in the supernatant (data not shown) of HaCaT keratinocytes after stimulation with EGF, TGF- α , FGF-7, and serum, and downregulation of the protein was found in the presence of TGF- β 1. These data suggest that keratinocyte mitogens present in wounded skin may be responsible for the increased expression of FGF-BP under these conditions and that they even overcome the inhibitory effect of TGF- β 1 on the expression of this protein.

Although FGF-BP has a signal peptide and should therefore be secreted, the protein was predominantly found in the lysate of HaCaT cells and also of primary

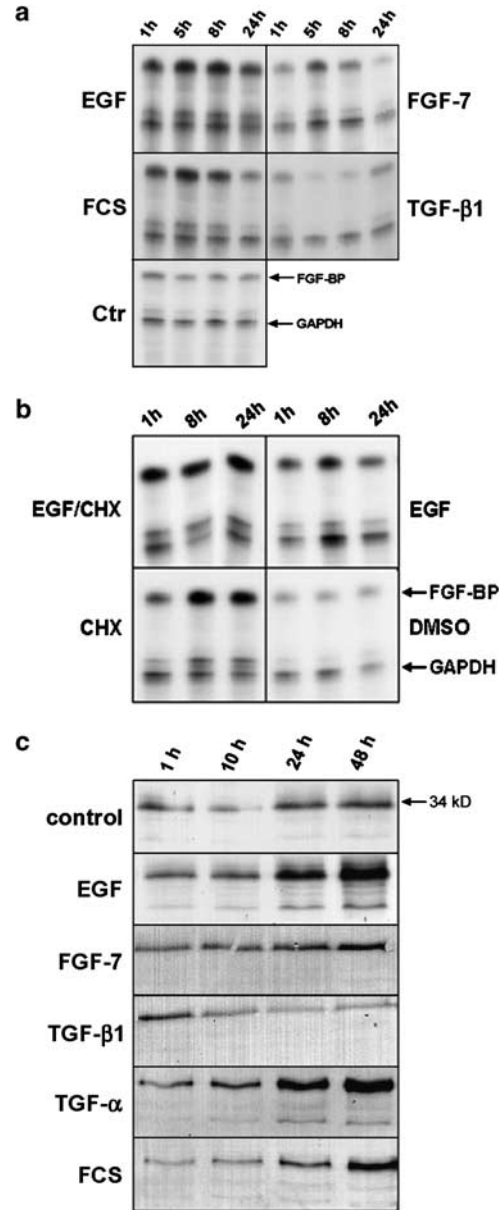


Figure 2 Regulation of FGF-BP expression by serum and growth factors in HaCaT keratinocytes. HaCaT keratinocytes were rendered quiescent by serum starvation and subsequently treated with EGF, FGF-7, FCS or TGF- β 1. Nontreated cells were used as a control. (a) Total cellular RNA was isolated from these cells at different time points after addition of serum or growth factors as indicated and analysed by RNase protection assay for the presence of FGF-BP mRNA. Hybridization of the same samples with a GAPDH riboprobe served as a loading control. (b) Serum-starved HaCaT cells were treated with 10 μ g/ml cycloheximide (final concentration). EGF was added 1 h later to some of the dishes. Control cells were treated with the solvent DMSO. Samples of 20 μ g RNA were analysed by RNase protection assay for the presence of *fgf-bp* and *gapdh* mRNAs. (c). Total cell lysates were prepared at different time points after addition of the growth factors. Samples of 50 μ g protein were analysed by Western blotting for the presence of FGF-BP

human keratinocytes (Figure 3). We therefore determined if it is bound to the cell membrane and if it can be released by addition of heparin. As shown in Figure 3

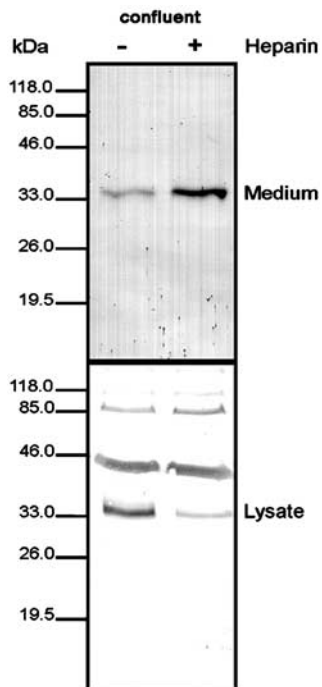


Figure 3 Heparin releases FGF-BP from the cell membrane. Confluent human primary keratinocytes were cultured in the presence or absence of 1 U/ml heparin as indicated on top of the gel. Proteins from the lysate (150 μ g) and the conditioned medium (15 μ l) were analysed by Western blotting for the presence of FGF-BP

for primary keratinocytes, the levels of the 34 kDa FGF-BP in the supernatant indeed increased upon addition of heparin, whereas the levels of cell-associated protein concomitantly decreased. This finding demonstrates that the protein is noncovalently bound to the cell membrane, most likely via heparansulfate proteoglycans. Proteins of 46 and 87 kDa, which were also detected in the lysate, are not FGF-BP variants, since they were also detected when the antibody was pre-incubated with the immunization peptide (data not shown).

