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A Novel Binding Protein for Fibroblast Growth Factors (FGF-BP2): Cloning, Expression Profile, Tumorigenic Activity and Regulation of Gene Expression by Fetal Bovine Serum and Retinoic Acid.

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1. INTRODUCTION

1.1 Cancer and Tumor Growth

The pathogenesis of tumor growth results from the disregulation of the normal mechanisms for regular cellular homeostasis in the context of the larger multicellular organism. Indeed, neoplasia by its very definition refers to cellular growth heedless to the signals provided by other, non neoplastic cells that would normally maintain the balance of cellular proliferation and death. Hanahan and Weinberg suggest that the vast catalog of cancer cell genotypes is a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000) It is proposed that these physiological changes are novel capabilities acquired during tumor development and that they are shared in common by most and perhaps all types of human tumors.

1.2 Tumor Angiogenesis and Metastasis

Angiogenesis, as one of the six proposed essential changes that are predictive for tumor growth, has been a major focus in cancer research. It is understood that critical to the process of tumor growth and metastasis is the persistent growth of new blood vessels, or angiogenesis, where new capillaries are induced to sprout from existing blood vessels. Angiogenesis is an early event in tumor formation and is often first detected during the preneoplastic stages of a tumor (reviewed by Hanahan and Folkman, 1996). The importance of angiogenesis for the growth of a tumor is reflected by the observation that most solid tumors are unable to grow beyond a microscopic size, no larger than about 2mm in diameter without a sufficient vasculature. Because angiogenesis is essential for tumor survival, an understanding of the mechanisms controlling new blood vessel formation have become a major focus of cancer research. In addition to the nourishing function of tumor blood vessels, they provide a route by which tumor cells metastasize to distant organs, metabolic waste is removed and, importantly, they supply growth factors to tumor cells.

Figure 1: Acquired Capabilities of Cancer

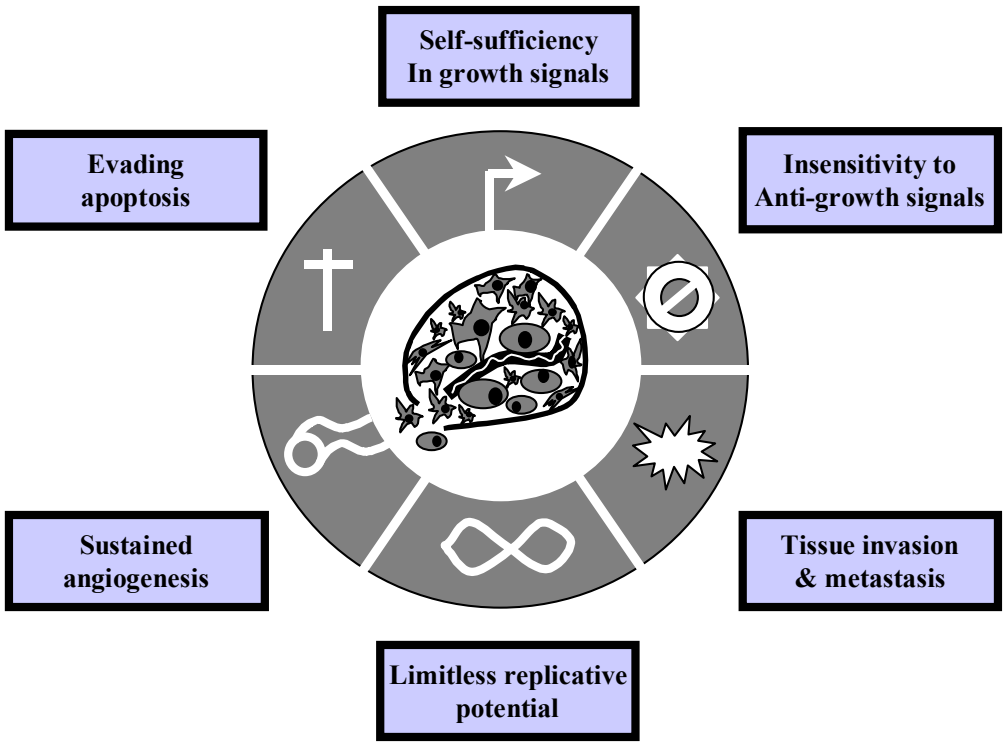

Figure 1: Acquired Capabilities of Cancer. The six essential alterations in cell homeostasis that are proposed to dictate malignant growth and to be shared by most of human tumors. (Adapted from Hanahan, D. and Weinberg, R.A. (2000) **The Hallmarks of Cancer.** *Cell*, 100, 57-70)

This doctrine is based on countless experiments using various tumor models and animal species (Folkman, and Shing, 1992; Folkman, 1991; Liotta, L.A. et al., 1991; Folkman and Klagsbrun, 1987; Cross and Dexter, 1991; Folkman, 1986; Fidler and Ellis, 1994).

Consequently, the amount of angiogenesis in a primary tumor, as measured by microvessel density, appears to be directly related to the rate of metastasis of many solid tumors. A direct correlation between blood vessel density in primary tumors and their metastasis has been reported for many different tumor types including breast

cancer (Horak et al., 1992; Weidner et al., 1991; Bosari et al., 1992; Weidner et al., 1992; Toi et al. 1993), lung cancer (Macchiarini et al., 1992), and squamous cell carcinoma of the head and neck (Gasparini et al., 1993). In these studies, the level of tumor angiogenesis was an independent prognostic indicator of the outcome of the disease.

In the adult, new blood vessel formation occurs primarily during highly regulated processes such as the female reproductive cycle. The induction of angiogenesis also occurs in certain pathological situations, including wound healing, diabetic retinopathy and tumor formation. Neoangiogenesis is a highly regulated process where quiescent endothelial cells are induced to degrade the extracellular matrix and basement membrane, migrate into the interstitial space, proliferate and organize tube-like structures. This process is mediated through a balance of both positive and negative angiogenic factors, the levels of which govern whether angiogenesis will be activated or inhibited (Hanahan and Folkman, 1996). With respect to positive angiogenic factors, more than a dozen distinct protein products are currently known to induce proliferation and migration of endothelial cells *in vitro* and/or angiogenesis *in vivo*, including Acidic Fibroblast Growth Factor (aFGF, FGF-1) und Basic Fibroblast Growth Factor (bFGF, FGF-2) (Christofori, 1997; Baird and Klagesbrun, 1991), Vascular Endothelial Cell Growth Factor (VEGF) (Kim et al., 1993), Hepatocyte Growth Factor (HGF) (Weidner et al., 1993), Epidermal Growth Factor (EGF) and Transforming Growth Factor Alpha (TGFα) (Schreiber et al., 1986) and Pleiotrophin (PTN) (Fang et al., 1992).

In contrast, angiogenic inhibitors such as Angiostatin (O'Reilly et al., 1994; O'Reilly et al., 1996), Endostatin (O'Reilly et al., 1997), Thrombospondin-1, Interleukin-1 and Protease inhibitors serve to counter the effects of angiogenic activators and keep the vasculature in a quiescent state. The regular turnover time of endothelial cells in adult tissue is measured in years.

Angiogenic factors, however, can induce endothelial cells to proliferate as fast as bone marrow cells, with resultant turnover times of only days. The ability of a tumor to shift the angiogenic balance in favor of angiogenic activators and turn on the 'angiogenic switch' provides the tumor with a survival advantage by recruiting new blood vessels from the surrounding vasculature.

1.3 The Role of Fibroblast Growth Factors (FGFs) and a Binding Protein for FGF (FGF-BP1)

Some of the most effective and best-studied angiogenic factors are members of the Fibroblast Growth Factor (FGF) family of polypeptides (Baird and Klagesbrun, 1991; Gospodarowicz et al., 1987). The FGFs are a family of at least 21 distinct growth factors which can interact with their membrane receptors coded for by four separate receptor genes and numerous protein products due to alternative splicing (reviewed in Schreiber et al., 1986; Powers C.J. et al., 2000). Members of the FGF family regulate various developmental processes and are potent stimulators of new blood vessel growth during wound healing and during tumor growth (Folkman and Klagsbrun, 1987; Burgess et al., 1989; Baird et al., 1990). FGF-1 (aFGF), FGF-2 (bFGF) and FGF-4 (K-FGF) have all been shown to be angiogenic activators (Burgess et al., 1989) and have therefore been a major focus of research in tumor vascularisation.

aFGF and bFGF are widely expressed in normal tissues and in tumors of different origin, although not at elevated levels (Burgess et al., 1989; Moscatelli et al. 1986). The observation that aFGF and bFGF are expressed in normal tissues, which are not undergoing proliferation or angiogenesis, suggests that mechanisms other than their expression exist which regulate FGF activation. Unlike other members of the FGF-family, aFGF and bFGF do not contain a signal sequence and are not secreted into the media in a classical way (Burgess et al., 1989; Mason, 1994). In fact, these growth factors are deposited into the extracellular matrix (ECM) where they are found tightly bound to membrane-attached heparansulfate proteoglycans. The immobilization of FGFs in the ECM quenches their biological activities by preventing them from reaching their high affinity receptors in the endothelial cell membrane (Vlodavsky et al., 1987; Rogelj et al., 1989; Saksela et al., 1988; Kiefer et al., 1990).

There are several possible mechanisms by which bFGF can be released from its matrix storage site and thus activated. One established mechanism is the proteolytic degradation of the matrix by heparanases (Vlodavsky et al., 1988; Bashkin et al., 1989; Moscatelli et al. 1992; Vlodavsky et al., 1991). Alternatively, active bFGF can be delivered from its ECM storage site to its receptors by an FGF binding protein, FGF-BP1.

1.4 FGF-BP1 is a Carrier for immobilized FGFs

FGF-BP1 is a secreted, heparin-binding protein of 17 kD molecular mass that was originally isolated from A431 human epidermoid carcinoma cells (Wu et al., 1991). FGF-BP1 binds to aFGF and bFGF in a non-covalent reversible manner. Furthermore, bFGF bound to this protein is protected from degradation and retains its mitogenic activity (Wu et al., 1991). These characteristics indicate that FGF-BP1 could be an important regulator that releases immobilized FGFs from their matrix storage site and thus activates them *in vivo* (Czubayko et al., 1994; Rak and Kerbel, 1997).

The role of FGF-BP1 in the activation of bFGF has been elucidated by several recent studies demonstrating the importance of this protein in regulating tumorigenesis and angiogenesis of several different tumor types. FGF-BP1 was transfected and overexpressed in a non-tumorigenic FGF-BP1 negative human adrenal carcinoma cell line (SW-13). Wild type SW-13 cells express bFGF but do not form colony in soft agar nor do they form tumors in athymic nude mice. The expression of FGF-BP1 in SW-13 cells resulted in a tumorigenic and angiogenic phenotype when these cells were injected into nude mice (Czubayko et al., 1994). It was also shown that these FGF-BP1 transfectants form colonies in soft agar and release the protein into their media together with bFGF in a non-covalently bound form. As expected, colony formation of FGF-BP1-transfected SW-13 cells can be blocked by a specific antibody for bFGF, demonstrating that the tumorgenic potential of FGF-BP is bFGF dependent (Czubayko et al., 1994).

As an alternative approach, the impact of depleting endogenous levels of FGF-BP1 with respect to its effect on angiogenesis and tumor growth was also investigated (Czubayko et al., 1997). In this study, FGF-BP1 positive cell lines ME-180 (human squamous cell carcinoma) and LS174T (human colon carcinoma) were transfected with ribozymes, which specifically target and degrade the endogenous FGF-BP1 mRNA. Reduced FGF-BP1 expression resulted in decreased levels of biologically active bFGF released from the cells in culture. In addition, the growth and angiogenesis of xenografted ME-180 and LS174T tumors in mice was decreased in parallel with the ribozyme-mediated reduction of FGF-BP1.

Figure 2. Model of FGF-BP1 Function.

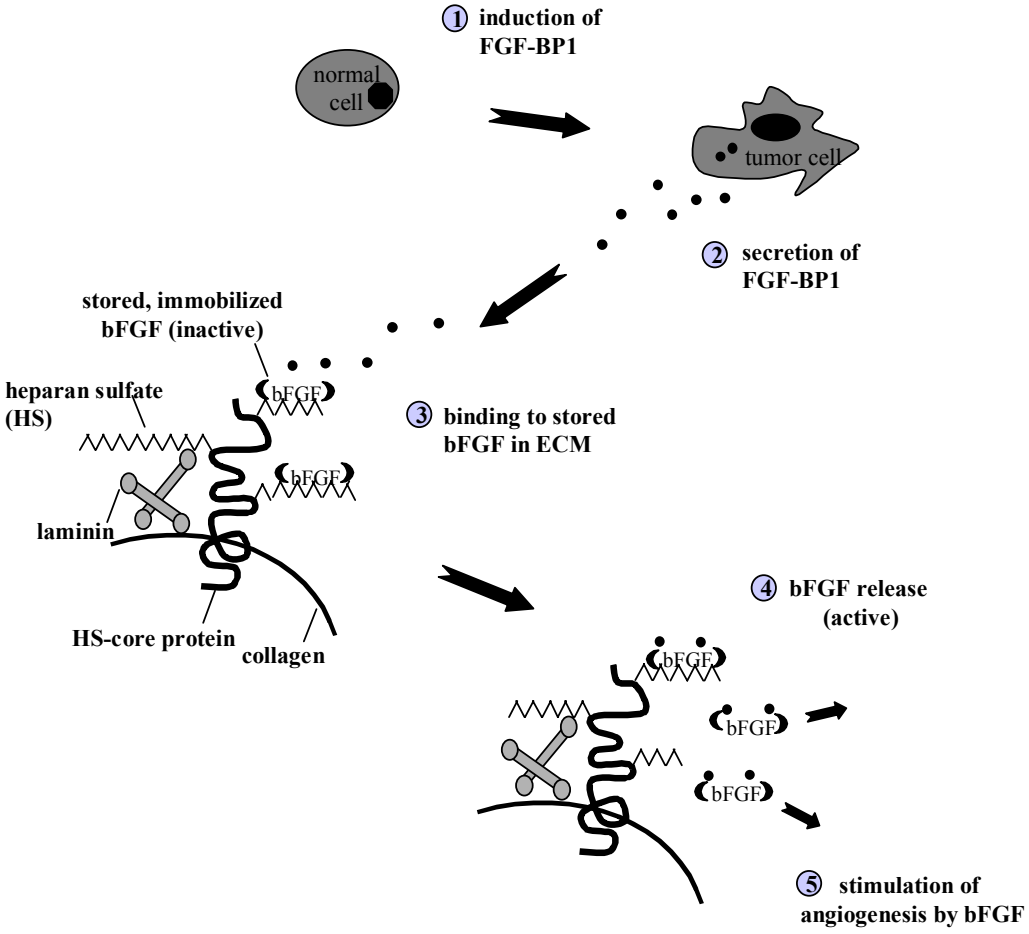

Figure 2. Model of FGF-BP1 Function. In some tumors, FGF-BP1 expression is upregulated (1) and FGF-BP1 is secreted (2) into the extracellular environment. (3) Secreted FGF-BP1 binds to immobilized bFGF, which is stored in the extracellular matrix. (4) bFGF is released in a soluble and bioactive form, (5) allowing it to reach its target cell receptor and stimulate angiogenesis. (From Rak, J. and Kerbel, R.S., *Nature Med* 3, 1083-1084, 1997).

Taken together, these studies point to a mechanism whereby FGF-BP1 expression facilitates the release of bFGF stored in the ECM, which then induces angiogenesis, as described in the model in Fig. 2 (Rak and Kerbel, 1997). According to this model, changes in the level of FGF-BP1 expression can shift the angiogenic balance in favor of bFGF activation and an angiogenic phenotype, suggesting that some human tumors can utilize FGF-BP1 as an 'angiogenic switch molecule'.

1.5 A novel secreted Protein with Similarities to FGF- BP1

Recently a human cDNA clone containing an open reading frame (ORF) for a protein, which has amino acid sequence similarity of 21% to FGF-BP1 and a homology of 41 % was discovered by a gene data bank blast search. No further significant homology was found with other known proteins. This certain gene sequence is located 22kb 5' upstream of the FGF-BP1 transcription start site on the short arm of human chromosome 4. It was derived from a BAC clone for human chromosome 4 whose genome sequencing is in progress.

Data on the protein, derived from this gene were exclusively published by Ogawa et al.(Ogawa et al., 2001) In this publication the protein is referred to as 'killer-specific secretory protein of 37 kDa'(Ksp37). Ksp37 is identified as a human Th1-specific protein, its expression being limited peripheral blood leukocytes, namely in Th1-type CD4+ T cells, effector CD8+ T cells, γδ T cells and CD16+ NK cells.

Ogawa et al. show a deduced amino acid sequence that displays a 24% identity to FGF-BP1. They describe a related conformation of the two proteins, concerning the positioning of eight cysteine residues in the signal-truncated form of Ksp37 that are completely conserved in FGF-BP1. They also state that calculated isoelectric points, which are 9.15 for Ksp37 and 9.28 for FGF-BP1 and hydrophobicity profiles are very similar to each other. From these data they suggest that like FGF-BP1, Ksp37 could bind to proteins to regulate their activity and thereby mediate in a yet unknown function.

These data led to the question if this protein has also functional similarity to FGF-BP1. Consequently in the further investigation this novel molecule was named FGF-BP2.

Ogawa et al. present further analysis of particular molecular characteristics of FGF-BP2, which reveal that the deduced primary structure of FGF-BP2 shows nine potential O-glycosylation sites as analyzed by the NetOGlyc program, but no possible N-glycosylation site. The protein was examined by hydropathy blot analysis, revealing two strongly hydrophobic regions at both termini. It is described that the amino-terminal hydrophobic region of the protein has a characteristic secretory sequence with a predicted cleavage site after the glycine of amino acid position 19. This implies that the carboxyl-terminal hydrophobic region, which consists of 14 amino acids, would seem too short to serve as a transmembrane domain. Ogawa et al. suggest from these findings, that FGF-BP2 might be secreted. Indeed, they show that pCMV/T48 transfected COS-7 cells secrete FGF-BP2 as a 37-kDa form, while they retain a 28-kDa species and a 37-kDa form. Also Th-1 cells secrete FGF-BP2 in as a 37-kDa form. From these results it is implied that FGF-BP2 is synthesized as a polypeptide with an apparent molecular mass of 28kDa, then modified, possibly by O-glycosylation, and secreted into the extracellular space as a 37-kDa form.

FGF-BP2 was detected in normal human sera and, consistent with differences in the absolute counts of blood T and NK cells between children and adults, the serum FGF-BP2 levels were significantly higher in children than in adults. It was shown that in patients with infectious mononucleosis, transient elevation of serum FGF-BP2 concentrations were detected during the early acute phase of primary EBV infection. It is postulated that FGF-BP2 must be involved essentially in the process of cytotoxic lymphocyte mediated immunity. Ogawa et al. state that FGF-BP2 did not show direct association with ^{125}I-labeled recombinant human basic FGF and no effect on the exogenous bFGF-dependent or the spontaneous colony growth of SW-13 cells in soft agar.