FGF-BP binds FGF-7, FGF-10 and FGF-22

The exclusive detection of FGF-BP in the epidermal compartment of the skin suggests that it interacts with an FGF, which binds to keratinocytes. The major type of FGF receptor expressed by these cells is FGFR2-IIIb, a splice variant of FGFR2, which mediates the functions of FGF-7 and FGF-10 (Miki *et al.*, 1992; Igarashi *et al.*, 1998). Due to the high homology of FGF-22 with the known FGFR2-IIIb ligands (Nakatate *et al.*, 2001), it may also bind this receptor. Therefore, we performed co-immunoprecipitation experiments to determine whether FGF-BP is a binding protein for these members of the FGF family. For this purpose, we first analysed if FGF-BP can be immunoprecipitated by our antiserum from lysates of COS-1 cells that had been transiently transfected with an FGF-BP expression vector and an empty control vector (pCG-HA). Due to the inefficient

secretion of FGF-BP (see above), we used the cell lysate instead of conditioned medium for our studies. Indeed, the antiserum precipitated 27 and 30 kDa variants of FGF-BP from the cell lysate of FGF-BP-transfected cells but not of control cells (Figure 4a). The different size of FGF-BP in COS-1 cells compared to keratinocytes is most likely due to differences in glycosylation in both cell types. As a positive control for our experiments, we subsequently analysed if the FGF-BP antibody can co-immunoprecipitate FGF-2 under our experimental conditions. For this purpose, COS-1 cells were co-transfected with the FGF-BP expression vector and an expression vector coding for FGF-2 that is tagged with an HA epitope at the carboxy-terminus. Although the two proteins may not physically interact within the cell *in vivo* due to their presence in different cellular compartments, our experimental conditions allow their *in vitro* interaction. The antibody directed against the epitope tag as well as the FGF-BP antibody precipitated the tagged version of FGF-2 from the cell lysate of transfected COS-1 cells, thus confirming the published interaction of FGF-2 and FGF-BP under our experimental conditions (Figure 4b). We then used the same conditions to determine if FGF-BP can interact with FGF-7, FGF-10 and FGF-22. Unlike FGF-2, these proteins have a signal peptide (Ornitz, 2000; Beyer *et al.*, 2003) and should therefore co-localize with FGF-BP within the ER/Golgi compartment or at the cell surface. As shown in Figure 4c, the FGF-BP antibody precipitated an HA-tagged version of FGF-7 (26 kDa) from COS-1 cells that had been co-transfected with FGF-BP and FGF-7-HA expression vectors. *Vice versa*, FGF-BP could be co-immunoprecipitated with an antibody against the HA epitope from the same lysates. In analogous experiments, HA-tagged versions of FGF-10 and FGF-22 were also shown to interact with FGF-BP (Figure 4d and data not shown). The HA epitope did not modify this interaction, since nontagged versions of FGF-7 and FGF-22 were also able to interact with FGF-BP (Figure 4e, f). Furthermore, the interactions were verified with the 17 kDa truncated variant of FGF-BP (Wu *et al.*, 1991), demonstrating that the carboxy-terminus is not required for binding to FGFs (data not shown). Taken together, these results demonstrate that FGF-BP can indeed interact with ligands of FGFR2-IIIb that are known to be important stimulators of keratinocyte proliferation (Rubin *et al.*, 1989; Igarashi *et al.*, 1998).

FGF-BP enhances the activity of low concentrations of FGF-7

Since FGF-BP was shown to enhance the mitogenic activities of FGF-1 and FGF-2 for fibroblasts (Tassi *et al.*, 2001), we determined whether FGF-BP is also able to stimulate the activity of FGF-7. Since keratinocytes express high levels of FGF-BP, we used L6 rat skeletal muscle myoblasts stably overexpressing FGFR2-IIIb for our studies (Dell and Williams, 1992). In these cells, FGF-BP was undetectable by Western blotting (data not shown). Cells were treated with