For further exploration of this protein with regard to functions potentially similar to FGF-BP1, one of the first steps is the analysis of its expression profile outside of Th-1 cells, namely in tumor cell lines. It therefore is beneficial to review the expression of FGF-BP1 in normal and neoplastic tissues, to facilitate a comparison of the expression patterns of both FGF-binding proteins.

1.6 FGF-BP1 Expression in Normal and Neoplastic Tissues

The expression pattern of FGF-BP1 in both normal and neoplastic cells has revealed that the expression of this protein is highly regulated and tissue-specific. The screening of FGF-BP mRNA expression in normal human adult and fetal tissues demonstrated that FGF-BP1 expression is highest in the trachea, colon, uterus and lung (Table 1) (Tuveson, 1998). Notably from these studies, FGF-BP1 mRNA expression is generally positive in the intestinal lining epithelia, particularly in the aerodigestive (trachea, lung, stomach, small intestine, appendix, colon, etc.) and genitourinary (uterus, bladder, prostate, kidney) systems. The observation that all screened neuroectodermal-derived tissues were negative for FGF-BP1 (Table 1) (Tuveson, 1998) is also noteworthy.

FGF-BP1 expression is also restricted to certain types of cell lines and primary tumors. A screening of different human tumor cell lines showed that FGF-BP1 mRNA was positive in most squamous cell carcinoma (SCC) cell lines derived from different origins including lung, bladder, skin, and cervix (Table1) (Czubayko et al. 1994). FGF-BP1 expression was also highly positive in clinical samples of primary and metastatic head and neck SCC (Czubayko et al. 1994). FGF-BP1 was also expressed in colonic adenocarcinoma cell lines and in tumor samples from colon cancer patients (45), as well as in breast carcinoma cell lines and tumor tissues. Most other cell lines of epithelial or mesenchymal origin did not show any detectable FGF-BP1 mRNA expression. The overall expression pattern of FGF-BP1 suggests a role for this growth factor as activator in the SCC as well as in colon cancer and breast cancer. The functional significance of FGF-BP1 expression in colon cancer has been demonstrated and discussed elsewhere (Tuveson, 1998).

Table 1: Expression of FGF-BP1 in Human Tissue, Tumors and Cell Lines.

	FGF-BP1 Positive	**FGF-BP1 Negative**
Normal Tissue	Fetal skin Trachea Lung Colon Stomach Small intestine Appendix Salivary gland Lymph node Thymus Spleen Uterus Bladder Kidney Prostate Testis Ovary Placenta Mammary gland	Brain Bone marrow Heart
Tumor Tissue	Head and neck SCC Colon adenocarcinoma Breast carcinoma (lobular and ductal)	
Tumor Cell Lines	Keratinocytes: Primary (HKc), Immortalized (HFK-SV40, HFK-VP16) SCC: Lung (SW900, NCI-H520, NCI-H596) Bladder (SCaBER) Pharynx (FaDu) Cervix (ME-180, SiHa, HeLa) Epidermoid (A431, A253) Tongue (SCC-25) Buccal mucosa (SqCC/Y1) Colon adenocarcinoma: (LS174T, LoVo, Sk-Co1, LS180) Breast carcinoma: (MDA-MB-231, BT549, T47D, MDA-MB-468, MCF/ADr, MDA-MB-445)	Fibroblasts: (NIH3T3) Adrenal carcinoma: (SW-13) Osteosarcoma: (HOS, MNNG/HOS, MG-63) Glioblastoma: (T98G) Astrocytoma: (U87, SW1088) Hepatocellular carcinoma: (HepG2) Liver adenocarcinoma: (SK-HEP-1)

Table 1: Expression of FGF-BP1 in Human Tumors, Tissues, and Cell Lines. The distinctive expression pattern of FGF-BP1 mRNA in human tumors, tissues, and cell lines. (Czubayko et al., 1994;Tueveson, 1998)

1.7 Mouse FGF-BP1 and its Regulation during Embryonic Development and Skin Carcinogenesis

In order to compare the two FGF-BP genes it is important to consider the mouse FGF-BP1 and its role in skin carcinogenesis. since new data presented here will show a certain significance of FGF-BP2 in this process.

Using sequence information from the human FGF-BP1 cDNA the mouse FGF-BP1 gene was previously isolated by screening a mouse genomic library (Kurtz et al., 1997). The overall nucleotide sequence homology between the mouse and the human cDNA sequences is 68%, and shows no homology with other sequences in genbank or other databases. The mouse FGF-BP1 cDNA predicts a 251 amino-acid protein that shows 63% identity and 74% similarity with the amino-acid sequence of the human FGF-BP1 protein. The high homology between the mouse and human suggests a high selection pressure on the function of this protein. The mouse and human also share similar charge and hydrophobic character. Furthermore, like the human protein, mouse FGF-BP1 is able to non-covalently bind bFGF and stimulate soft agar colony formation when expressed in SW-13 cells (Kurtz et al., 1997)

Similar to the human, expression of the mouse FGF-BP1 in the adult is highly tissue-specific, with high levels in the lung and placenta and somewhat lower levels in the intestine, skin and ovaries (Kurtz et al., 1997). Furthermore, mouse FGF-BP1 expression is also highly regulated during mouse embryonic development. *In situ* analysis of mouse FGF-BP1 mRNA in embryonic tissues showed that expression during all stages of development was highly restricted to the skin, lungs and intestine, with a continuous increase during development, which peaked perinatally and dropped significantly in the adult. In the skin, FGF-BP1 was found in the basal and suprabasal cell layers and hair follicles and expression increased in association with the development of the skin layers. In the adult skin, when morphogenic changes are minimal, FGF-BP1 expression decreased dramatically. These studies demonstrate a strong correlation between FGF-BP1 expression and skin development where it presumably acts in a paracrine manner by mobilizing bFGF stored in the dermal layer of the skin where it plays an inductive role in skin development (du Cros, 1993).

Due to the fact that FGF-BP1 is expressed in a variety of SCCs, in normal human keratinocytes, and in the mouse embryonic skin (Wu et al., 1991; Czubayko et al.,

1994; Kurtz et al., 1997; Vellucci et al., 1995), the role of FGF-BP1 during skin carcinogenesis was explored (Kurtz et al., 1997). The mouse model of skin carcinogenesis describes a predictable multistage process of tumor formation from the clonal expansion of a normal epidermal cell into a benign squamous papilloma, which progresses into a malignant squamous cell carcinoma (reviewed by Yuspa, 1994). In this experimental protocol, a carcinogen induced mutational event is generated through the topical application of DMBA (7,12-dimethylbenz(a)anthracene) to the mouse skin, leading to an initiation event in the cell where altered response to signals for growth and differentiation result in a selective growth advantage. The mouse skin is subsequently treated with repeated applications of a tumor promoter, such as the phorbol ester TPA (12-O-tetradecanoyl phorbol 13-Acetat), causing a selective clonal outgrowth of initiated cells into multiple squamous cell papillomas. Finally, conversion of a papilloma to a malignant carcinoma in this model is generally a spontaneous process, involving a number of genetic and epigenetic aberrations.

Figure 3: Mouse model of skin carcinogenesis.

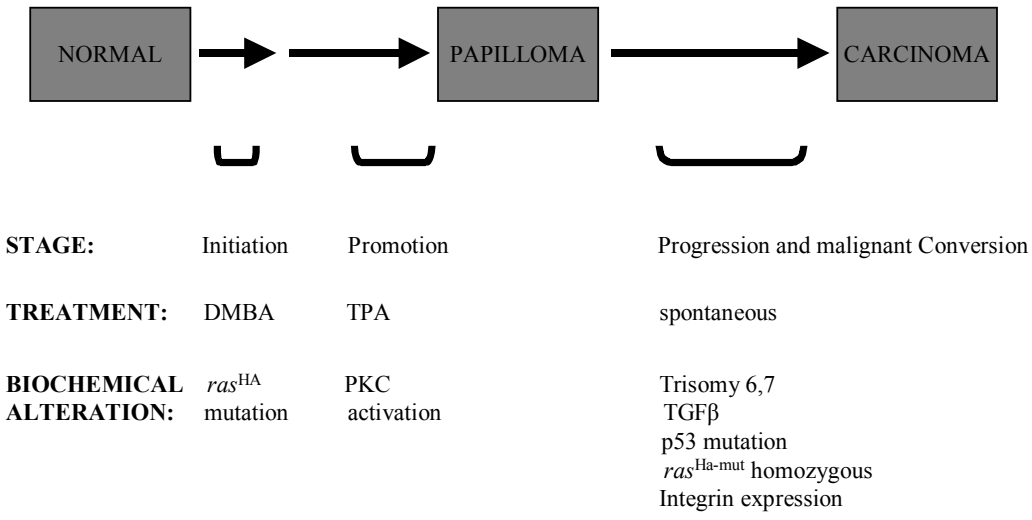

Figure 3: Mouse Model of Skin Carcinogenesis. Multi-stage process of skin carcinogenesis as described through experimental treatment with DMBA/TPA. (Adapted from Yuspa, S., *Cancer Res* 54, 1178-1189, 1994).

Experimental skin carcinogenesis in the mouse, as shown in Fig 2, provides an insightful model for the molecular analysis of multistage carcinogenesis of other epithelial cancers, including other SCCs and also colon cancer (Kinzler and Vogelstein, 1996).

Using this as a model, the role of FGF-BP1 expression during tumor progression was investigated. In DMBA/TPA-induced papillomas, levels of FGF-BP1 mRNA were 4-5 fold higher than in untreated skin and remained upregulated after malignant conversion into SCC (Kurtz et al., 1997). Moreover, FGF-BP1 mRNA was upregulated in cell lines derived from DMBA/TPA-induced papillomas and a cell line derived from DMBA-initiated skin and selected for resistance to Ca^{2+}-induced terminal differentiation (Kurtz et al., 1997). These studies demonstrated a potential function of FGF-BP1 during the early, premalignant stages of skin tumorigenesis, which coincides with the activation of angiogenesis as an early and necessary event in tumor formation.

The role of FGF-BP1 in the formation of human skin SCC was also examined using a similar model to the mouse, where human skin was xenografted onto SCID mice and treated with the DMBA/TPA carcinogen protocol. Similar to the mouse, human FGF-BP1 protein was dramatically upregulated upon carcinogen treatment compared to untreated human skin (Aigner et al., in review). Interestingly, the upregulation of FGF-BP2 was already detectable even before the histological neoplastic change was visible. Furthermore, FGF-BP1 upregulation was paralleled with an increase in microvessel density and positive p53 staining, which is indicative of the onset of carcinogenesis. Both in the mouse as well as in the human, FGF-BP1 is associated with the early neoplastic stages of skin SCC, a stage where angiogenesis is thought to play a critical role.

1.8 Regulation of FGF-BP1 by Fetal Bovine Serum, EGF and TPA

Fetal Bovine Serum (FBS) and mitogens such as Epidermal Growth Factor (EGF) and 12-O-tetradecanoyl phorbol 13-Acetat (TPA) are known to be inductive for the FGF-BP1 mRNA expression as to be determined by Northern blotting analysis.

It was shown that FGF-BP1 mRNA is upregulated in its gene expression by treatment with fetal bovine serum (FBS) (Harris et al., 2001). Treatment of serum starved ME-180 cells with FBS results in a rapid increase in steady-state levels of FGF-BP1 mRNA and in the rate of FGF-BP1 gene transcription. A time-dependence of FGF-BP1 mRNA induction by serum and EGF was shown. Treatment of serum-starved ME-180 cells with 10% FBS resulted in a rapid and transient increase in FGF-BP1 mRNA, which peaked after 6 h. This time course of serum induction is very similar to the induction mediated by EGF (Harris et al., 2000a, Harris et al., 2000b). Also the upregulation of FGF-BP1 gene expression by TPA, which was described earlier (Harris et al., 1998) revealed a similar time course.

From the rapid (within 1h) induction of FGF-BP1 mRNA by serum a direct transcriptional mechanism of activation was suggested and proved by nuclear run on analysis and a transcriptional inhibition of the FGF-BP1 expression by actinomycin D whereas in contrast inhibition of *de novo* protein synthesis with cycloheximide did not block the serum effect. Because both EGF and serum treatment increase FGF-BP mRNA with similar kinetics, it was questioned whether the serum effect was dependent on the EGF receptor. It was shown that this is not the case but that the serum induction of FGF-BP1 must be mediated through an alternate receptor pathway(s). The activation of these pathways together has no additive or synergistic effect on the gene expression of FGF-BP1. Treatment of ME-180 cells with EGF or FBS in combination caused a maximal fourfold induction. Combined treatment with EGF and FBS did not induce FGF-BP1 mRNA higher than fourfold.

Phorbol esters, such as TPA, are potent tumor promoters as pharmacological activators of the protein kinase C (PKC) pathway (Blumberg, 1988). Many of the PKC enzyme family members play an important role in cell growth and differentiation for different cell types, including a well-characterized regulation during normal keratinocyte differentiation (Stabel and Parker, 1991; Nishizuka, 1992; Dlugosz, 1995). PKC activation by TPA or growth factors has pleiotrophic effects. During neoplastic transformation, PKC activation is often a central and critical event in the early stages of tumor promotion, as seen in the model of epidermal carcinogenesis.

Recent studies demonstrated that FGF-BP1 is upregulated during DMBA/TPA treatment of both mouse and human skin (Kuetz et al., 1997; Aigner et al., submitted).

This observation led to the hypothesis that FGF-BP1 may be regulated directly by TPA activation of PKC in human squamous cell carcinoma, where FGF-BP1 is commonly overexpressed. Upon TPA treatment of ME-180 cells, FGF-BP1 mRNA amounts (as determined by Northern Blot analysis) increased in a time- and dose-dependent manner (Harris et al., 1998).

1.9. Regulation of FGF-BP1 by Retinoids

Former studies revealed a well-defined *in vitro* regulation of FGF-BP1 expression by all-*trans* retinoic acid (tRA). Vitamin A (retinol) and its derivatives (retinoids) are necessary for normal growth and development of vertebrates. Experimental models of carcinogenesis have demonstrated that retinoids can suppress carcinogenesis in various epithelial tissues, such as the oral cavity, bladder, lung, prostate and mammary gland (Bertram et al., 1987; Lotan, 1994), as well as directly inhibit the effects of tumor promotion during skin carcinogenesis (Moon et al., 1994; De Luca et al., 1994). More importantly, clinical trials have indicated that retinoids are useful as cancer chemopreventive agents for SCC of the upper aerodigestive tract, skin and cervix, as well as cancers of the breast and ovaries (Hong and Itri, 1994; Lippman et al., 1995; De Palo et al., 1995; Lotan, 1996).

Retinoids have been shown to inhibit the growth and reverse aberrant differentiation of SCC cells in vitro, as shown by changes in expression of squamous differentiation markers (Lotan, 1994). Other studies have shown that retinoids markedly inhibit angiogenesis in the chorioallantoic membrane model (Oikawa et al., 1989) and in an experimental in vivo model of cutaneous angiogenesis in the mouse (Majewski et al., 1993; Majewski et al., 1994).

The mechanism by which retinoids can act to inhibit angiogenesis is currently unknown, although it is likely that retinoids affect multiple targets along the angiogenic process. One target by which retinoids may act to inhibit angiogenesis is the transcriptional inhibition of matrix degrading proteins, such as collagenase or stromelysin, which are required for the matrix remodeling that coincides with new blood vessel formation (Brinckerhoff et al., 1984; Quinones et al., 1989). In addition, retinoids may decrease levels of angiogenic activators, resulting in a shift in the

angiogenic balance in favor of inhibition. Classical angiogenic factors, however, such as FGFs or VEGF, are not known to be regulated by retinoids.

FGF-BP1, however, has been found to be a target for downregulation by retinoids. It was demonstrated that in six different human SCC cell lines FGF-BP1 mRNA is down-regulated by 39-89% within 24 hours (Liaudet-Coopman et al., 1996). In the ME-180 cell line it was shown that tRA down-regulates FGF-BP1 mRNA in a time- and dose- dependent manner. The maximum effect was reached 8-12 h after tRA exposure in a concentration of 10^{-5} M. Since tRA massively decreases the FGF-BP1 mRNA half life from >16 hours to 5 hours and also decreases gene transcription by 2- to 3-fold within 1hour it was suggested that the down-regulation of FGF-BP1 mRNA is achieved through a combination of transcriptional and posttranscriptional mechanisms (Liaudet-Coopman et al., 1996). Furthermore, it was demonstrated that in vivo tRA treatment of SCC xenografts in athymic nude mice reduces FGF-BP1 mRNA expression, correlating with inhibition of angiogenesis, induction of apoptosis and a decrease in tumor growth rate (Liaudet-Coopman et al., 1997). These results indicate that the tRA-induced downregulation of FGF-BP1 contributes to the reduction of angiogenesis in SCC, presumably due to the inability of FGF to become activated. Further studies will have to answer the question if this regulation also applies to FGF-BP2.

Most of the actions of retinoids are mediated through high affinity nuclear retinoic acid receptors (RARs) and retinoic ‘X’ receptors (RXRs) (reviewed by Chambon, 1996; Mangelsgorf and Evans, 1995). Several of the human epidermal keratin genes have been shown to be transcriptionally downregulated by retinoids through RAR binding in their promoter regions (Tomic et al., 1990; Stellmach et al., 1991; Tomic-Camic et al., 1992; Tomic-Camic et al., 1996). Also the downregulation of the stromelysin and of the collagenase genes by retinoids is mediated through binding sites in their respective promoters (Nicholson et al., 1990; Lafyatis et al. 1990; Kamei et al., 1996). It seems likely that many of the therapeutic effects of retinoids are related to its RAR-mediated competing inhibition of transcription factor activity. In that respect, several synthetic retinoids have been found which transrepress as efficiently as retinoic acid, but are totally ineffective for transactivation (Chen et al., 1995; Fanjul et al., 1994; Nagpal et al., 1995). Such “dissociated” synthetic retinoids promise to be interesting candidates for further drug design.

2.10 The objective of this study

FGF-BP2 is a new interesting candidate with potential similar functions to FGF-BP1

One can hypothesize that in addition to structural homologies, FGF-BP2 also displays functional analogies to FGF-BP1. Therefore the expression profile of FGF-BP2 in human tissues and tumor cell lines was analyzed by Northern blotting, and serum, TPA, EGF or retinoid-mediated regulation of FGF-BP2 expression was analyzed. Finally, the tumorigenic potential was determined by FGF-BP2 overexpression.

Specific Tasks:

1. Cloning the FGF-BP2 cDNA
2. Construction of FGF-BP2 expression vector
3. Screening tissues and various tumor cell lines for FGF-BP2 expression
4. Comparison of the expression patterns of FGF-BP1 and FGF-BP2
5. Analyzing FGF-BP2 regulation in two melanoma cell lines under treatment with drugs and growth factors
6. Transfecting the FGF-BP2 cDNA for overexpression
7. Performing a soft agar assay with FGF-BP2 transfected cells

2. MATERIAL

2.1 Chemicals

Agarose, Electrophoreses Grade	Ultra Pure, Life Technologies, USA
Ampicillin (aminobenzylpenicillin)	USB, USA
Chloroform	EM Science, USA
DEPC	Quality Biological Inc., USA
DMSO	Mallinckrodt AR ®, USA
Ethidium bromide	Oncor, USA
Ethanol	SIGMA, USA
Formaldehyde (37 % v/v Solution)	SIGMA, USA
Formamide	Boehringer Mannheim Corp., USA
Isopropanol	Fischer Scientific, USA
SDS	SIGMA, USA
Sodium Chloride	Fischer Scientific, USA
Sodium Citrate	EM Science, USA
EDTA	Biofluids, Inc., USA
Tryptone	Difco Laboratories, USA
Yeast Extract	Difco Laboratories, USA
Tris-Base	Life Technologies, USA
Boric Acid	SIGMA, USA
Geniticin® (G418 Sulphate)	Life Technologies, USA
Glycerol	SIGMA, USA
Bromphenol Blue	Fischer Scientific, USA
Bacto-Agar	Difco Laboratories, USA
BSA	SIGMA, USA

2.2 Working Material and Apparatus

MicroSpin™ S-200 HR columns	Amersham, UK
Ultracentrifuge Sorvall RC-5B	Du Pont Instruments, USA
Centrifuge tubes	Seton, USA
Speedvac concentrator	Savant, USA
Hybridization oven	Hybaid, Labnet, USA
Liquid scintillation counter 1209	Rackbeta Pharmacia, USA
DNA gel apparatus	OWL Scientific, USA
RNA gel apparatus	OWL Scientific, USA
Spectrophotometer	Pharmacia, USA
PCR machine Robocycler 40	Stratagene, USA
Image analyzer Eagle Eye II	Stratagene, USA
UV light box	Fischer Scientific, USA
Micro centrifuge 5415 C	Eppendorf, USA
Nitrocellulose membrane	Micron Separations Inc., USA
Whatman paper	Whatman International, Ltd., USA
Parafilm	American National Can ™,USA
Gel electrophoresis camera OSP	IBI, USA
Incubator Shaker Innova 4000	New Brunswick Scientific, USA
Heat Blocks	Fischer Scientific, USA
UV Stratalinker 1800	Stratagene, USA
Electrophoresis power source EPS 600	Pharmacia, USA
Phosphor Imager 445 SI	Molecular Dynamics,USA

2.3 Enzymes

Restriction Endonucleases	Concentration	Reaction Buffer	Source
BamH I	20 U/µl	NEBuffer 2	New England Biolabs Inc
Xba I	20 U/µl	NEBuffer 2	New England Biolabs Inc.
Hind III	20 U/µl	NEBuffer 2	New England Biolabs Inc.

2.4 Molecular Weight Standards

1 kb DNA ladder	Life Technologies, USA

2.5 Vectors

pCR® 3.1 (5.0 kb)	Invitrogen®, USA
pcDNA3.1/*Myc*-His (5.5 kb)	Invitrogen®, USA

2.6 cDNA Probes for Nothern Blot Analysis

GAPDH - cDNA	Clontech (1.1 kb)
FGF-BP 2 ORF cDNA	BamH I/Xba I Fragment (700 bp) isolated from pCR ® 3.1 /FGF-BP2 Vector
FGF-BP1 cDNA	Dr. Violaine K. Harris, Lombardi Cancer Center

2.7 Molecular Biology Reagents

Salmon Sperm DNA	Life Technologies. USA
T4 DNA Ligase	Invitrogen®, USA
10x Ligation Buffer	Invitrogen®, USA

2.8 Kits and Reagents

RNA Stat-60	TEL-TEST Inc., USA
QIAEX II Gel Extraction Kit (150)	Qiagen Inc., USA
Qiagen Plasmid Midi/ Maxi Kit	Qiagen Inc., USA
Rediprime ™ II random prime labeling system	Amersham, UK
Scintillation Fluid Bio-Safe II ™	RPI Corp., USA
LIPOFECTAMINE™	Life Technologies, USA

2.9 Radioisotopes

Radioisotope	**Half life (t ½)**	**Specific Activity**	**Concentration**	**Source**
α-(^{32}P) dCTP	14.3 days	6000 Ci/mmol	10 µCi/µl	Amersham, USA

2.10 Bacterial Cells

Escherichia coli DH5α	Life Technologies, USA

2.11 Bacterial Growth Media and Plates

LB Medium	1 % (w/v) Tryptone 0.5 % (w/v) Yeast extract 1 % (w/v) NaCl pH adjusted to 7.5
LB Plates with Ampicillin	LB medium 1.5 % (w/v) Bacto-Agar Ampicillin 50 µg/ml

2.12 Cell Culture Materials

IMEM	Biofluids, Inc., USA
Leibovitz's L15 Medium	Life Technologies, USA
OPTI–MEM® I Reduced Serum Medium	Life Technologies, USA
Keratinocyte-SF Medium	Life Technologies, USA
Fetal Bovine Serum	Life Technologies, USA
Trypsin/EDTA	Life Technologies, USA
Cell Culture Freezing Medium with DMSO	Life Technologies, USA
Cell Culture Flasks (Vent, 75 & 162 cm^2)	Costar, USA
Cell Scraper	Costar, USA
Bottle Filter System	Corning, Inc., USA

2.13 Mammalian Cell Lines

Table 2: Source and Classification of the Cancer Cell Lines

Cell Type	Cell Line	Source
Melanoma	1205LU	Dr. M. Herlyn, Wistar Institute, Philadelphia
	SK- MEL- 5	ATCC (HTB 70)
	SK- MEL- 24	ATCC (HTB 71)
	SK- MEL- 31	ATCC (HTB 73)
Prostate Carcinoma	LNCaP- FGC- 10	ATCC (CRL 1740)
	DU 145	ATCC (HTB 81)
	PC-3	ATCC (CRL 1435)
Colon Carcinoma	COLO 357	Dr. H. Juhl, Lombardi Cancer Center, Washington, DC
	LS 180	ATCC (CL 187)
	HT-29	ATCC (HTB 38)
	SW 480	ATCC (CCL 228)
	SW 680	Lombardi Cancer Center Tissue Culture Core Facility
Breast Carcinoma	BT- 20	ATCC (HTB 19)
	BT- 549	ATCC (HTB 122)
	MCF- 7	ATCC (HTB 22)
	MDA- 231	ATCC (HTB 26)
	MDA- 435	ATCC (HTB 129)
	SK- BR- 3	ATCC (HTB 30)
	MDA- 468	ATCC (HTB 132)
	MDA- 460	Lombardi Cancer Center Tissue Culture Core Facility

Cell Type	Cell Line	Source
Adrenal Carcinoma	SW-13	ATCC (CCL105)
Cervical Carcinoma	ME-180	ATCC (HTB 33)
Choriocarcinoma	JAR	ATCC (HTB 144)
	JEG- 3	ATCC (HTB 36)
Pancreatic Carcinoma	PANC- 89	Lombardi Cancer Center Tissue Culture Core Facility
Leukemia	HL-60	Lombardi Cancer Center Tissue Culture Core Facility
	Jurkat	ATCC (CRL 8163)
	K-562	ATCC (CCL 243)
Hepatocellular carcinoma	Hep G2	ATCC (HB8065)
Glioblastoma	U-87	ATCC (HTB 14)
	U-138	ATCC (HTB 16)
	U-373	ATCC (HTB 17)
	T-98	Lombardi Cancer Center Tissue Culture Core Facility
	A-172	ATCC (CRL 1620)
	Hs 683	ATCC (HTB 138)
Other	WI-38 (Lung)	ATCC (CCL 75)
	HU-VEC (Human umbilical vein endothelial cells)	ATCC (CRL 1730)
Neonatal melanocytes		Lombardi Cancer Center Tissue Culture Core Facility

Table 2: Source and Classification of the Cancer Cell Lines. All cell lines that were screened for FGF-BP2 expression are listed for their type and origin.

2.14 Buffers and Solutions

10 x TBE	1 M Tris-Base 0.8 M Boric Acid 20 mM EDTA
10 x MOPS	Quality Biological, Inc., USA
DNA Running Buffer	1 x TBE
DNA Loading Buffer	0.1 mM EDTA 0.2 % (w/v) SDS 50% (v/v) Glycerin 0.005 % (v/v) Bromphenol Blue in 1 x TBE
RNA Running Buffer	1 x MOPS in DEPC-H_2O
RNA Loading Buffer	50 % (v/v) Formamide 17.5 % (v/v) Formaldehyde 1 x MOPS 10 % (v/v) Bromphenol Blue in DEPC- H_2O
50x Denhardt's Buffer	2 % (w/v) Polyvinyl-Pyrrolidone 2 % (w/v) BSA 2 % (w/v) Ficoll
20 x SSC	3 M Sodium Chloride 0.3 M Sodium Citrate
DEPC-H_2O	0.1 % (v/v) DEPC

2.15 Northern Blot Solutions

Prehybridization Solution	50 % (v/v) Formamide 5 x SSC 1% (w/v) SDS 5 x Denhardt's Buffer 100 µg/ml Denatured salmon sperm DNA
Washing Solutions	(1) 2 x SSC (2) 2 x SSC/1 % (w/v) SDS (3) 0.1 x SSC
Stripping Solution	0.1 x SSC

2.16 Compounds for Cell Treatment

12-O-tetradecanoyl phorbol 13-Acetat (TPA), Solvent: 100% DMSO	SIGMA, USA
all-*trans*-retinoic acid, Solvent 100% DMSO (tRA)	SIGMA, USA
human recombinant Epidermal Growth Factor (EGF)	Clonetics, USA
human recombinant Basic Fibroblast Growth Factor (bFGF)	Life Technologies, USA

3. METHODS

3.1 General Laboratory Techniques

3.1.1 Sterilization of Solutions and Work Materials

Solutions, tubes, glassware, plastics, deionized H_2O and other items were sterilized by autoclaving for 45 min at 121^{o} C

3.1.2 Determination of DNA and RNA Concentrations

A spectrophotometer was used to measure the concentration of nucleic acids in solutions. 2µl of DNA or RNA samples were added to 98 µl sterile H_2O (1:50 dilution) for an optical density (OD_{260nm}) reading. The DNA concentration was determined based on the fact that an absorption of 1 at 260nm is equivalent to a DNA concentration of 50µg/ml. Absorption (OD_{260nm}) x 50 µg/ml x Dilution Factor (50) = µg/ml DNA. For RNA, an absorption of 1 at 260nm corresponds to an RNA concentration of 40 µg/ml. Absorption (OD_{260nm}) x 40 µg/ml x Dilution Factor (50) = µg/ml RNA. The purity of nucleic acid in solution can be determined by dividing the absorption's ($OD_{260/280\ nm}$), as peptides bonds absorb UV light at 280nm. Protein contaminated DNA or RNA solutions have a ratio of < 1.7.

3.1.3 Work with RNA

Precautions were used while isolating and handling RNA in order to prevent degradation. All used solutions, glassware and plastics were sterilized to ensure the absence of RNase, which is an extremely stable enzyme requiring no cofactors to exert its effect of RNA digestion and degradation. Everything was handled only while wearing gloves. Workbench, RNA gel apparatus, gel tray and gel combs were cleaned with RNaseZAP™ (RNase-Inhibitor). RNA was always dissolved in DEPC-H_2O and stored at –80^{o} C or on ice while handling.

3.1.4 Work with DNA

Although DNA is by far not as sensitive to degradation as RNA it was also kept on ice while working with it and stored in a freezer at –20^{o} C.

3.1.5 Work with Radioactive Isotopes

The only isotope used in this study was ^{32}P, which was purchased as an incorporated component of the deoxyribonucleotide Cytosine Tri-Phosphate (dCTP). Radioactive phosphorous (^{32}P) emits β-radiation and has a maximum principle emission of 1.709 MeV. Radioactive nucleotides were kept in a safety container at –20^{o} C and thawed behind a Plexiglas shield immediately before usage. All radioactive contaminated waste, liquid and solid, was disposed in special radioactive waste containers.

3.1.6 Gel Electrophoresis of Nucleic Acids

DNA Agarose Gel Electrophoresis

1 % (w/v) agarose was added to100ml 1x TBE buffer and heated until the agarose was completely dissolved. Next, 5µg/ml ethidium bromide was added to the gel solution for DNA visualisation and the gel was poured into a gel tray. Before its polymerization (20 min.) combs were placed in the gel to create wells. The DNA solutions (i.e. plasmid DNA or PCR product) were mixed with 20µl of DNA loading buffer and loaded in the wells. The DNA fragments were separated by electrophoresis in parallel to a 1 kb DNA ladder for 1-2 hours at 80-100 volts in DNA running buffer. DNA bands were detected under UV light in the image analyzer or on an UV light box.

RNA Formaldehyde Gel Electrophoresis

1 % (w/v) agarose (1g according to a total volume of 100ml) was heated in 87 ml DEPC H_2O until the agarose had completely dissolved. 10 ml 10x MOPS buffer were added to achieve a final dilution of 1x MOPS. This was followed by the addition of 3ml formaldehyde to make it a 3% solution and 5µg/ml ethidium bromide. The gel was poured under the chemical hood due to the toxicity of formaldehyde. Combs were stuck in the gel to form loading wells. Once the gel was polymerized it was allowed to set for 1 hour in 1x MOPS buffer. The RNA, which was dissolved in 20 µl RNA loading buffer was denatured at 65^o C for about 10 min and loaded into the wells. The RNA was separated at 60-80 volts for about 3 hours in RNA running buffer. Native RNA molecules exist in a folded confirmation as partially double stranded molecules which would interfere with their electrophoretic mobility as well as their accessibility to hybridization. To prevent this, the denaturing agent formaldehyde was used in the gel.

3.1.7 Work with Bacteria

Inoculation

A streak of transformed (see section 3.2.4) bacterial cells taken from an overnight culture was spread onto a Bacterial Stam Plate to generate single colony growth. Bacteria were picked from a mother colony into 5ml LB medium, containing 50µg/ml Ampicillin, and incubated in a 37^o C shaker at 220-rpm overnight. Inoculation of bacterial cells into LB growth medium was performed close to an open flame fire to prevent air borne bacteria from contaminating the media. These overnight cultures were used for Qiagen Mini Prep, or as a starter culture for Qiagen Maxi Prep and transferred into 250 ml LB medium for another overnight incubation at the same conditions.

3.1.8 Work with Mammalian Cell Lines

Handling of Human Cell Lines

Handling and propagation of all cell lines were performed in a cell and tissue culture laminar-flow hood under sterile conditions. All solutions were stored at 4^{o} C and warmed up to 37^{o} C in a water-bath before using. All solutions were only opened under sterile conditions in a hood. Cell culture media and FCS used for all cell lines were sterile filtered in the hood. All cell lines were grown in IMEM 10 % FCS, except the cell line1205 LU was grown in ¾ Keratinocyte-SF-Medium, ¼ Leibovitz's L15 Medium, 5 % FBS.

Thawing of Cultured Human Cell Lines

The cell line aliquots were stored in 1 ml freezing medium at –80^{o} C. To grow up a cell line the freeze down was thawed quickly in a 37^{o} C water-bath then transferred into a 15ml tube and mixed with 10 ml of medium. The suspended cells were centrifuged at 1,000 g for 5 min. in order to isolate a cell pellet free of DMSO residues from the freezing medium. After removing the supernatant the pellet was resuspended in 10 ml medium and transferred into a T75 cm^{2} cell culture flask. The cells were stored in a 5 % CO_2, 37^{o} C incubator.

Maintenance of Human Cell Lines

All cell lines were grown in a 37^{o} C incubator and split in certain ratios depending on the stage of confluence and the proliferation rate of each cell line. The amount of medium added to the flask was dependent on its size. 10 ml were added into T75 flasks whereas 20 ml were added to the T 164 flasks. The medium was changed every other day.1/10 of the amount of culture medium was used as the amount of Trypsin/EDTA supplied to a culture flask in order to detach the cells. To split cells the medium was completely

aspirated from the flask and the cells were washed briefly with 2 ml of Trypsin to remove traces of Antitrypsin originating from the serum in the media. After removing the Trypsin another 2ml Trypsin were placed into the T 164 and 1 ml into the T 75 flasks. The cells detached after about 5 min with occasional gentle tapping. The Trypsin was then immediately inactivated by adding 3ml of medium to each flask. This suspension was transferred into a 50ml tube and centrifuged at 1,000 g for 5 min. The supernatant was aspirated and the cell pellet was resuspended in medium and split into 3 to 5 new flasks. For each experiment, each individual cell line was pooled during the splitting process to assure equal amounts of cells plated into each flask.

Freezing Cultured Human Cell Lines

To freeze cells for long term storage, cells were harvested (at least one T75 flask of 80 % confluent cells) and centrifuged at 1,000 g for 5 minutes. The media was then aspirated and cells were resuspended in 1 ml of cell culture freezing medium. The resuspended cell solution was transferred to Cryo-tubes and placed at -70^{o} C in an isopropanol-containing cell freezing container, which guarantees a slow freezing process. After 24 hours the tube can be stored at -70^{o}C in a regular box.

3.2 The BP2 cDNA BAC clone

The FGF-BP2 full length sequence was obtained from a human chromosome 4 BAC clone C0024K08 (GenBank accession number AC005598), where it is covering the sequence between nucleotides 132697 – 135582. The BP2 open reading frame cDNA extending from an ATG start codon at nucleotide 64 to a TGA stop codon at nucleotide 736 was already cloned into the BamHI/XbaI site of the pC4 vector which was kindly provided by Dr. Yanggu Shi, Human Genome Sciences, Rockville, MD, USA. The pC4 vector is a Human Genome Sciences in-house vector. This construct consists of a CMVmi promotor driving expression of the full length ORF of FGF-BP2.

3.3 Cloning of the FGF-BP 2 cDNA into the Vector pCR ® 3.1

For further work with the FGF-BP2 cDNA, the BamHI/XbaI fragment from the vector pC4, containing the FGF-BP2 cDNA, was subcloned into pCR ® 3.1 which is a commercially available vector. The pCR® 3.1 vector contains BamHI and XbaI sites in its multiple cloning site. It was ensured that neither of the restriction endonucleases would cut inside of the insert by checking the nucleotide sequence for the enzymes' restriction sites. The new construct was named pCR3.1/ FGF-BP2.