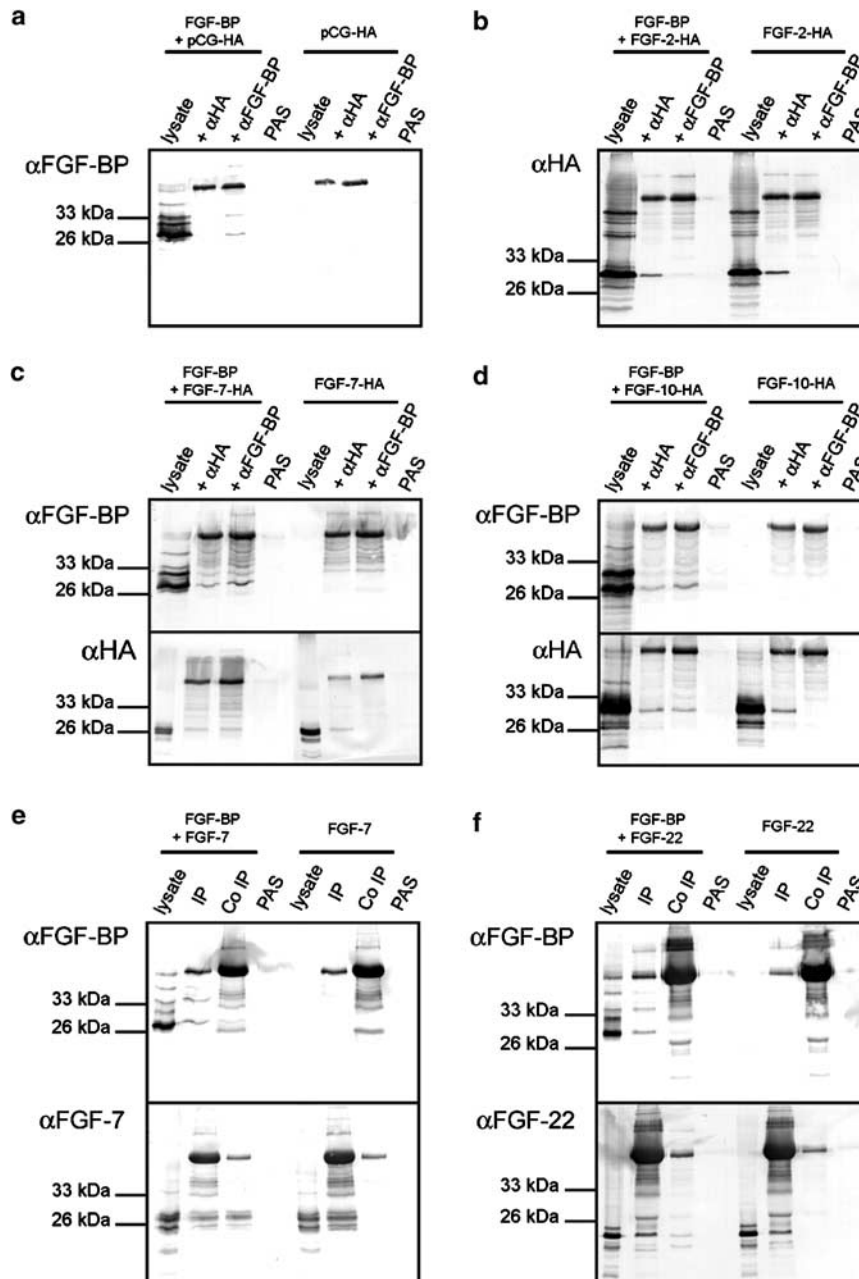


Figure 4 FGF-BP interacts with FGF-2, FGF-7, FGF-10, and FGF-22. COS-1 cells were transfected with an empty expression vector (pCG-HA) (a) or with expression vectors containing HA-tagged versions of an FGF-2 (b), FGF-7 (c), and FGF-10 (d) or untagged versions of FGF-7 (e) and FGF-22 (f) cDNAs as indicated on top of the gels (underlined). Transfection with these vectors was performed with and without an FGF-BP expression plasmid. Total lysates from these cells were immunoprecipitated with antibodies against the HA tag (α HA) or FGF-BP (α FGF-BP) (a–d) or with antibodies against FGF-7 (α FGF-7) or FGF-22 (α FGF-22) and FGF-BP (e, f). Total lysates from the transfected cells as well as immunoprecipitates were analysed by Western blotting using the antibodies indicated on the left-hand side of the gels. Incubation of the lysates with protein A sepharose (PAS) but without antibodies was used as a negative control

different concentrations of FGF-7 together with purified full-length FGF-BP in the presence of an excess of bovine serum albumin (BSA). The effect of FGF-7 and FGF-BP on the proliferation rate of L6-FGFR2-IIIb cells was determined by 3 H-thymidine incorporation. Addition of FGF-BP (10 ng/ml) to serum-free medium without FGF-7 had no effect (Figure 5a, left panel). However, FGF-BP enhanced the mitogenic activity of

low concentrations of FGF-7 (0.01 and 0.1 ng/ml; Figure 5a, right panels). By contrast, it even inhibited the mitogenic effect of higher concentrations of the growth factor (Figure 5a). This result was repeated in three independent experiments and was also observed when primary human keratinocytes were used instead of L6 cells (data not shown). The mitogenic activity of different concentrations of FCS was not altered by