3.3.1 Restriction Digest of the Expression Vector pC4

For subcloning purposes 20 μg of the plasmid DNA of the pC4 vector was digested with two restriction endonucleases to obtain the FGF-BP2 ORF insert. The restriction reaction was carried out in a total volume of 50 μl, containing the plasmid DNA, 30 U of the restriction endonuclease BamHI, 30 U of the restriction endonuclease XbaI and 10% of NEBuffer 2. To start the reaction, the restriction endonucleases were added last and the reaction was incubated at 37^{o} C for 1 hour to allow the enzymes to work at optimal condition. After that 1μl of the sample was loaded on a DNA gel for electrophoresis. Samples were run on the gel with a 1 kb DNA marker at 40 Volts for 120 min. The gel was checked on an UV light box for a correct, complete digest and the presence of a 700bp insert. The total amount of the restriction reaction was loaded on a DNA gel and separated.

3.3.2 Gel Extraction

The FGF-BP2 cDNA insert was obtained by gel extraction according to the Qiagen Gel Extraction Kit protocol. The insert was then cut out of the gel using a sterile razorblade and placed into a microfuge tube. The total weight of the gel containing the BP2 fragment was about 200 mg. The first step was to solubilize the gel slice by adding 3 volumes (μl) of Buffer QX1 to 1 volume (mg) of gel. Next DNA was bound to the silica particles in

the QIAEX II suspension by adding 30µl of QIAEX II to the sample and incubating at 50° C for 10 min. The sample was vortexed every other minute to keep the silica particles in suspension. The buffer QX1 contains a pH indicator, which allowed the optimal pH at < 7.5 for DNA adsorption to be determined. Next, the sample was centrifuged for 30 sec and the supernatant was removed. To remove residual agarose 500 µl of Buffer QX1 were added, the pellet was resuspended by vortexing, centrifuged for 30 sec and the supernatant was removed. To remove residual salt contaminants the pellet was washed twice in 500 µl of Buffer PE. After vortexing the sample to resuspend the pellet and subsequent centrifugation all traces of supernatant were removed. The pellet was air-dried for 10 min. Afterwards the DNA was eluded by resuspending the pellet in 20 µl H_2O. The sample was incubated for 5 min at room temperature. The resuspention was then centrifuged and the supernatant, now containing the purified DNA, was removed. The DNA concentration was determined by spectrophotometry.

3.3.3 Linearization of the Vector pCR® 3.1

The BP2 cDNA digested with BamHI and XbaI was ligated into the BamHI site of the expression vector pCR® 3.1. To accomplish this, the vector first had to be cut at its BamHI and XbaI sites to linearize it, allowing ligation of the insert at this site. The linerization of the vector was performed in a total volume of 50 µl. 20µg of the vector were digested in 10 % NEBuffer2 with 30 U BamHI and 30 U XbaI under the same conditions as described in 3.3.1. Next, the linearized vector was purified by gel extraction in the same procedure as described in 3.3.2. The concentration was determined.

3.3.4 Ligation of theFGF-BP2 ORF into the Vector pCR® 3.1

The FGF-BP2 insert was ligated into the BamHI/XbaI site of the pCR® 3.1 vector. The ligation reaction was carried out in a total volume of 10µl. The ligation was set up in a 1 : 23 molar ratio of vector : insert. 50 ng of the linearized vector were resuspended in 2µl H_2O, 150 ng of the insert were resuspended in 1µl H_2O. 5 µl of H_2O, 1 µl of 10x

Ligation Buffer and 1μl of T4 DNA Ligase were mixed with the vector and the insert and the ligation were incubated at 16 ^{o}C for 6 hours.

3.4 Transformation of the DH5α™ Cells with the FGF-BP2 Plasmid

In order to obtain plasmid DNA for purposes of transfection, sequencing and generating probes, the vector containing the cloned FGF-BP2 ORF was transformed into a clone of DH5α ™ bacterial cells. The competent cells were removed from a -70^{o} C freezer and thawed on ice. Next, 2 μl of mercaptoethanol and 5ng of DNA, in 2 μl of the ligation reaction, were added to a 100 μl aliquot of the cells. The tube was gently tapped to mix and then incubated on ice for 30 min. To heat shock the cells after the incubation, the sample was placed in a 42^{o} C water bath for 45 sec, and then placed on ice for two minutes. 450 μl of LB medium was added, and the sample was amplified by incubating for 1 hour at 37^{o} C while shaking at 225 rpm. A 1:10 dilution of the sample was made and 100 μl of the dilution was spread out on LB agar growth plates containing 100μg/ml Ampicillin. The plate was incubated in a bacterial cell incubator at 37^{o} C overnight. Transformed bacteria form a single colony growth on the Ampicillin selective milieu. The growth plates were stored at 4^{o} C

3.5 DNA Plasmid Purification

DNA plasmids were purified for purposes of analytical restriction digests, sequencing and generation of probes. The plasmid purification was achieved by using Qiagen QIAprep Miniprep Kit for plasmid screening of transformed bacterial colonies and Qiagen Plasmid Maxiprep Kit for purification of larger amounts of DNA for sequencing and generation of probes.

3.5.1 Qiagen Miniprep DNA Plasmid Isolation

Bacterial cells were inoculated and incubated (3.2.1) in 5 ml LB medium containing 50µg/ml Ampicillin. The cultured cells were centrifuged at 3,000 x g for 10 min in order to form a bacterial cell pellet. The medium was aspirated and the pellet was resuspended in 250µl Buffer P1, containing RNase A, and transferred into a fresh tube. For alkaline cell lysis 250µl Buffer P2 (NaOH/SDS) were added and mixed with the solution by gently inverting the tube. The lysis reaction, that solubilizes the phospholipids and proteins of the cell membrane (SDS) and denatures the chromosomal and plasmid DNA (NaOH), was allowed to proceed for 5 min. Then 350µl of Buffer N3 were added for neutralization purposes. To avoid any localized precipitation, the solution was mixed immediately after the addition of buffer. The sample was centrifuged at 3,000 x g for 15 min. The cell debris and SDS precipitant form a pellet. The plasmid DNA is in suspension in the supernatant, which was then transferred to a QIAprep spin column and centrifuged at 3000g for 1 min. The silica-gel membrane ensures a selective absorption of plasmid DNA in high salt buffer. To wash the column salt contaminants, 750µl Buffer PE were added and centrifuged for 1 min at 3000g. To elute plasmid DNA, the column was placed on a clean microfuge tube and 30µl H_2O were added in the center of the column and collected. After waiting for 3 min the column was centrifuged at 3000g for 1min. Due to the low salt concentration of the H_2O the plasmid DNA is eluted from the column. The DNA yield was defined by spectrophotometric reading.

3.5.2 Qiagen Maxiprep DNA Isolation

The plasmid DNA purification principle according to the Qiagen Maxiprep is similar to the process of the Qiagen Miniprep. Bacterial cells were incubated in 250 ml LB medium, containing 100µg/ml Ampicillin. To harvest the bacterial cells, the culture was centrifuged at 6,000 x g for 15 minutes. All traces of supernatant were removed and the bacterial cell pellet was resuspended in 4ml Buffer P1 (RNase added). The suspension was transferred into a fresh tube and 4 ml of Buffer P2 were added. The solution was

mixed and incubated at room temperature for 5 min. Next, 4 ml of chilled Buffer P3 were added and immediately mixed with the solution. The sample was incubated on ice for 15 min and then centrifuged at 20,000 x g for 30 min at 4^{o} C. Meanwhile Qiagen-tip 100 columns were equilibrated by applying 4 ml Buffer QBT on the column and allowing it to empty by gravity flow. The supernatant from the centrifugation was immediately removed and applied to the equilibrated column and allowed to enter a resign by gravity flow. Afterwards the column was washed twice with 10 ml of Buffer QC. Adding 15 ml of Buffer QF onto the column eluted the plasmid DNA. The DNA then was precipitated by adding 10 ml isopropanol. The sample was mixed and subsequently centrifuged at 15,000 x g for 30 min at 4^{o} C. The supernatant was decanted and the DNA pellet was washed with room-temperature 70% ethanol and centrifuged at 15 000g for 10 min. The supernatant was aspirated and the pellet was air- dried for 5-10 min. The plasmid DNA then was dissolved in 50-100 µl H_2O and the concentration was determined.

3.6 Sequencing of theFGF- BP2 ORF

To verify the correct subcloning of the FGF-BP2 insert the plasmid DNA was sequenced. The sequencing was provided by the Lombardi Cancer Center Sequencing Core Facility. The sequencing was done by automated cycle sequencing (ABI PRISM Dye Terminator Cycle Sequencing, Perkin-Elmer). The pCR® 3.1 vector contains a T7 promotor/priming site at the 5'end of the multiple cloning site which was used as the sequencing primer. An amount of 500 ng of the purified plasmid DNA was used for the sequencing.

3.7 Stable Transfection of the Cell Line SW13

The expression vector pCR® 3.1 containing the FGF-BP2 cDNA was stably transfected into the SW-13 cell line. This cell line originates from an adrenal cell carcinoma and is non-tumorgenic. The SW 13 cells were transfected with the pCR ® 3.1 vector containing

the FGF-BP2 ORF whose expression is driven by the CMV promoter. The cells were also transfected with an empty pCR3.1 vector as a negative control. The stably transfected cells were selected after Geneticin® (G418) treatment. The SW 13 cells were grown to 50-70 % confluency (2 x 10^5) in T75 cm^2 flasks. Then 1µg of the plasmid DNA was diluted in 100µl OPTI–MEM® I reduced serum medium. In a second tube 7µl of LIPOFECTAMINE was diluted in 100µl of OPTI–MEM® I. Both solutions were combined, gently mixed and incubated at room temperature for 30 min to allow DNA-liposome complexes to form. Meanwhile the cells were rinsed in serum-free medium. 0.8 ml OPTI-MEM® I was added to the tube containing the complex. The sample was mixed gently and the diluted complex solution was overlaid onto the rinsed cells. The cells were incubated with the complexes for 5 hours at 37^o C in a 5% CO_2 incubator. The transfection medium was then replaced with IMEM 10 % FCS and incubated for 24 hours. The stably transfected cells were selected by culturing them in IMEM 10% FCS in the presence of 500µg/ml Geneticin® with a medium change every other day. The expression of the BP 2 ORF was shown by Northern Blot Analyses.

3.8 Soft agar growth assay

In order to examine a potential role of FGF-BP2 in tumor growth the SW-13 cell line was used as model system. This cell line expresses high levels of FGF-2, but lacks both FGF-BP1 and FGF-BP2 expression. (Introduction 2.5 Results 4.6). Furthermore, wild type SW-13 cells do not form colony in soft agar unless they are supplemented with exogenous FGFs or transfected with FGF genes with secreted signal peptides (Wellstein et al. 1990). To study an anchorage-independent growth of the FGF-BP2 transfected SW-13 cells a soft agar assay was carried out. The SW-13 cells, transfected with the empty vector were used as a control. Melted, pre-sterilized Bactoagar in a concentration of 1.2g inn 50ml water was mixed with 100ml of IMEM, 50ml of 2x IMEM, and 20% of FCS and kept at 42-44 C. From this stock, a 1-ml bottom layer of 0.6% agar was formed in 35-mm dishes and allowed to solidify at room temperature. The transfected SW-13 cells

were cultured, grown and harvested as previously described and suspended in a concentration of about 10,000 – 20,000 cells in 0.5 ml total volume. This cell suspension mixed with an equal volume of culture medium, in some experiments containing FGF-1 or FGF-2 in various concentrations (0.05, 0.10, 0.25, 0.50, 1.00 ng/ml) and 1.5g of the agar stock solution. This suspension (0.36% of agar) was layered onto the bottom layer (0.8 ml/dish), and allowed to solidify. The dishes were then incubated at 37 C for 10 – 14 days. The number of colonies of 40 – 60 μm in diameter were hereafter counted using an image analyzer. All experiments were carried out in triplicate sets of dishes.

3.9 Preparation of cDNA Probes for Northern Blot Analysis

The aim of the Northern Blot Analysis is to pick up a mRNA signal out of the total RNA of a cell line or tissue homogenate. To accomplish this the single stranded denatured RNA is exposed to a radioactive labeled and denatured DNA probe in order to form a hybridization between the two complementary nuclic acids strings. The two cDNA molecules used were the FGF-BP2 ORF cDNA , the FGF-BP1 cDNA and the cDNA coding for glyceraldehyde-3 phosphate dehydrogenase (GAPDG). GAPDH is an essential enzyme and ubiquitously expressed in every used cell line. Therefore, the GAPDH mRNA expression was used as a loading control and the FGF-BP2 mRNA expression level was normalized to it.
The GAPDH cDNA from Clontech is commercially available. The FGF-BP2 cDNA open reading frame is 700bp long, and was obtained by digesting it out of the multiple cloning site of the pCR ® 3.1 vector, where it was previously cloned. (Methods 3.3)

3.9.1 DNA Isolation

A single colony of FGF-BP2 transformed E.coli DH5α was picked from an Ampicillin selective LB bacterial growth plate inoculated and incubated (Methods 3.1) in order to

perform a DNA Isolation according to Qiagen Plasmid Maxi Kit (Methods 3.5). The total plasmid DNA was diluted in 100 µl H_2O.

3.9.2 Digestion of the FGF-BP2 Fragment

For generation of the hybridization probe plasmid DNA was digested with the restriction endonucleases BamH I and Xba I which cut at either end of the FGF-BP2 ORF in the multiple cloning side, producing the 700 bp FGF-BP2-ORF insert as a fragment. The restriction was performed in a total volume of 50 µl, containing 50 µg of the plasmid DNA, 10 % NEBuffer2, 30 U of the restriction endonuclease BamH I and 30 U of the restriction endonuclease Xba I. The restriction reaction was carried out in the same conditions as described in 3.2.3. To test for complete digestion a sample of 1µl of the reaction was loaded on a DNA gel for electrophoresis. After electrophoresis the DNA fragments were visualized on an UV light box to judge the correct and complete digest. The now linearized plasmid runs at 5 kb where as the FGF-BP 2 open reading frame runs at its size of 700 bp, identified by comparison with the 1kb DNA molecular weight marker. The total digest was loaded on the gel, separated and the insert was cut out from the gel by using a sterile razorblade. Any excess agarose was carefully removed. The isolated gel section, containing the FGF-BP 2 insert, was placed in a tube.

3.9.3 Gel Extraction

The gel extraction was accomplished by using the Qiagen QIAEX II Gel Extraction Kit. The estimated amount of the restricted FGF-BP 2 cDNA insert, 1/8 of the total volume, was about 6 µg. The total amount of gel containing the cDNA was about 200 mg. The gel extraction was accomplished as described in 3.2.3. As a result the FGF-BP2 ORF cDNA was obtained.

3.9.4 Radioactive Labeling of the Probe for Northern Blot Analysis

For hybridization purposes the FGF-BP2 cDNA fragment was radioactive labeled. The incorporation of radioactivity in the cDNA was achieved by random prime labelling according to the Amersham *redi*prime™ II random prime labelling system protocol. At first 50-100 ng of cDNA were diluted in 45 µl H_2O. The dilution was denatured by incubating it at 90° C for 10 min and a subsequent cold shock on ice. The denatured DNA was added to the labeling reaction mix, which consists of a buffered solution of dATP, dGTP, dTTP, Klenow enzyme and random primers in a dried stabilized form. The tube was flicked until a complete suspension was achieved. Then 5 µl of α-(^{32}P) dCTP were added and mixed with the solution. The sample was incubated at 37° C for 30 min in order to allow the nucleotides to form a complementary strand to the single stranded DNA. The primers bind randomly to the DNA strand and allow the Klenow enzyme, an exonuclease free particle of the DNA polymerase I, to polymerize the nucleotides complementary to the ssDNA fragment, incorporating the radioactive dCTP. The next step is to purify the DNA fragments from the free nucleotides. For this gel-filtration chromatography is used. A micro-spin S-200® spin column was centrifuged for 1 min at 3000g to eliminate the equilibration buffer. Then the sample was loaded onto the spin column and centrifuged for 3 min at 3,000 x g. The larger DNA fragments are eluded into a tube beneath the column, whereas the free nucleotides get stuck in the gel particles of the chromatography column. The eluted DNA strands were denatured to ssDNA by heating to 90° C and immediate shocking on ice. The amount of incorporated radioactivity was determined before using the ssDNA as a hybridization probe. For quantification 2µl of the sample were diluted in 2ml-scintillation fluid and the activity in cpm was determined in a scintillation counter.

3.10 RNA Isolation from Mammalian Cell Lines

3.10.1 Homogenization

In order to isolate the total RNA for a subsequent Northern Blot Analysis, the cells were grown in T75 cm^2 flasks in a monolayer to 80 % confluency. After aspirating the medium, the cells were directly lysed in the flask by adding 1 ml RNA STAT-60™. This latter is a phenol/guanidinium isothiocyanet (GTC) solution, which acts to homogenize the cells by denaturing proteins and disrupting membrane structures. The solution was distributed equally in the flask and an immediate cell lyse was visible. The total harvest was collected by scrapping, then homogenized by repetitive pipetting and transferred into a tube. The homogenate was stored at room temperature for 5 min. to permit the complete dissociation of nucleoprotein complexes.

3.10.2 Extraction

For the extraction of the RNA 0.2 ml of chloroform per 1ml of RNA Stat-60™ were added, the sample was vortexed for 15 sec and left at room temperature for 3 min. Next, it was centrifuged at 12,000 x g for 15 min at 4 C. Following the centrifugation, the homogenate separates into three phases, a lower organic phenol/chloroform phase, an interphase and an upper aqueous phase. This step serves to extract proteins by confining them to the organic phase, separating the DNA into the interphase while leaving the ribonucleic acid remaining exclusively in the aqueous phase.

3.10.3 Precipitation

For precipitation of the RNA, the aqueous phase was transferred to a fresh tube and mixed with 0.5 ml isopropanol per 1 ml RNA Stat-60™ used for homogenization by vortexing the sample for 15 sec. The sample was kept at room temperature for 10 min.

and then centrifuged at 1,200 x g for 10 min. at 4 C. Afterwards the RNA precipitate forms a visible pellet at the bottom of the tube.

3.10.4 Washing

The supernatant was removed and the pellet was washed in 75 % ethanol by vortexing and subsequent centrifugation at 10,000 x g at 4^{o} C. 1 ml ethanol was used per1 ml RNA Stat-60™ used initially. The supernatant was aspirated from the pellet and the RNA was briefly air-dried. Each sample was dissolved in 50 µl DEPC-H_2O.

3.11 Northern Blotting Analysis

By Northern Blot Analysis, the expression stage of a gene from a certain cell line can be identified. A Northern Blot Analysis can not only define the presence of a particular mRNA, but also quantify its amount of expression in a certain cell line or tissue homogenate.

3.11.1 Preparation and Electrophoresis of RNA Samples

RNA samples for Northern Blot Analysis were prepared so that each sample contained 20µg of total RNA harvested from a single cell culture flask, diluted in a volume of 20 µl loading buffer. For this purpose the speedvac concentrator was used to reduce the volume of each RNA sample to a volume of approximately 2 µl, using caution not to dry the sample completely, as it will greatly decrease its solubility. Then 20 µl of RNA loading buffer were added to each sample, mixed and incubated at 65 C for 15 min. to linearize the RNA. The samples were loaded on an RNA gel and separated by electrophoresis. Afterwards the gel was inspected under UV light to ensure the RNA was separated. The very visible rRNA bands (18s at1874bp and 28s at 4718bp) were used as a good

indication of the fractionation status of the RNA. Any possible degradation of the RNA was checked for under UV light, detectable as a smear in the lane. A photograph of the gel next to a ruler was taken.

3.11.2 RNA Transfer to Nylon Membrane

For hybridization purposes, the RNA has to be transferred from the Formaldehyde gel to a nylon membrane. This transfer was performed via capillary action from the agarose gel to a solid support using 10 x SSC as a transfer buffer. The buffer is drawn from a reservoir and passes through the gel into a stack of paper towels. The RNA is eluded from the gel by the moving stream of buffer and deposited on a nylon membrane, which was placed between the gel and the paper towels. A weight applied to the top of the paper towels ensured a tight connection between all layers of material used in the transfer system, which is important for a sufficient and equal transfer. Two pieces of Whatman paper were cut to equal width but greater length of the gel. They were placed on a plate above a bowl with its longer ends hanging into the bowl filled with 1 L 10x SSC transfer buffer. The gel was placed upside down on top of the Whatman paper. To remove bubbles a sterile pipette was rolled over the gel. Parafilm stripes were used to surround the gel ensuring the buffer would pass only through the gel to the paper towels. The Nylon Membrane was cut to the size of the gel, soaked in the transfer buffer and placed on top of the gel. Any occurring air bubbles were also removed. Additional two layers of Whatman paper, cut to the size of the gel, were arranged on top of the membrane, followed by a stack of gel sized paper towels and a weight. Gel and membrane were exposed to this transfer system overnight. To verify a complete and even transfer of the total RNA, gel and membrane were checked under UV light to determine if any RNA remained in the gel and to mark the 18s and 28 s bands on the membrane.

3.11.3 RNA Fixation to the Nylon Membrane

After the transfer was carried out the RNA was immobilized on the Nylon Membrane. The RNA was fixed to the membrane by crosslinking it via an exposure to low doses of ultraviolet irradiation. This was accomplished by the use of an UV crosslink apparatus at an energy level of 120 000 microjoules/cm^2, utilizing a 254-nm light source. This procedure forms a covalent bond between the positively charged amino groups of the nylon and the uracil bases of the RNA. Caution was used not to overirradiate, which otherwise would result in the covalent attachment of a higher proportion of uracil, with a consequent decrease in hybridization signal.

3.11.4 Prehybridization

The crosslinked Nylon membrane was placed rolled inside a hybridization tube with the RNA side facing inward to get a maximum contact with the solution. Before it is possible to hybridize the membrane it is essential to complete a prehybridization to prevent nonspecific binding signals. The 5 x Denhardt's buffer and 10 µg/ml denatured Salmon Sperm DNA were supplemented to a total of 10 ml prehybridization solution. To denature the Salmon Sperm DNA it was placed in a 90^o C heat block for 10 min and chilled quickly on ice before it was pipetted into the prehybridization solution. The solution was transferred into the hybridization tube and incubated for 2-5 hours at 42 C in a Hybridization oven under permanent agitation.

3.11.5 Hybridization

After sufficient prehybridization to minimize unspecific binding the membrane was hybridized with the specific probe. The radioactive labeled strand cDNA used to detect the desired specific signal was denatured in order to allow hybridization with its complementary RNA on the membrane. Denaturing of the labeled cDNA was achieved by heating it in a 90^o C heat block for 10 min and subsequent cold shock on ice before

adding the probe into the existing prehybridization solution. The final concentration of the labeled probe was always greater than 10^6 cpm/ml hybridization solution. The hybridization was carried out overnight at 42^o C under constant agitation in the hybridization oven.

3.11.6 Washing

Different washing steps were carried out to carefully remove background radioactivity, by eliminating unspecific binding. The stringency of the washing steps were varied, with increases in higher temperature and decreases in lower salt concentration. For each specific signal the right conditions were determined according to the strength of the signal, using caution to wash enough background but not the specific signal. The three performed washing rounds were ordered in an increasing stringency. Subsequent to removing the hybridization solution, the blot was first rinsed in 100ml of 2x SSC for 5 min at room temperature. The next step was washing in 100ml 2x SSC, 1.0% SDS at 50^o C under constant agitation in the hybridization oven for 30 min. Finally, the blot was washed in 100 ml 0.1 x SSC at room temperature for 30 min. The hybridized membrane was removed from the bottle and immediately sealed in a plastic bag.

3.11.7 Autoradiography of Hybridized Membranes

The sealed radiolabelled membranes were placed between two intensifying screens and exposed to an X-ray film to obtain an autoradiographic image. The exposure time was varied between 6-24 hours according to the intensity of the signal. For maximum intensity the film cassette was placed at – 70 C. The quantitation of mRNA levels for the cell lines 1205 LU, SK-MEL 5 and the FGF-BP2 transfected SW 13 were done by using a Phosphor imager screen. The blot was exposed to the screen for 24-48 hours at room temperature, scanned and then analyzed by the quantitation software program ImageQuant.

3.12 Cell Line Treatment with EGF, TPA, Fetal Bovine Serum and all-*trans*-retinoic Acid

For the purpose of the FGF-BP2 mRNA regulation study, the 1205LU, SK-MEL 5 and the FGF-BP2 transfected SW 13 cell lines were treated in culture with growth factors and drugs in certain concentrations and at different time points. The cell lines were treated with bFGF, EGF, TPA and all-*trans*-retinoic Acid. The mRNA expression level corresponding to the effect of the treatment was visualized by Northern Blot Analysis. The cells were equally distributed in a determined number of T75 flasks and grown to 80% confluency in their regular FCS containing medium. All residues of serum were washed off before adding the drug. This was achieved by washing the cells twice in serum free medium, then incubating them in serum free medium overnight and an additional serum free medium change before adding serum again or a drug or a growth factor. In this study fetal bovine serum (FBS) was used in concentrations of 5 % and 10 %. All-*trans*-retinoic acid (tRA) was used in concentrations of 10^{-5} M, 10^{-6} M, 10^{-7} M, 10^{-8} M and 10^{-9} M. The growth factors Basic Fibroblast Growth factor (bFGF) and Basic Epidermal Growth Factor (EGF) as well as the Phorbol ester TPA were also used to treat the cell lines, although the obtained data is not shown in the results. The different agents were all added directly into the 10 ml medium of the T75 flasks in a final volume of 10µl. It was ensured that the drug or growth factor was equally distributed in the flask by carefully shaking it. Since all-*trans*-retinoic acid and the phorbol ester TPA are diluted in 100% DMSO this vehicle achieves a final concentration of 0.1% in the medium. Therefore it was obligatory to add the same concentration of DMSO to a control flask in each experiment, which was not treated with either tRA or TPA. This was necessary to determine the effect to the drug and exclude the possibility that the effect was due to the vehicle. The flasks were stored in the incubator until the time point of harvesting. In this study different time points of harvesting the cells were taken. Cells were harvested after 30 min, 1 hour, 2 hours, 3 hours, 5 hours, 6 hours 12 hours 20 hours and 24 hours.

4. RESULTS

4.1 Genomic Sequence Analysis of FGF-BPs

The FGF-BP1 gene is located on one of the six unordered genomic DNA pieces derived from the BAC clone C0024K08 for human chromosome 4 (Accession number AC005598). On the basis of the human FGF-BP1 cDNA sequence, which was published by Wu et al (Wu et al., 1991), an amino acid sequence search revealed that about 22kb 5' upstream of the FGF-BP1 transcription start site lies a gene with the closest homology (Figure 4). We named this gene FGF-BP2. This gene is structured into 2 exons, that are separated by an 1.8 kb intron. The entire ORF is located on exon 1. Both genes are in the same transcription orientation. The deduced amino acid sequence of FGF-BP2 revealed that the translated protein is composed of 233 amino acid residues with a M_r of 24.5 kDa, including a putative 19 amino acid signal peptide. Using GeneMap of human genome, both human FGF-BP1 and FGF-BP2 genes are found to reside on the short arm of human chromosome 4 between D4S412 and D4S1601 (3.7 – 28.2 cM) microsatellite anchor markers.

Figure 4: Gene Structures of FGF-BP1 and FGF-BP2

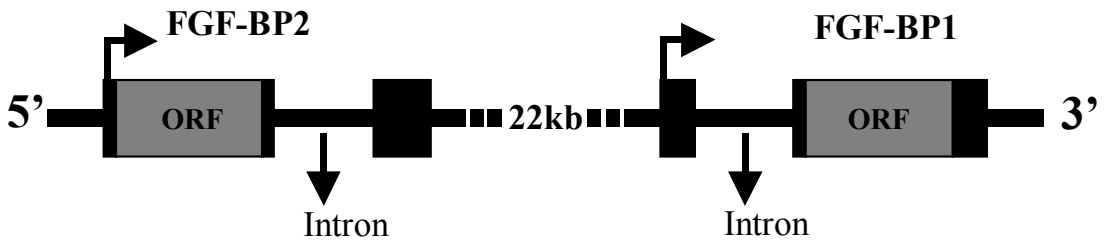

Figure 4: Gene Structures of FGF-BP1 and FGF-BP2. The genes for FGF-BP1 and FGF-BP2 were located on a BAC clone for human chromosome 4. The open reading frame (ORF) of FGF-BP1 and its 3' untranslated region are located on exon 2. Exon 1 contains the 5' untranslated region separated from exon 2 by an intron of 1.6 kb. About 22kb 5' upsteam of the FGF-BP1 transcription start site is exon 2 of FGF-BP2 containing the 3' untranslated region. The entire ORF of FGF-BP2 is located on exon 1 separated from exon 2 by an intron of 1.8kb.

4.2 Cloning of the FGF-BP2 ORF into the Vector pCR® 3.1

The FGF-BP2 open reading frame was received as a gift from Dr. Yanggu Shi of Human Genome Sciences. The gene was cloned into the BamHI/XbaI restriction site of the pC4 expression vector, which is a Human Genome Sciences in house vector. For further work with the gene the FGF-BP2 ORF it was now subcloned into the commercially available expression vector pCR® 3.1 (Figure 5). The FGF-BP2 ORF was released from the pC4 vector by restriction digest reaction using the restriction endonucleases BamHI and XbaI (Methods 3.3.1). The insert was purified (Methods 3.3.2), the vector pCR® 3.1 was lineraized (Methods 3.3.3) and the insert was ligated into the BamHI/XbaI sites of the multiple cloning site of the vector pCR® 3.1 (Methods 3.3.4).

Figure 5: Cloning of the FGF-BP2 cDNA

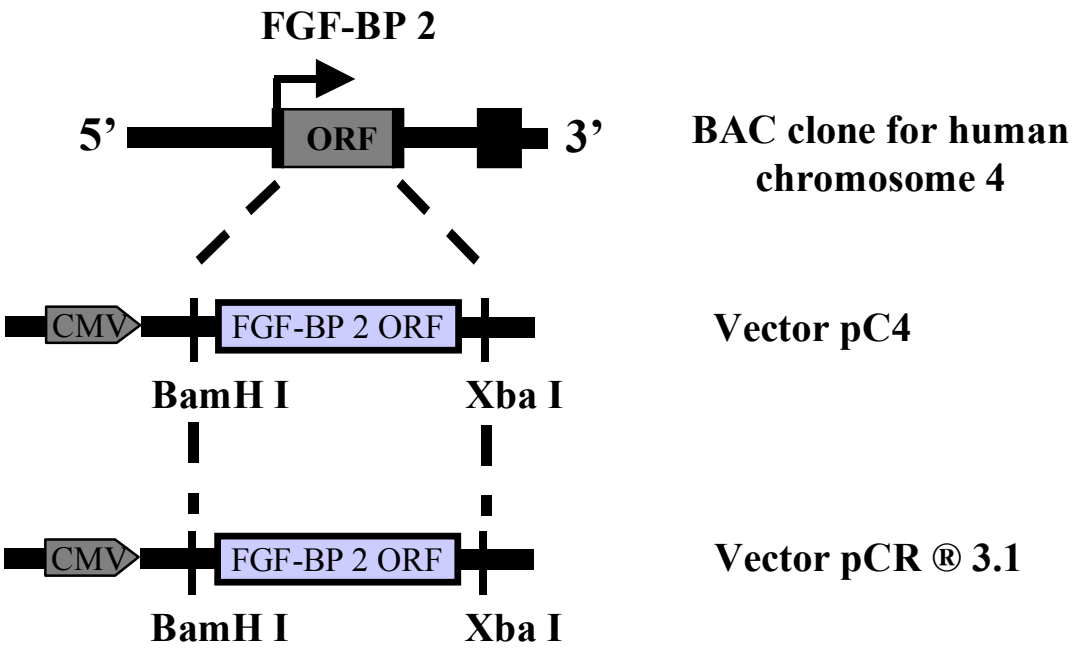

Figure 5: Cloning of the FGF-BP2 cDNA. The FGF-BP2 open reading frame was cut out of the BAC clone for human chromosome four and cloned into the BamHI/XbaI site of vector pC4. For further studies the ORF was then cloned into the BamHI/XbaI site of the pCR ® 3.1 expression vector.

4.3 Transformation of the DH5α ™ Cells with the FGF-BP2 Plasmid and Purification of the Plasmid

The subcloned FGF-BP2 plasmid was transformed into DH5α ™ Cells to amplify the plasmid DNA (Methods 3.4). Selected bacterial colonies were picked and grown, and the plasmid DNA was purified (Methods 3.5). Analytical restriction digest reactions were performed to show a correct insertion of the insert.

4.4 Sequence Analysis of the subcloned FGF-BP2 and Comparison to FGF-BP1

The newly subcloned FGF-BP2 ORF was sequenced for its nucleotide structure (Methods 3.6), both to verify the correct subcloning and to compare to the FGF-BP1 gene. The isolated full length FGF-BP2 cDNA contains 1120 base pairs excluding the poly-(A) tail, and its sequence is mapped between nucleotides at position 132636 and 135582 on the BAC clone described above. The ORF extends from an ATG start codon at nucleotide 64 to a TGA stop codon at nucleotide 736. There is no homology between FGF-BP1 and FGF-BP2 nucleotide sequences. The deduced amino acid sequence of FGF-BP2 contains 223 amino acids with a M_r of 24.5 kDA and a pI of 9.15. Using a computer program predicting prokaryotic and eucaryotic signal peptides and their cleavage sites (Nielsen et al., 1997), the signal peptide for FGF-BP2 would consist of the first 19 amino acid residues with the potential cleavage site occurring between $G_{19} - Q_{20}$. Deduced amino acid sequences of FGF-BP1 and FGF-BP2 are aligned for comparison (Figure 6). This comparison shows that FGF-BP2 has a 21% amino acid identity and a 41% homology with respect to FGF-BP1. Both FGF-BPs contain 8 conserved cysteine residues. This might suggest that these disulfide bridges involved in the formation of a tertiary structure are critical of the function of the proteins.

Figure 6: Deduced Amino Acid Sequence of FGF-BP2

```
FGF-BP 2:   24   QKQGSTGEEFHFQTGGRDSCTMRPSSLGQGAGEVWLRVDCRNTDQTYWCEYRGQPSMCQA   83
                 +++   G +  F T  + +C    +   +G   + L+V+C   D  + C + G P+ C
FGF-BP 1:   49   KQKSRPGNKGKFVTKDQANCRWAATEQEEG---ISLKVECTQLDHEFSCVFAGNPTSCLK   105

FGF-BP 2:   84   FAADPKPYWNQALQELRRLHHACQ-GAPVLRPSVCREAGPQAHMQQVTSSLKGSPEPNQQ   142
                    D + YW Q  + LR     C+     ++  VCR+  P++ ++ V+S+L G+ +P ++
FGF-BP 1:  106   L-KDERVYWKQVARNLRSQKDICRYSKTAVKTRVCRKDFPESSLKLVSSTLFGNTKPRKE   164

FGF-BP 2:  143   PEAGTPSLRPKATVKLTEATQLGKDSMEELGXXXXXXXXXXXXXXXGPRPGGNEEAKKKA   202
                       + P+  +K       GK++                     P        +K A
FGF-BP 1:  165   ----KTEMSPREHIK-------GKETTPSSLAVTQTMATKAPECVEDPDMANQ---RKTA   210

FGF-BP 2:  203   WEHCWKPFQALCAFLIS    219
                  E C + + +LC F +S
FGF-BP 1:  211   LEFCGETWSSLCTFFLS    227
```

Identities = 42/197 (21%), Positives = 82/197 (41%), Gaps = 19/197 (9%)

Figure 6: Deduced Amino Acid Sequence of FGF-BP2. The deduced amino acid sequence of FGF-BP2 is aligned with that of FGF-BP1 for comparison. Identical amino acids are matched by their short form in the middle, amino acids with homology to each other are matched by (+) and conserved cysteine residues are in bold letters.

4.5 Generating a Hybridization Probe for Northern Blot Analysis

The FGF-BP2 ORF insert was cut out of the purified vector pCR® 3.1 by a restriction digest, using the endonucleases BamHI and XbaI (Methods 3.2.8.2). The insert was isolated, purified (Methods 3.2.8.) and used as a hybridization probe for further Northern Blot Analyses (Figure 7).

Figure 7: Isolated FGF-BP2 cDNA Fragment

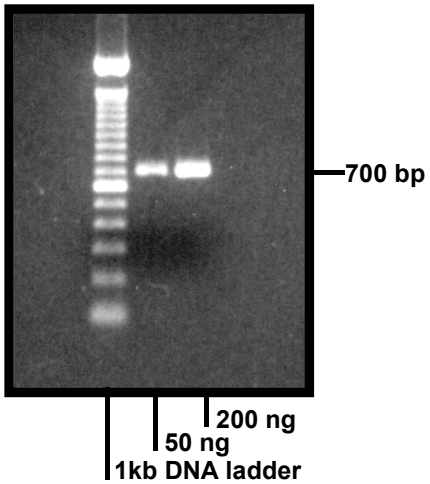

Figure 7: Isolated FGF-BP2 cDNA Fragment. The isolated FGF-BP2 fragment (50µg and 200µg) is shown on an agarose gel next to a 1kb DNA ladder.