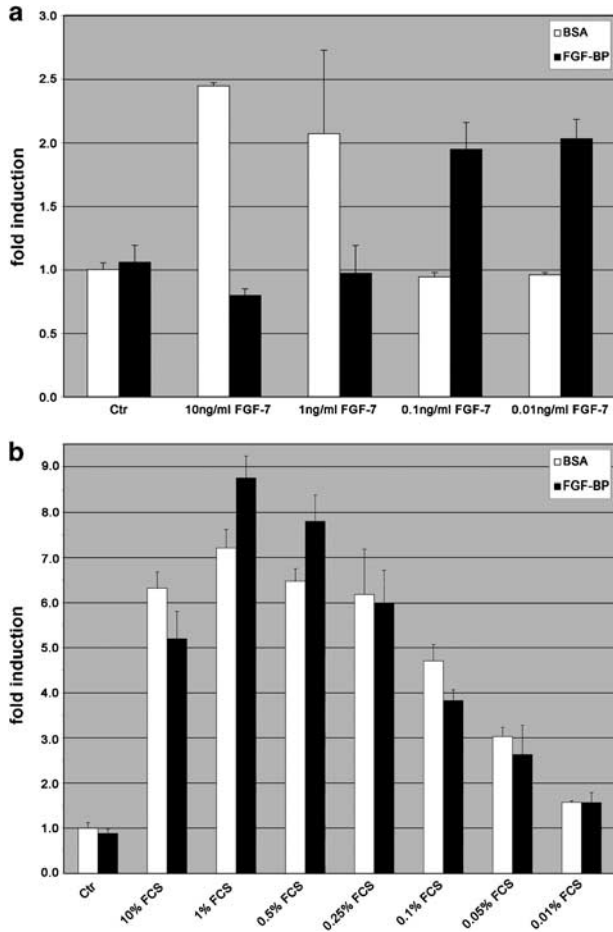


Figure 5 FGF-BP enhances the activity of low concentrations of FGF-7. Rat L6 skeletal muscle myoblasts stably expressing FGFR2-IIIb were treated with different concentrations of FGF-7 (a) or FCS (b) as indicated, in the presence or absence of purified FGF-BP (10 ng/ml). The mitogenic activity of the cells was determined by ^3H -thymidine incorporation. Results obtained with cells treated with BSA alone were arbitrarily set as 1. Radioactivity was counted in triplicate wells. The result was repeated in three independent experiments

addition of FGF-BP, demonstrating the specificity of FGF-BP for FGF (Figure 5b). These findings suggest that FGF-BP expression may enhance FGF-7 activity in tissues, where low levels of the growth factor are present.

Discussion

Injury to adult skin initiates a series of events, which finally lead to at least partial reconstruction of the wounded tissue (Werner and Grose, 2003). Of particular importance for the regeneration of the injured epidermis are members of the FGF family, as shown by over-expression of a dominant-negative FGF receptor mutant in the epidermis of transgenic mice. These animals were characterized by a major delay in wound re-epithelialization (Werner *et al.*, 1994a). The FGFs,

which are responsible for re-epithelialization, are most likely FGF-7, FGF-10, and FGF-22, since these mitogens are present at high levels at the wound site (Werner *et al.*, 1992; Marchese *et al.*, 1995; Beer *et al.*, 1997; Beyer *et al.*, 2003; Tagashira *et al.*, 2001), and since at least FGF-7 and FGF-10 activate the major FGF receptor on keratinocytes, FGFR2-IIIb (Miki *et al.*, 1992; Igarashi *et al.*, 1998). In addition to wound healing, FGFR2-IIIb ligands play crucial roles in the repair of other epithelial tissues, including intestine and liver (Chen *et al.*, 2002; Steiling *et al.*, 2003). Furthermore, FGF-7 is a potent cytoprotective factor, which is used for the treatment of mucositis in cancer patients receiving chemotherapy and/or radiotherapy (Finch and Rubin, 2004). Therefore, it is of particular interest to identify the mechanisms which regulate the activity of these FGFs.

Previous studies have shown that the biological activity of FGF-2 can be enhanced by FGF-BP, a secreted protein, which binds to this FGF and thereby mobilizes it from the extracellular matrix (Aigner *et al.*, 2001; Tassi *et al.*, 2001). By contrast, no binding to EGF was observed, suggesting that FGF-BP is specific for FGFs (Tassi *et al.*, 2001). Since FGF-BP is also expressed in keratinocytes, in particular during early stages of carcinogen-induced transformation (Kurtz *et al.*, 1997; Mongiat *et al.*, 2001), we speculated about a role of this protein in normal skin and in the hyperproliferation of keratinocytes observed after skin injury. Indeed, we found a strong expression of this protein in the normal epidermis and a further enhancement of its expression after skin injury. This injury-mediated upregulation was recently confirmed with another wound model (Kurtz *et al.*, 2004). These data strongly suggest that FGF-BP is not only important for tumorigenesis, but also for tissue homeostasis and repair.