4.6 Stable Transfection of the Cell Line SW-13 with the FGF-BP2 cDNA

The human adrenal carcinoma cell line SW-13 was chosen for a stable transfection with the FGF-BP2 plasmid. SW-13 cells express high levels of FGF but lack both FGF-BP1 (Introduction 2.6) and FGF-BP2 mRNA expression (Figure 8). The cells were transfected with the eukaryontic pCR® 3.1 vector containing the FGF-BP2 ORF and with an empty vector as a negative control (Methods 3.7). The expression in the pCR® 3.1 vector is driven by the CMV promoter. Earlier studies with SW-13 cells had shown that the CMV promoter supports high levels of gene expression in this cell line. The stably transfected cells were selected after G418 treatment. High levels of FGF-BP2 mRNA in the FGF-BP2 transfectants and no FGF-BP2 signal in the mock transfected cells were detected by Northern Blot Analysis (Figure 8). The total RNA harvest from the FGF-BP2 transfected SW-13 cells was later also used as a positive control on all further Northern Blot Analyses to detect the FGF-BP2 mRNA expression signals.

Figure 8: Expression of FGF-BP2 mRNA in SW-13 transfected Cells.

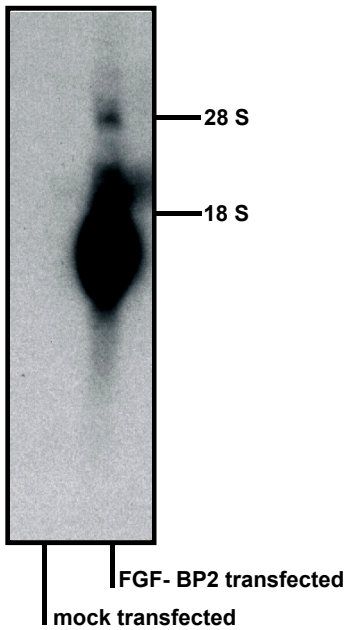

Figure 8: Expression of FGF-BP2 mRNA in SW-13 transfected Cells. A Northern Blotting analysis of SW-13 transfected cells. Total RNA (20µg) derived from mock and FGF-BP2 transfected cells were separated and probed as described in Methods 3.2.11.

4.7 FGF-BP2 Expression in Normal Tissues and Tumor Cell Lines and Comparison with FGF-BP1 Expression

After the significance of FGF-BP2 as a tumor promoter had been shown in soft agar assays it was of major interest to study the FGF-BP2 expression pattern in physiological and pathological conditions. Therefore, the FGF-BP2 mRNA expression in normal adult tissues as well as in 36 human cancer cell lines was examined by Northern Blot Analysis.

4.7.1. FGF-BP2 Expression in Normal Tissues

Analysis of a ClonTech Multiple Tissue Northern Blot revealed an abundance of FGF-BP2 mRNA expression in peripheral blood leukocytes, heart, spleen, liver and lung. The highest level was detected in blood leukocytes (Figure 9). The detected signal for liver appeared predominantly at a higher M_r, which is not present in other tissues. Lower FGF-BP2 mRNA message levels were also detected in other tissues: skeletal muscle, colon, thymus, kidney and small intestine samples. No signal, however, could be observed in brain and placenta samples.

Figure 9: Expression of FGF-BP2 mRNA in Normal Adult Tissue I

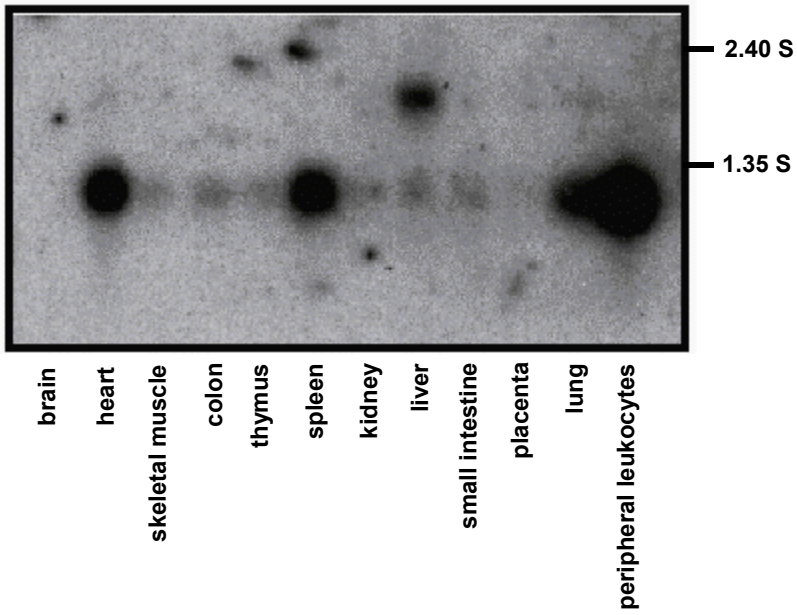

Figure 9: Expression of FGF-BP2 mRNA in Normal Adult Tissue I. A ClonTech Multiple Tissue Northern Blot was probed with the FGF-BP2 cDNA fragment

The distinct mRNA expression pattern of FGF-BP2 was also apparent upon analysis of a ClonTech RNA Master Dot Blot, which contains poly-(A) RNA samples of 50 human tissues (Figure 10). The Dot Blot reveals an FGF-BP2 mRNA expression in high levels in peripheral leukocytes and spleen, as also shown in the Multiple Tissue Northern Blot. It also shows a high expression level of FGF-BP2 in trachea, spinal cord and occipital lobe and a weaker signal in skeletal muscle, colon, bladder, pancreas, uterus, pituitary gland, adrenal gland, salivary gland, mammary gland, stomach, bone marrow and heart.

Figure 10: Expression of FGF-BP2 mRNA in Normal Adult Tissue II

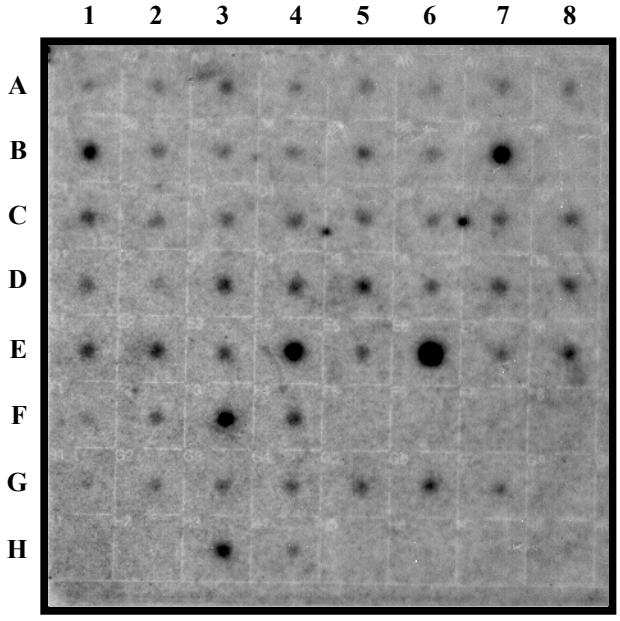

Figure 10: Expression of FGF-BP2 mRNA in Normal Adult Tissue II. A ClonTech Human RNA Master Blot was probed with the FGF-BP2 cDNA fragment, revealing an intense positive staining for peripheral leukocytes (E6), spleen (E4), spinal cord (B7), occipital lobe (B1) and trachea (F3).

4.7.2. Comparison of FGF-BP1 and FGF-BP2 Expression in Normal Tissue

After stripping the FGF-BP2 signal of both blots, the membranes were reprobed with an FGF-BP1 ORF probe, to show the FGF-BP1 expression pattern in comparison to FGF-BP1. On the ClonTech Multiple Tissue Northern Blot no signal for FGF-BP1 was detectable (data not shown), similar to previous observations (Czubayko et al., 1994). The ClonTech RNA Master Dot Blot though showed a distinct FGF-BP1 expression pattern (Figure 11). FGF-BP1 is expressed in a high level in trachea, colon and uterus. It shows a lower expression in pituitary gland, adrenal gland, salivary gland, stomach, heart, peripheral leukocytes, spleen, spinal cord and occipital lobe.

Figure 11: Expression of FGF-BP1 mRNA in Normal Adult Tissue

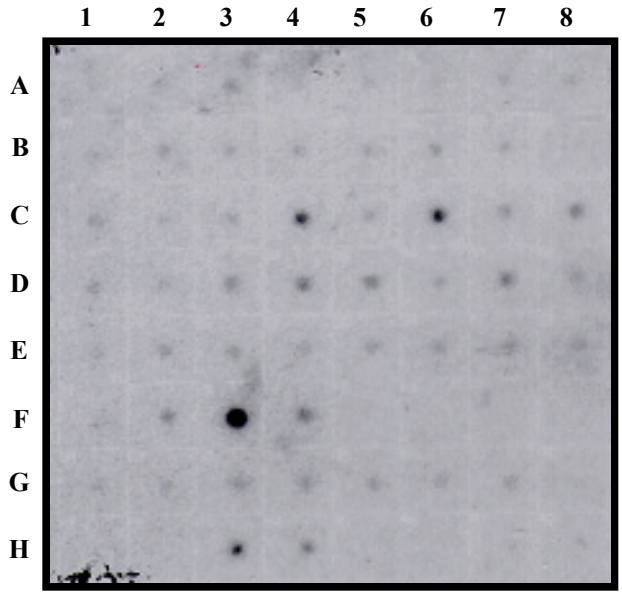

Figure 11: Expression of FGF-BP1 mRNA in Normal Adult Tissue. A ClonTech Human RNA Master Blot was probed with the FGF-BP1 cDNA fragment, revealing an intense staining for trachea (F3), colon (C4) and uterus (C6)

The distribution and comparison of both FGF-BPs in the presented tissues of the ClonTech RNA Master Dot Blot is shown in Figure 12. The only tissue examined which shows high mRNA levels of both FGF-BPs is trachea. Interestingly the GAPDH-normalized expression levels of both FGF-BPs in pituitary gland, adrenal gland, and salivary gland appear to have similar ratios, proportional to each other in their intensity.

Figure 12: Distribution and Comparison of FGF-BP1 and FGF-BP2 mRNA Expression

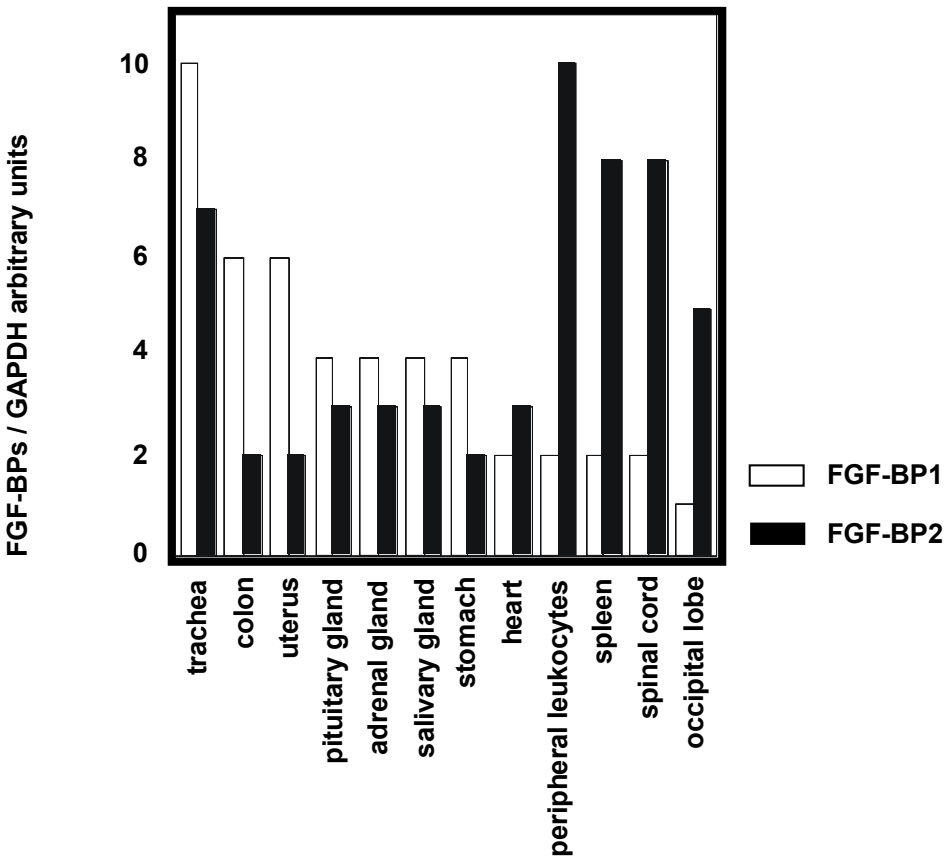

Figure 12: Distribution and Comparison of FGF-BP1 and FGF-BP2 mRNA Expression. The distribution of FGF-BP1 (open bars) and FGF-BP2 (solid bars) mRNA in selected tissues are blotted. Signal intensities are assigned with arbitrary units.

4.7.3. FGF-BP2 Expression in Tumor Cell Lines

After examining the FGF-BP2 expression under physiological conditions the next step was to study the expression of FGF-BP2 under pathological conditions. Therefore human cancer cell lines were investigated for the expression of mRNA signals of FGF-BP2. The expression of the FGF-BP2 mRNA was studied by Northern Blot Analysis.

36 tumor cell lines of various tumor types were examined, including breast carcinoma cell lines (BT-20, BT-549, MCF-7, MDA-231, MDA-435, SK-BR-3, MDA-460, MDA-468), colon carcinoma cell lines (LS 180, HT-29, SW480, SW680), prostate carcinoma cell lines (LNCaP-FGC-10, DU 145, PC-3), melanoma cell lines (1205LU, SK-MEL-5, SK-MEL-24, SK-MEL-31), an adrenal carcinoma cell line (SW 13), a cervical carcinoma cell line (ME 180), choriocarcinoma cell lines (JAR, JEG-3), pancreatic carcinoma cell lines (PANC-89, COLO 357), leukemia cell lines (HL-60, Jurkat, K-562), a liver cell carcinoma cell line (Hep G2), glioblastoma cell lines (U-87, U-138, U373, T-98, A-172, Hs 683) and a lung carcinoma cell line (WI-38). Also a human umbilical vein endothelial cell line (HU-VEC) and neonatal melonocytes were studied for their FGF-BP2 mRNA expression.

Of all the probed cell lines the FGF-BP2 mRNA signal could distinctively be detected in all of the melanoma cell lines (Figure 13). Interestingly, a specific mRNA-binding running at a higher M_r was found in the liver cell carcinoma cell line Hep-G2 (Figure 13), somehow similar to what was observed in the liver tissue of the ClonTech Multiple Tissue Northern Blot (Figure 9). All other 32 cell lines were negative for FGF-BP2 mRNA expression, including normal human neonatal melanocytes.

Figure 13: FGF-BP2 mRNA Expression in Tumor Cell Lines.

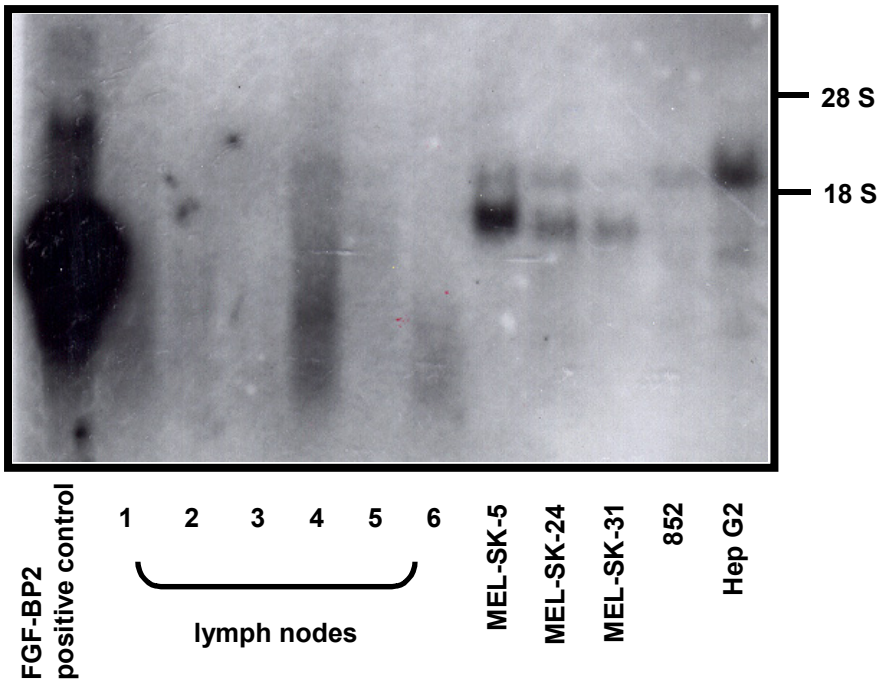

Figure 13: FGF-BP2 mRNA Expression in Tumor Cell Lines. A Northern Blotting analysis of tumor cell lines. Total RNA derived from tumor cell lines and lymph node tissue was separated and probed with the FGF-BP2 cDNA. On this blot a positive signal for FGF-BP2 mRNA is detectable for the Melanoma Cell Lines MEL-SK-5, MEL-SK-24, MEL-SK-31, and for the Liver Cell carcinoma Cell Line Hep G2, running at a higher M_r.

4.7.4. Comparison of FGF-BP1 and FGF-BP2 Expression in Tumor Cell Lines

This very distinct expression pattern of FGF-BP2 is different from what was detected before for FGF-BP1. In fact, FGF-BP1 shows mRNA expression in numerous tumor cell lines (Introduction 2.6, Table 1), including prostate carcinoma, breast carcinoma, cervix carcinoma, pancreatic carcinoma, lung carcinoma and colon carcinoma. Conversely the investigated melanoma cell lines that are positive for FGF-BP2 mRNA expression do not show a positive signal for FGF-BP1. As a matter of fact the expression patterns of FGF-BP1 and FGF-BP2 are not overlapping among the cell lines studied (Table 3).

Table 3: FGF-BP1 and FGF-BP2 mRNA Expression in Human Cell Lines

Cell Type	Cell Line	FGF-BP1	FGF-BP2
Melanoma	1205LU,SK-MEL-5 SK-MEL-24, SK-MEL-31	-	+
Prostate Carcinoma	LNDaP-FGC-10	-	-
	DU 145, PC-3	+	-
Colon Carcinoma	LS-180	+	-
	HT-29, SW480, SW680	-	-
Breast Carcinoma	BT-20, MCF-7, MDA-231, MDA-435, SK-BR-3	-	-
	MDA-460, MDA-468, BT-549	+	-
Adrenal Carcinoma	SW-13	-	-
Cervix Carcinoma	ME 180	+	-
Choriocarcinoma	JAR, JEG-3	-	-
Pancreatic Carcinoma	PANC-89, COLO 357	+	-
Leukemia	HL-60, Jurkat, K-562	-	-
Liver Carcinoma	Hep G2	-	(+)*
Glioblastoma	U-87, U-138, U-373, T-98, A-172, Hs 683	-	-
Other	WI-38 (Lung Carcinoma)	-	-
	HU-VEC (Human umbilical vein endothelial cells) Neonatal melanocytes	-	-

Table 3: FGF-BP1 and FGF-BP2 mRNA Expression in Human Cell Lines. All human cell lines which were examined for FGF-BP2 mRNA expression by Northern blotting analysis are listed and compared to their expression of FGF-BP1 mRNA.
* In the Liver Cell Carcinoma Hep G2 a higher M_r FGF-BP2 mRNA species was distinguished.

4.8. The Regulation of FGF-BP2 mRNA Expression by Serum and all-*trans*-retinoic Acid.

As reviewed in the introduction, former studies have revealed a well-defined *in vitro* regulation of FGF-BP1 mRNA. In particular the regulation of FGF-BP1 expression by all-*trans* retinoic acid (tRA) was examined. Therefore the next step in this study was to answer the question if this regulation also applies for FGF-BP2. For further studies the

two melanoma cell lines 1205LU and SK-MEL-5 were chosen, both expressing FGF-BP2 mRNA on a adequate level.

4.8.1. FGF-BP2 Serum Regulation

Since recently a significant upregulation of FGF-BP1 mRNA by fetal bovine serum (FBS) was shown (Harris et al., 2001) the initial experiment for a regulation study of the FGF-BP2 mRNA was the examination of FGF-BP2 serum regulation by Northern Blotting analyses.

The 1205LU melanoma cells were grown in medium containing 5% FBS. Before the experiment the cells were washed twice with serum-free medium (Methods 3.10). Hereafter the cells were incubated with 5% serum medium for 3 hours, 6 hours and for 24 hours. A control group with serum-free medium was harvested after 3 hours. The total RNA (20μg) of each group was separated and probed by Northern Blotting analysis. The FGF-BP2 mRNA signal intensities were quantified and normalized for GAPDH expression (Figure 14). Conversely to what was shown for FGF-BP1 in the ME-180 SCC cell line, the result exposed no significant up- or downregulation of FGF-BP2 mRNA in the 1205LU melanoma cell line when incubated with serum (Discussion 5.7). The experiment was repeated with different time points of harvesting (2 hours, 5 hours, 20 hours), revealing the same results (data not shown). The effect of serum-containing medium in contrast to serum-free medium was also studied in the MEL-SK-5 melanoma cell line, displaying the same result (data not shown).

Figure 14: Fetal Bovine Serum Treatment of 1205LU Melanoma Cells

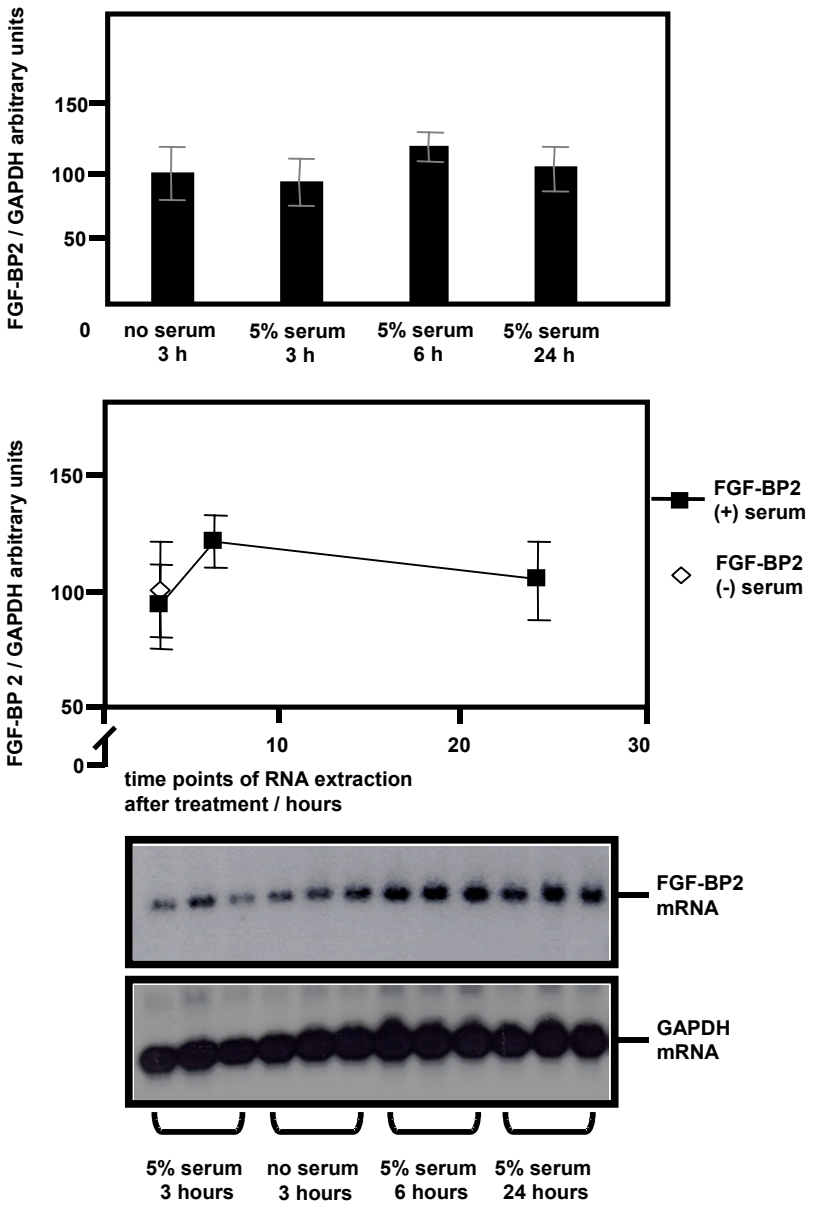

Figure 14: Fetal Bovine Serum Treatment of 1205LU Melanoma Cells. Northern blotting analysis of the expression of FGF-BP2 mRNA in the 1205LU cell line incubated with serum containing medium compared to the FGF-BP2 mRNA expression of a serum-starved control group. Cells were harvested at 3 different time points after serum exposure. Signal intensities were quantified by phosphoimaging and normalized for GAPDH. The results are presented in percentage of the control group. The experiment was carried out in triplicates of each group.

4.8.2 FGF-BP2 Regulation by all-*trans* Retinoic Acid (tRA)

As described in the Introduction section FGF-BP1 mRNA shows a very clear downregulation induced by all-*trans*-retinoic acid (tRA). FGF-BP1 downregulation in the SCC cell line ME-180 was most significant in the first 6 hours after tRA exposure and remained constant in the continuous presence of tRA for another 24 hours. The maximum effect was reached with a tRA concentration of 10^{-5} M.

Initially the effect of tRA on the FGF-BP2 mRNA expressing 1205LU melanoma cell line was tested at two different time points, 5 hours and 20 hours after exposure. The tRA concentration was chosen to be 10^{-5} M, similar to what was known from the regulation of FGF-BP1. Control groups were harvested either after 5 hours or 20 hours. Since tRA is solubilized in 0.1% DMSO, the same amount of DMSO was added to the control groups to exclude a possible effect of the vehicle.

In contrast to the tRA effect on the ME-180 cell line, no major effect of tRA on the 1205 cells was detectable (Figure 15). A slight decrease in the FGF-BP2 mRNA in the tRA treated cells appears to be within the range of the standard deviation and therefore does not seem to be significant. The effect of tRA was also tested in the MEL-SK-5 melanoma cell line after an exposure time of 5 hours. Here as well no significant effect of tRA on the FGF-BP2 mRNA was measurable (Figure 15).

During this series of experiments, the effect of TPA, EGF and FGF on the mRNA expression of FGF-BP2 was tested in both cell lines and at various time points. No significant or consistent effect of either of the compounds was detectable (Discussion 5.8).

Figure 15: tRA Treatment of 1205LU and MEL-SK-5 Melanoma Cells

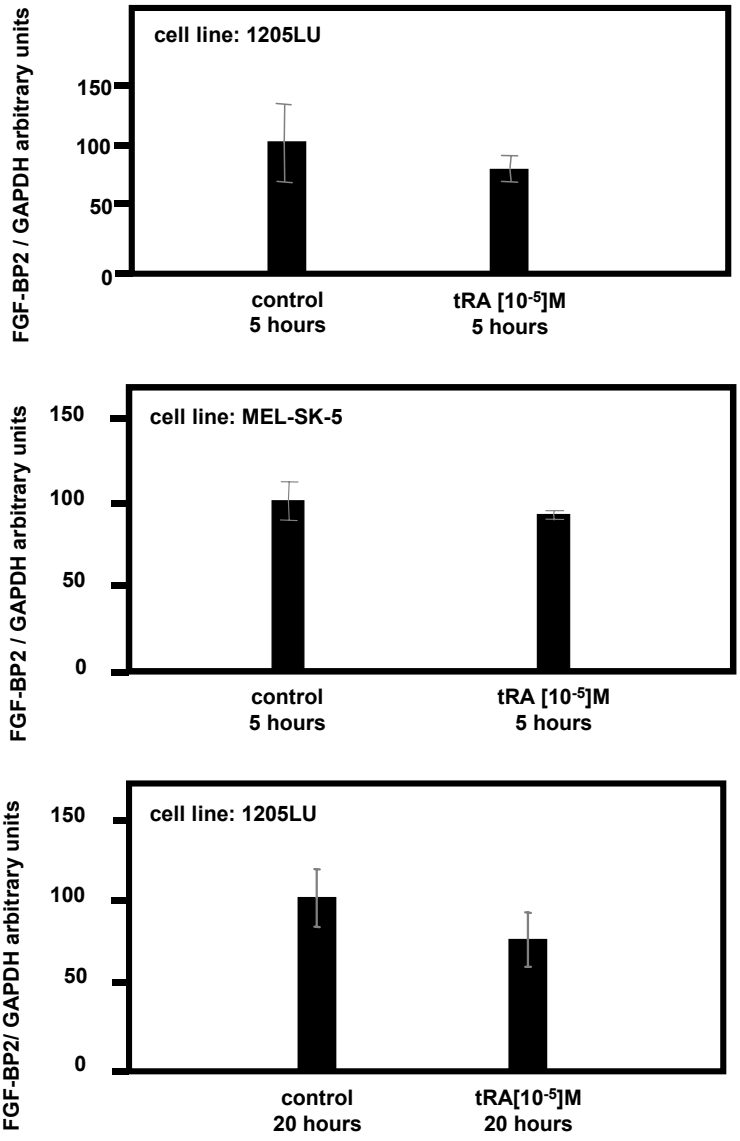

Figure 15: tRA Treatment of 1205LU and MEL-SK-5 Melanoma Cells. Northern blotting analysis of the tRA effect on 1205LU and MEL-SK-5 melanoma cells after exposure to tRA in a concentration of 10^{-5}M. 1205LU cells were harvested after an exposure time of 5 and 20 hours. MEL-SK-5 cells were harvested after tRA exposure of 5 hours. Signal intensities were quantified by phosphoimaging and normalized to GAPDH. The results are presented in percentage of a tRA untreated control group. The experiment was carried out in triplicates of each group.

Since the tRA effect on the expression of FGF-BP1 mRNA was described to be most significant in early hours after exposure (Liaudet-Coopman and Welllstein, 1996) the next step to precise these preliminary data was to particularly study the time course of the tRA effect in early time points. These experiments were completed in both melanoma cell lines and, with regard to the possibility of a posttranscriptional regulation as described for the ME-180 cells, also in the transfected SW-13 cell line (Figures 16,17,18).

The 1205LU melanoma cells were exposed to tRA in concentrations of 10^{-5} M and 10^{-7} M for ½ hour, 1 hour, 2 hours, 3 hours, and 5 hours before harvesting the RNA. The cells of the tRA untreated control group (0.1% DMSO) were harvested after ½ hour, 3 hours and 5 hours (Figure 16).

In the first experiment a minor upregulation of the FGF-BP2 mRNA appears to become visible after tRA exposure of 2 hours in a concentration of 10^{-7} M. All other groups show only slight variations of their signal intensity, almost within the range of the standard deviation of the control groups. In the second experiment, which was performed under the exact same conditions again no significant effect of tRA in time of its exposure was measurable (Figure 16). All variations of signal intensity are within the standard deviation and show no statistically significant effect. No effect of tRA on the amount of mRNA expression of FGF-BP2 in the melanoma cell line 1205LU was detectable by Northern Blotting analysis.

Figure 16: Time Course of tRA Treatment of 1205LU Melanoma Cells

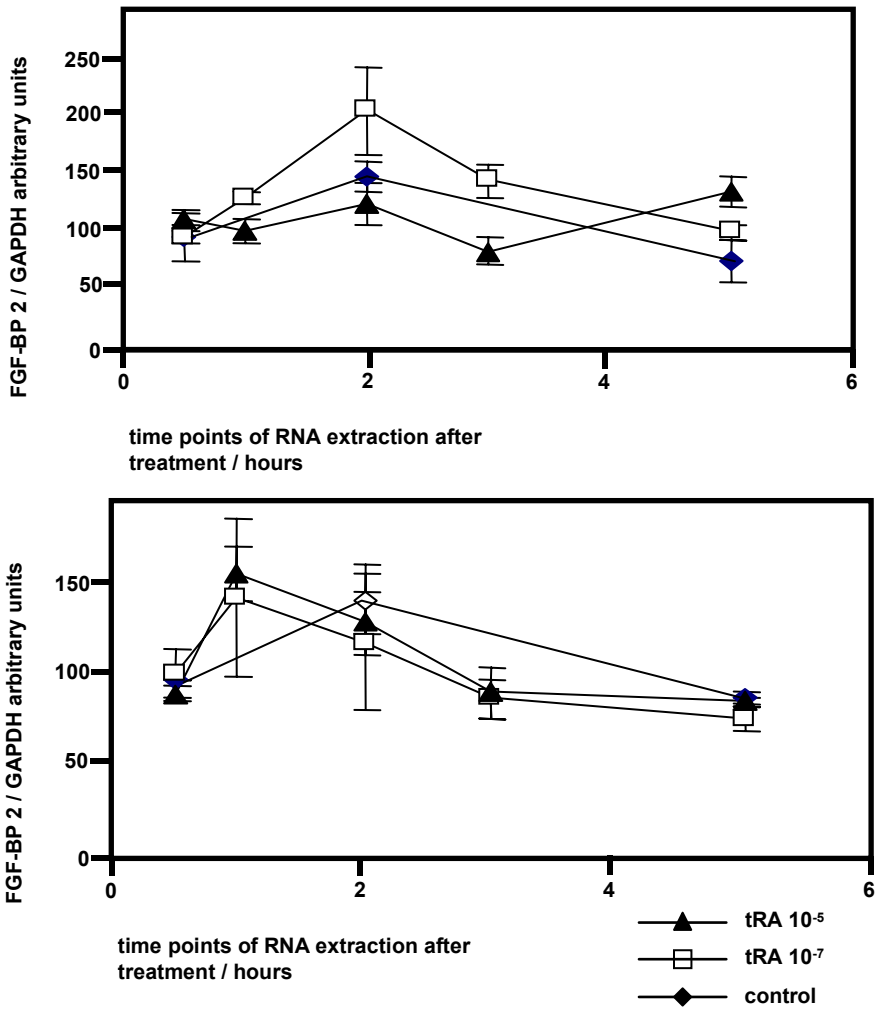

Figure 16: Time Course of tRA Treatment of 1205LU Melanoma Cells. Northern blotting analysis of the tRA effect on 1205LU melanoma cells after exposure to tRA in concentrations of 10^{-5} M and 10^{-7} M in time. RNA of the treated cells was collected after an exposure time of ½ hour, 1 hour, 2 hours, 3 hours, and 6 hours. Untreated cells were harvested after ½ hour, 2 hours and 5 hours. RNA signal intensities were quantified by phosphoimaging and normalized to GAPDH. The results are presented in percentage of the tRA untreated control group harvested after ½ hour. The experiment was carried out in triplicates of each group.

Figure 17: Time Course of tRA Treatment of MEL-SK-5 Melanoma Cells

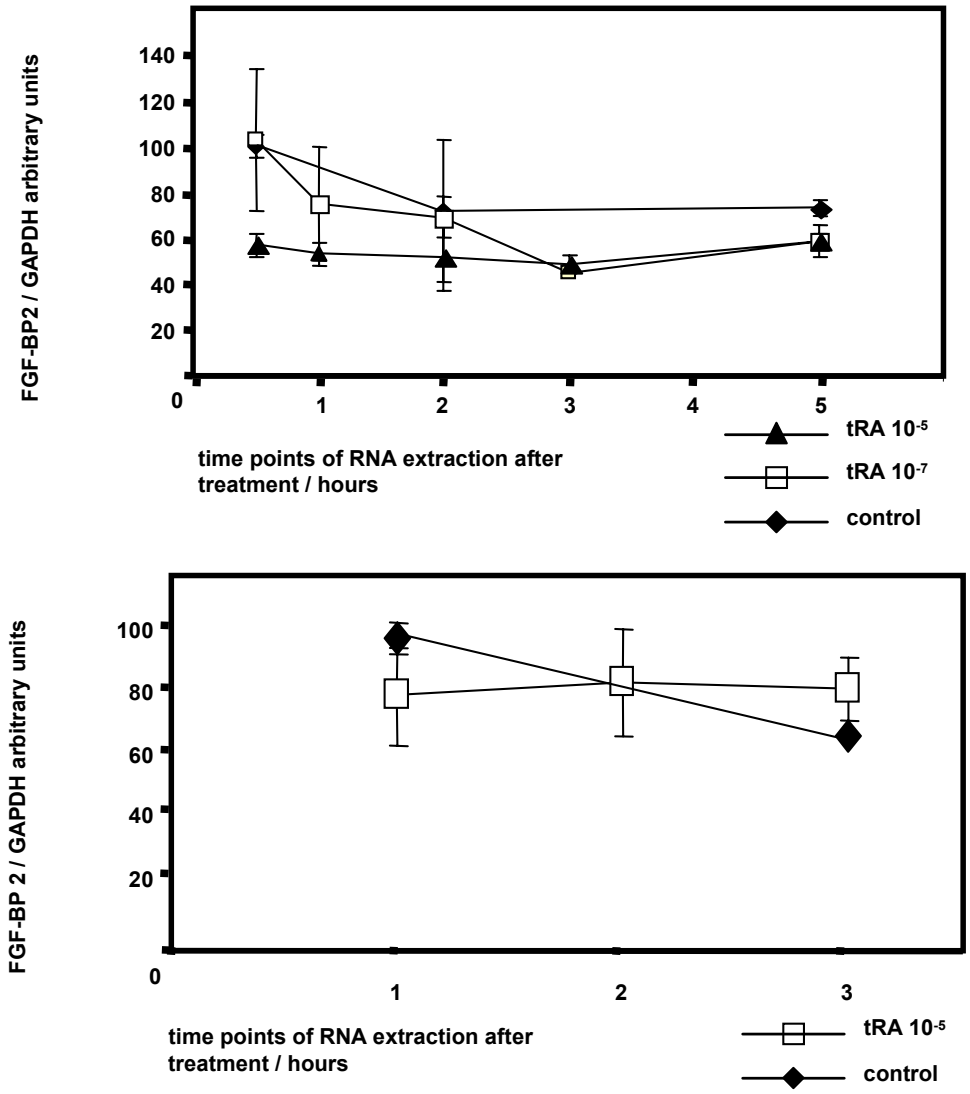

Figure 17: Time Course of tRA Treatment of MEL-SK-5 Melanoma Cells. Northern Blotting analysis of the tRA effect on MEL-SK-5 cells after exposure to tRA in concentrations of 10^{-5} M and 10^{-7} M in time. In the first experiment treated cells were harvested after an exposure time of ½ hour, 1 hour, 2 hours, 3 hours, and 6 hours. Untreated cells were harvested after ½ hour, 2 hours and 5 hours. In the second experiment RNA of the treated cells was collected after 1 hour, 2 hours and 3 hours. Control groups were harvested after 1 hour and after 3 hours. Signal intensities were quantified by phosphoimaging and normalized to GAPDH. The results are presented in percentage of the tRA untreated control group harvested at the first time point. The first experiment was carried out in duplicates of each group, the second one in quadruplicates.