Which factors might be responsible for the upregulation of FGF-BP expression after injury? To address this question, we determined the effect of various growth factors present at the wound site on expression of FGF-BP in cultured keratinocytes. Consistent with previous results obtained with squamous cell carcinoma cells (Harris *et al.*, 2000), EGF strongly enhanced the levels of FGF-BP mRNA and protein in HaCaT keratinocytes. By contrast, TGF- β 1, a potent inhibitor of keratinocyte proliferation, suppressed *fgf-bp* gene expression. Thus, induction of *fgf-bp* expression is obviously a characteristic feature of keratinocyte mitogens. Since the latter are abundant at the wound site (Werner and Grose, 2003), these growth factors might also enhance FGF-BP expression *in vivo*.

The FGF-BP present in proliferating keratinocytes had an apparent molecular weight of approximately 34 kDa, which is similar to the size found in other cell types (Aigner *et al.*, 2001; Liu *et al.*, 2001; Sauter *et al.*, 2001). The slight differences in molecular weight between different cell types are most likely the result of different glycosylation. Although FGF-BP has a putative signal peptide, only low amounts were detectable in the supernatant. However, the 34 kDa protein

was released into the medium upon treatment with heparin, suggesting that it is normally bound to the cell surface via heparan sulfate proteoglycans.

FGF-BP is well known for its enhancement of the mitogenic activity of FGF-2 (Tassi *et al.*, 2001). However, this type of FGF is a poor mitogen for keratinocytes, since it binds the major receptor on these cells, FGFR2-IIIb, with very low affinity (Miki *et al.*, 1992). Therefore, the high expression of FGF-BP in keratinocytes suggested that it also interacts with other FGF family members *in vivo*. This hypothesis is supported by recent findings by Aigner *et al.* (2002a), who demonstrated that the positive effect of FGF-BP on colony formation of prostate cancer cells in soft agar is at least in part independent of FGF-2. Rather, other FGFs expressed in prostate cancer might be responsible. Furthermore, Mongiat *et al.* (2001) suggested that FGF-BP can also interact with FGF-7, possibly via their common binding partner perlecan. Therefore, we investigated if FGF-BP can bind to FGF-7 and FGF-10, which act via FGFR2-IIIb on epithelial cells, and/or to the recently identified and highly homologous growth factor FGF-22 (Nakatake *et al.*, 2001). Indeed, our studies revealed that full-length FGF-BP and also the carboxy-terminally truncated variant bind to all of these FGF family members. Most importantly, purified FGF-BP stimulated the mitogenic activity of low concentrations of FGF-7 for rat skeletal muscle myoblasts expressing FGFR2-IIIb. The stimulatory effect is obviously not due to stabilization of an FGF in the conditioned medium by FGF-BP, since the enhanced mitogenicity was only observed in the presence of FGF-7. Rather, FGF-BP appears to enhance the activity of the exogenously added FGF-7, possibly via stabilization of the growth factor and/or via presentation of the ligand to its transmembrane receptor. Interestingly, FGF-BP inhibited the mitogenic effect of high concentrations of FGF-7. Since high concentrations of a growth factor cause receptor downregulation, as shown, for example, for FGF-7 and its receptor FGFR2-IIIb in human wounds (Marchese *et al.*, 1995), the enhancement of FGF-7 activity by FGF-BP may already result in receptor endocytosis at lower concentrations of FGFs. Independent of the mechanisms of action, our results suggest that FGF-BP enhances FGF activity under conditions where the ligand is present in limited amounts. For FGF-7, this is obviously the case *in vivo*, even after injury-induced expression. For example, it has as yet not been possible to detect FGF-7 in wound lysates by Western blotting, indicating that the concentrations are very low (SW, unpublished data). Therefore, the enhanced expression of FGF-BP in wounded skin may help to increase the mitogenic activity of endogenous FGFs. Generation of FGF-BP knockout mice and the analysis of the wound-healing process in these animals will be required to elucidate the function of FGF-BP in the healing process. Finally, our findings indicate that FGF-BP could be therapeutically explored for the enhancement of endogenous FGF activity at the wound site and thus for the treatment of impaired wound healing.

Materials and methods

Plasmids

The full-length cDNAs of murine FGF-2, FGF-7, FGF-10, and FGF-22 with or without a carboxy-terminal in-frame epitope of the influenza virus hemagglutinin (HA) were cloned into the pCG eukaryotic expression vector. In the latter, expression of cDNAs is driven by the cytomegalovirus promoter. The FGF-22 plasmid had been described previously (Beyer *et al.*, 2003). The full-length human FGF-BP cDNA was amplified by RT-PCR using RNA from human skin, 5'-GCTCTAGAATGAAGATCTGTAGC-3' as 5'-primer and 5'-GCGATATCTTAGCATGACGTGTCCTGCAC-3' as 3'-primer. The cloned cDNAs were fully sequenced.

Cell culture

Human foreskin keratinocytes were cultured in K-SFM (Gibco, Invitrogen, Basel, Switzerland). The human keratinocyte cell line HaCaT (Boukamp *et al.*, 1988) as well as FGFR2-IIIb-transfected L6 rat skeletal muscle myoblasts (Dell and Williams, 1992) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were grown to confluence without changing the medium, rendered quiescent by a 16–20 h serum starvation and subsequently incubated in fresh DMEM containing 10% FCS, FGF-7 (5 ng/ml of medium), EGF (20 ng/ml), TGF-α (50 ng/ml), transforming growth factor β1 (TGF-β1) (1 ng/ml), or without additives. They were harvested at different time points after growth factor addition and used for RNA isolation or preparation of cell lysates. DMEM was purchased from Sigma (Munich, Germany), FCS was from Amimed-BioConcept (Allschwil, Switzerland), and growth factors were from Roche (Basel, Switzerland) or from R&D systems (R&D Systems, Minneapolis, MN, USA). Rat FGF-BP was purchased from R&D Systems. Cycloheximide (Sigma) was dissolved at 10 mg/ml in dimethylsulfoxide (DMSO), heparin (Sigma) at 1 U/ml in the appropriate growth medium.

Preparation of keratinocyte lysates

HaCaT cells or primary human keratinocytes were lysed in 400 µl (per 10 cm dish) of urea buffer (10 mM Tris/HCl pH 8.0, 9.5 M urea, 2 mM EDTA, 2 mM PMSF). Cells were scraped off the dish and sonicated. After centrifugation the protein concentration was determined with the BCA kit (Pierce, Rockford, IL, USA).

Western blot analysis

Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose filters. Antibody incubations were performed in 3% nonfat dry milk in TBS-T (10 mM Tris/HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20). A polyclonal antibody against FGF-BP was generated in rabbits using the peptide QKDTLGNTQIKQKSR, and the antibody was affinity-purified. The specificity of the FGF-BP antibody was analysed by Western blotting using recombinant FGF-BP and lysates of FGF-BP-transfected and vector-transfected COS-1 cells, as well as by immunofluorescence using these transfected COS-1 cells. Membranes were probed with FGF-BP antibody or with a polyclonal antibody directed against the HA epitope (Santa Cruz, Santa Cruz, CA, USA), followed by alkaline-phosphate-conjugated anti-rabbit IgG (Promega, Heidelberg, Germany). Antibody-binding proteins were visualized using nitro blue

tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Promega). The polyclonal antibodies against FGF-7 and FGF-22 were described previously (Brauchle *et al.*, 1994; Beyer *et al.*, 2003). Equal loading and transfer efficiency were controlled by Ponceau S staining of the membrane before antibody treatment.

Transfection of COS-1 cells

COS-1 cells were cultured in DMEM containing 10% FCS. For transient transfection, LipofectAMINE 2000 was used according to the manufacturer's instructions (Invitrogen, Paisley, UK). Plasmid DNAs used for transfection were isolated using the Endo-Free kit (Qiagen, Hilden, Germany).

Immunoprecipitation

Transfected COS-1 cells were lysed in immunoprecipitation buffer (50 mM Tris, pH 7.5, 15 mM EDTA, 100 mM NaCl, 0.1% (w/v) Triton X-100, 1 mM DTT, 1 mM PMSF). After sonication and centrifugation, the supernatant was incubated with antibodies against FGF-BP (1:50 diluted) or the HA epitope tag (1:20 diluted) for 90 min at 4°C. After centrifugation, antigen-antibody complexes were precipitated with protein A sepharose slurry (Amersham Biosciences, Braunschweig, Germany) for 1 h at 4°C and subsequently pelleted. Pellets were washed 3 times with immunoprecipitation buffer and incubated for 5 min at 95°C in Laemmli sample buffer. Eluates were analysed by SDS-PAGE and subsequent Western blotting.

FGF-BP activity assay

L6 rat skeletal muscle myoblasts stably expressing FGFR2-IIIb or primary human keratinocytes were seeded into 24-well plates (20 000 cells per well), and grown for 3 days in DMEM/10% FCS. They were subsequently starved in serum-free medium for 24 h, and then treated with different concentrations of FGF-7 or FCS in fresh medium. The medium was further supplemented by BSA (1 µg/ml) with or without 10 ng/ml FGF-BP. After 24 h, 1 µl (methyl-³H)thymidine (1 mCi/ml; Amersham Biosciences) was added to each well and incubated for 1 h. Plates were cooled on ice, washed with ice-cold 5% trichloroacetic acid and incubated on ice for 15 min. Cells were lysed in 750 µl 0.25% NaOH/0.1% SDS and incubated for 20 min on a rocker at room temperature. In all, 500 µl of the suspension were neutralized with 50 µl 6 N HCl. Subsequently, the radioactivity in each sample was determined in a ³H channel.

Wounding and preparation of wound tissues

Four full-thickness excisional wounds of 4 mm diameter were generated on the back of BALB/c mice (all female, 10–12 weeks old) by excising skin and *panniculus carnosus* as described (Werner *et al.*, 1994a,b). Wounds were left uncovered without a dressing and harvested at different time points after injury. For expression analysis, the complete wounds including 2 mm of the epithelial margins were excised, immediately frozen in liquid nitrogen and stored at –80°C until used for RNA isolation. Nonwounded back skin served as a control. All experiments with animals were carried out with permission from the local veterinary authorities.

Immunofluorescence

Frozen sections from normal and wounded human skin (7 µm) were incubated overnight at 4°C with the affinity-purified

rabbit polyclonal FGF-BP antibody (diluted 1:100 in PBS with 1% BSA), followed by a 1 h incubation at room temperature with anti-rabbit IgG-Cy3™ diluted in PBS with 12% BSA (Jackson ImmunoResearch Laboratory Inc., West Grove, PA, USA). Subsequently, slides were washed in PBS containing 0.1% Tween-20, prior to mounting in Mowiol (Hoechst, Frankfurt, Germany) and photographing on a Zeiss Axioplan fluorescence microscope.

Human biopsies

Punch biopsies (4 mm) were taken from normal skin and from full-thickness excisional wounds of healthy adult volunteers at different time points (1 h, 24 h, 5 days, and 15 days) after wounding. For real-time RT-PCR, six biopsies from each time point were pooled. Each measurement per time point was repeated with two independent pools. For immunohistochemistry, 5-day wounds were directly frozen in tissue-freezing medium (Jung, Nussloch, Germany) for subsequent preparation of cryosections. All procedures were approved by the local ethics committee of the Technical University of Munich.

RNA isolation, RNase protection assay, and RT-PCR

Total cellular RNA was isolated by a modified method of Chomczynski and Sacchi (1987), including a DNaseI digestion step.

For cDNA synthesis MultiScribe reverse transcriptase (50 U/µl) (Applied Biosystems, Weiterstadt, Germany) and random hexamers (2.5 µM) were used. Quantitative real-time RT-PCR was performed using a SYBR green PCR master mix (Applied Biosystems). Quantifications were carried out relative to cyclophilin A as a housekeeping gene. AmpErase UNG (1 U/µl) (Applied Biosystems) treatment was performed to avoid contaminations with PCR products. The following primers were used: 300 nM of FGF-BP forward primer (5'-GAGCTCTCTCTGCACATTCTTCC-3') and 50 nM reverse primer (5'-TAGCATGACGTGTCTCCTGCACTA-3'), as well as 300 nM cyclophilin A forward primer (5'-GGAATGGCAA GACCAGCAAG-3'), and 300 nM cyclophilin A reverse primer (5'-GGATACTGCGAGCAAATGGG-3'). Each PCR reaction was measured in triplicate.

RNase protection assays were performed as described (Werner *et al.*, 1993). All protection assays were carried out at least in duplicate with different sets of RNAs from independent experiments. The following templates were cloned: a fragment of the murine *fgf-bp* cDNA (Kurtz *et al.*, 1997), including the start codon and the following 350 nucleotides as well as a fragment of the human *fgf-bp* cDNA (Wu *et al.*, 1991), including the start codon and the following 205 nucleotides. As a loading control, the RNA was hybridized to a probe for glyceraldehyde phosphate dehydrogenase (murine GAPDH; nucleotides 566–685 of the cDNA; GenBank™ M32599; human GAPDH; nucleotides 580–695; GenBank™ BC001601).

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References

- Aigner A, Butscheid M, Kunkel P, Krause E, Lamszus K, Wellstein A and Czubayko F. (2001). *Int. J. Cancer*, **92**, 510–517.
- Aigner A, Malerczyk C, Houghtling R and Wellstein A. (2000). *Growth Factors*, **18**, 51–62.
- Aigner A, Ray PE, Czubayko F and Wellstein A. (2002b). *Histochem. Cell Biol.*, **117**, 1–11.
- Aigner A, Renneberg H, Bojunga J, Apel J, Nelson PS and Czubayko F. (2002a). *Oncogene*, **21**, 5733–5742.
- Beer HD, Florence C, Dammeier J, McGuire L, Werner S and Duan DR. (1997). *Oncogene*, **15**, 2211–2218.
- Beyer TA, Werner S, Dickson C and Grose R. (2003). *Exp. Cell Res.*, **287**, 228–236.
- Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A and Fusenig NE. (1988). *J. Cell Biol.*, **106**, 761–771.
- Brauchle M, Angermeyer K, Hubner G and Werner S. (1994). *Oncogene*, **9**, 3199–3204.
- Chen Y, Chou K, Fuchs E, Havran WL and Boismenu R. (2002). *Proc. Natl. Acad. Sci. USA*, **99**, 14338–14343.
- Chomczynski P and Sacchi N. (1987). *Anal. Biochem.*, **162**, 156–159.
- Czubayko F, Liaudet-Coopman ED, Aigner A, Tuveson AT, Berchem GJ and Wellstein A. (1997). *Nat. Med.*, **3**, 1137–1140.
- Czubayko F, Smith RV, Chung HC and Wellstein A. (1994). *J. Biol. Chem.*, **269**, 28243–28248.
- Dell KR and Williams LT. (1992). *J. Biol. Chem.*, **267**, 21225–21229.
- Finch PW and Rubin JS. (2004). *Adv. Cancer Res.*, **91**, 69–136.
- Harris VK, Coticchia CM, Kagan BL, Ahmad S, Wellstein A and Riegel AT. (2000). *J. Biol. Chem.*, **275**, 10802–10811.
- Igarashi M, Finch PW and Aaronson SA. (1998). *J. Biol. Chem.*, **273**, 13230–13235.
- Johnson DE and Williams LT. (1993). *Adv. Cancer Res.*, **60**, 1–41.
- Kurtz A, Aigner A, Cabal-Manzano RH, Butler RE, Hood DR, Sessions RB, Czubayko F and Wellstein A. (2004). *Neoplasia*, **6**, 595–602.
- Kurtz A, Wang HL, Darwiche N, Harris V and Wellstein A. (1997). *Oncogene*, **14**, 2671–2681.
- Liu X-H, Aigner A, Wellstein A and Ray PE. (2001). *Kidney Int.*, **59**, 1717–1728.
- Marchese C, Chedid M, Dirsch OR, Csaky KG, Santanelli F, Latini C, LaRochelle WJ, Torrisi MR and Aaronson SA. (1995). *J. Exp. Med.*, **182**, 1369–1376.
- Miki T, Bottaro DP, Fleming TP, Smith CL, Burgess WH, Chan AM and Aaronson SA. (1992). *Proc. Natl. Acad. Sci. USA*, **89**, 246–250.
- Mongiati M, Otto J, Oldershaw R, Ferrer F, Sato D and Iozzo RV. (2001). *J. Biol. Chem.*, **276**, 10263–10271.
- Nakatake Y, Hoshikawa M, Asaki T, Kassai Y and Itoh N. (2001). *Biochim. Biophys. Acta*, **1517**, 460–463.
- Okamoto T, Tanaka Y, Kan M, Sakamoto A, Takada K and Sato JD. (1996). *In vitro Cell. Dev. Biol. Anim.*, **32**, 69–71.
- Ornitz DM. (2000). *BioEssays*, **22**, 108–112.
- Ornitz DM and Itoh N. (2001). *Genome Biol.*, **2**, Reviews 2005.
- Ornitz DM, Xu J, Colvin JS, McEwen DG, MacArthur CA, Coulter F, Gao G and Goldfarb M. (1996). *J. Biol. Chem.*, **271**, 15292–15297.
- Orr-Urtreger A, Bedford MT, Burakova T, Arman E, Zimmer Y, Yayon A, Givol D and Lonai P. (1993). *Dev. Biol.*, **158**, 475–486.
- Powers CJ, McLeskey SW and Wellstein A. (2000). *Endocr. Relat. Cancer*, **7**, 165–197.
- Rubin JS, Osada DP, Finch PW, Taylor WG, Rudikoff S and Aaronson SA. (1989). *Proc. Natl. Acad. Sci. USA*, **86**, 802–806.
- Sauter ER, Nesbit M, Tichansky D, Liu ZJ, Shirakawa T, Palazzo J and Herlyn M. (2001). *Int. J. Cancer*, **92**, 374–381.
- Shipley GD, Pittelkow MR, Wille Jr JJ, Scott RE and Moses HL. (1986). *Cancer Res.*, **46**, 2068–2071.
- Steiling H and Werner S. (2003). *Curr. Opin. Biotechnol.*, **14**, 533–537.
- Steiling H, Wustefeld T, Bugnon P, Brauchle M, Fassler R, Teupser D, Thiery J, Gordon JI, Trautwein C and Werner S. (2003). *Oncogene*, **22**, 4380–4388.
- Tagashira S, Harada H, Katsumata T, Itoh N and Nakatsuka M. (2001). *Gene*, **197**, 399–404.
- Tassi E, Al-Attar A, Aigner A, Swift MR, McDonnell K, Karavanov A and Wellstein A. (2001). *J. Biol. Chem.*, **276**, 40247–40253.
- Werner S, Breeden M, Hübner G, Greenhalgh DG and Longaker MT. (1994b). *J. Invest. Dermatol.*, **103**, 469–473.
- Werner S, Duan DS, de Vries C, Peters KG, Johnson DE and Williams LT. (1992). *Mol. Cell. Biol.*, **12**, 82–88.
- Werner S and Grose R. (2003). *Phys. Rev.*, **83**, 835–870.
- Werner S, Smola H, Liao X, Longaker MT, Krieg T, Hofschneider PH and Williams LT. (1994a). *Science*, **266**, 819–822.
- Werner S, Weinberg W, Liao X, Peters KG, Blessing M, Yuspa SH, Weiner RL and Williams LT. (1993). *EMBO J.*, **12**, 2635–2643.
- Wu DQ, Kan MK, Sato GH, Okamoto T and Sato JD. (1991). *J. Biol. Chem.*, **266**, 16778–16785.