In the MEL-SK5 cell line the first time course experiment was completed under exactly the same conditions as the 1205LU time course experiments. Again no major signal intensity changes were measurable among the treated groups. All minor changes were more or less within the range of the standard deviation of the control groups. However, the 10^{-5} M tRA treated group appeared to be with a slight signal decrease compared to the control groups. In order to confirm a possible effect a second time course experiment with the MEL-SK-5 cell line was performed. RNA of 10^{-5} M tRA treated and untreated (DMSO) cells was collected after 1 hour, 2 hours and 3 hours (Figure 17). This time clearly no effect of tRA was visible. It seems that there is no tRA effect on the mRNA expression of FGF-BP2 in the MEL-SK-5 cell line as detectable by Northern blotting analysis.

Since in the ME-180 cell line a posttranscriptional downregulation of the FGF-BP1 mRNA is described a final time course tRA experiment was done with the FGF-BP2 transfected SW-13 cells. The expression vector pCR® 3.1 is driven by the CMV promoter. The 10^{-5} M tRA treated cells were harvested after 1, 2, and 3 hours of treatment. The DMSO treated control groups were harvested after 1 and 3 hours (Figure 18). Once again it was not possible to show an effect of tRA on the RNA expression of FGF-BP2.

Finally, to verify that adequate concentrations of tRA were used, a dose response experiment was completed. MEL-SK-5 cells were treated with tRA concentrations of -log[9], -log[8], -log [7], -log [6], and -log [5]. RNA from all cells, including an untreated control group was collected after 2 hours (Figure 19). The result reveals that there is no dose dependent effect of tRA on the FGF-BP2 mRNA in MEL-SK-5 melanoma cells.

Overall speaking it seems obvious that in both studied melanoma cell lines it was impossible to show an effect of tRA or fetal bovine serum treatment on the expression of FGF-BP2 mRNA. Also no posttranscriptional regulation mechanisms in the FGF-BP2 transfected adrenal carcinoma cell line SW-13 were detectable whatsoever by quantification of Northern Blotting signals (Discussion 5.8).

Figure 18: Time Course of tRA Treatment of FGF-BP2 transfected SW-13 Cells

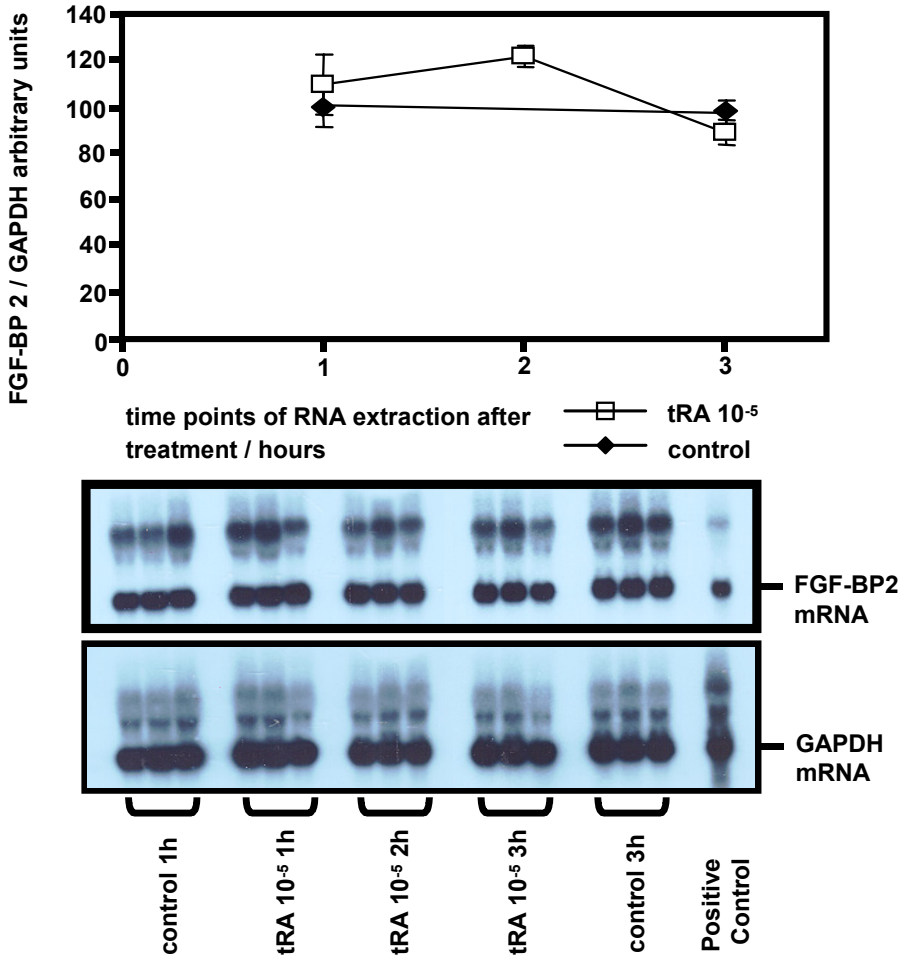

Figure 18: Time Course of tRA Treatment of FGF-BP2-transfected SW-13 Cells. Northern blotting analysis of the tRA effect on FGF-BP2-transfected SW-13 cells after exposure to tRA in a concentration of 10^{-5}M and in time. The total RNA of the treated cells was collected after 1 hour, 2 hours and 3 hours of exposure time. Control groups were harvested after 1 and 3 hours. The total RNA was separated and probed. Signal intensities were quantified by phosphoimaging and normalized to GAPDH. The results are presented in percentage of the tRA untreated control group harvested at 1 hour. The experiment was carried out in triplicates of each group.

Figure 19: Dose Response of tRA Treatment of MEL-SK-5 Cells

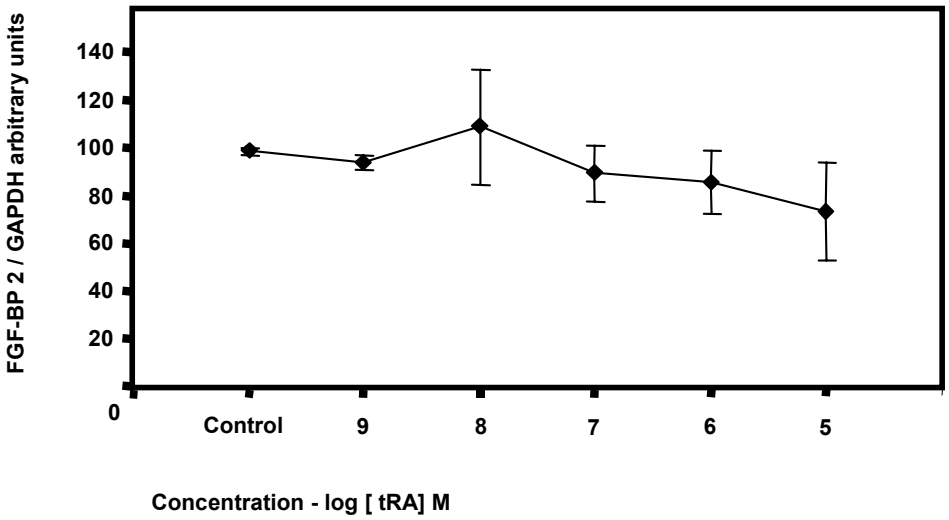

Figure 19: Dose Response of tRA treatment of MEL-SK5 cells. Northern blotting analysis of tRA effect on the FGF-BP2 mRNA expression under different concentrations. Cells were exposed to tRA in concentrations of -log [9], -log [8], -log [7], -log [6], and -log [5]. All cells including a tRA free control group were harvested after 2 hours. Their total RNA was separated and probed with the FGF-BP2 cDNA. Signal intensities were quantified by phosphoimaging and normalized to GAPDH. The results are presented in percentage of the tRA untreated control group harvested at 1 hour. The experiment was carried out in triplicates of each group.

4.9 Biological activity of FGF-BP2 transfected SW-13 Cells in Soft Agar Assays

To examine the potential role of FGF-BP2 in tumor growth, the FGF-BP2 transfected SW-13 cell line was selected as a model system. The wild type SW-13 cell line is tumorigenic but does not form colonies in soft agar, nor does it form tumors in athymic nude mice unless the cells are supplemented with exogenous FGFs or transfected with FGF genes with secreted signal peptides (Czubayko et al., 1994, Wellstein et al., 1990). The cells transfected with the empty pCR ® 3.1 vector were used as a negative control. The transfected cells were plated on agarose dishes and incubated for 10-14 days (Methods 3.8).

Figure 20: Biological Activity of in SW-13 FGF-BP2 transfected Cells

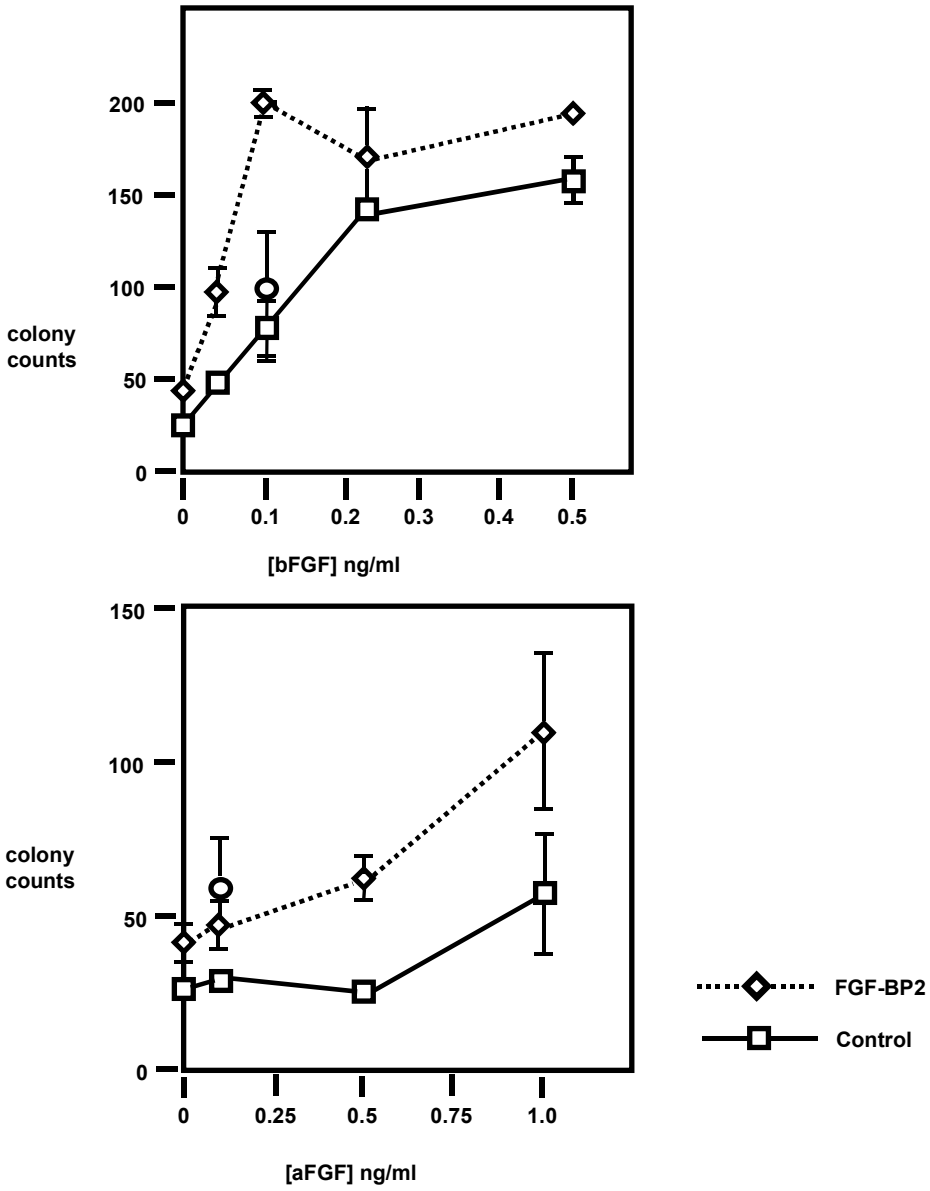

Figure 20: Biological Activity of in SW-13 FGF-BP2 transfected Cells. Colony formation in soft agar by FGF-BP2 transfectants (dashed line) and by control mock transfected cells (solid line) in the presence of various concentrations of free exogenous bFGF or aFGF. A circle indicates treatment of FGF-BP2 transfected cells with a neutralizing monoclonal antibody against bFGF (1μg/ml).

Hereafter, the number of colonies of 40 - 60μm in diameter were counted. The results revealed that twice as many colonies were formed in soft agar by FGF-BP2 transfectants as compared to the mock transfected cells (Figure 20), indicating that FGF-BP2 promotes colony formation. However, both transfected cell populations were stimulated to form more colonies in presence of exogenous bFGF (FGF-2), but consistently twice as many colonies could be observed to be formed by the FGF-BP2 transfectants relative to the control cells at low exogenous bFGF concentrations (0.05 – 0.10 ng/ml). At high exogenous bFGF concentrations (> 0.25 ng/ml) the detected difference of the two cell populations greatly diminished. In one experiment an bFGF antibody was added to the FGF-BP2 transfected cells. In the presence of 1ng/ml bFGF, treatment with an α-bFGF antibody (1μg/ml) effectively reduced colony formation in FGF-BP2 transfectants to levels comparable to untreated mock transfected cells. Similar observations concerning colony formation induced by FGF-BP2 transfectants were made when exogenous aFGF (FGF-1) was used instead of bFGF, although higher aFGF concentrations were required. Treatment with an α-bFGF antibody (1μg/ml) had no apparent effect on colony formation for FGF-BP2 transfected cells in the presence of 0.1 ng/ml of aFGF, indicating the specificity of the antibody used.

5. DISCUSSION

5.1 Genomic Sequence analysis of FGF-BPs

In this study the gene structure, tumorigenic activity, expression profile and *in vitro* mRNA regulation by serum and retinoids of a novel fibroblast growth factor binding protein (FGF-BP2) was examined. In the beginning of the study little was known about the gene and the protein that was later named FGF-BP2. A gene bank BLAST search on the basis of the FGF-BP1 cDNA sequence published by Wu et al. (Wu et al., 1991) revealed the homology of this gene to FGF-BP1. By performing sequence similarity searches we were able to map both cDNA sequences of human FGF-BP1 and FGF-BP2 on a chromosome 4 genomic fragment of 91.5 kb in length. On the basis of this genomic sequence and other related sequences available in the GenBank data base, the gene structures for both FGF-BPs are deduced as shown in Figure 4.

Since the promoter region of FGF-BP2 has not been defined, one can not preclude the possibility that the FGF-BP2 gene may contain another exon encoding for its 5'-untranslated region. Apparently the 200 bp region immediately 5' upstream of the putative transcription start site of the FGF-BP2 gene does not contain any TATA box or homologous regulatory elements such as Ets, AP-1, SP1 and CAATT/enhancer binding protein C/ERP sites which is in contrast to the human FGF-BP1 promoter (Harris et al., 1998). This already suggests that the two FGF-BP genes may be regulated by different mechanisms, which was furthermore shown in this study for the regulation of FGF-BP2 by serum and retinoids in contrast to the known mechanisms for FGF-BP1.

5.2 Biochemical Characterization of Recombinant Human FGF-BP2

Parallel to this study, Dr. Q. Nguyen performed further experiments revealing additional data that are relevant to this discussion.

Ogawa et al., the first to publish data on this novel protein (Introduction 1.5, Ogawa et al., 2001), affirm that FGF-BP2 would not show direct association with ^{125}I-labeled

recombinant human basic FGF and therefore, besides all biochemical similarity see no functional association with FGF-BP1.

In contrast to these findings Nguyen was able to demonstrate that actually FGF-BP2 is capable of binding FGF-2 and heparin. First of all he constructed and expressed FGF-BP2 containing a Myc/His tag at its carboxyl terminus in the model of stable transfected SW-13 cells. Proteins in serum-free culture media of FGF-BP2/Myc/His or mock transfectants were incubated with Ni-NTA resins, then subjected to immunoblotting analysis using a monoclonal antibody specific for the His tag. A reactive band of M_r 38 kDa was only observed for samples of FGF-BP2/Myc/his transfected cells (Q. Nguyen and A. Wellstein, unpublished data). A similar reactive band was detected on the blots that were probed separately with polyclonal antibodies raised against FGF-BP2 peptides whose sequence is shown in Figure 3. This finding is somewhat comparable to the data from Ogawa et al, who detected FGF-BP2 in a 37 kDa form secreted by transfected COS-7 cell line and also by Th-1 cells. The transfected COS-7 cell line also retained a major 28 kDa form, which the authors claim to be identical in size with an in vitro transcription/translation product, and also a minor 37 kDa form. From their findings the authors suggest that FGF-BP2 is synthesized as a polypeptide with an apparent molecular mass of 28 kDa then modified and secreted into the extracellular space as a 37 kDa form (Introduction 1.5; Ogawa et al., 2001). However, in Dr. Nguyen's work it appeared as if the recombinant FGF-BP2 is secreted into the supernatant as a dimer. Upon incubation of the samples with Ni-NTA resins in the presence of ^{125}I-FGF-2, in addition to the immunoreactive band, a strong radiolabeled band of M_r 17 kDa was detected in samples of FGF-BP2 transfectants, whereas a faint radioactive band was observed for mock control samples (Q. Nguyen and A. Wellstein, unpublished data). These data demonstrate that FGF-BP2 can interact with FGF-2 (bFGF). An immunoreactive band of M_r 38 kDa was also detected for FGF-BP2/Myc/His samples eluted from heparin-Sepharose beads, indicating that FGF-BP2 is a heparin binding protein (Q. Nguyen and A. Wellstein, unpublished data).

The deduced amino acid sequence of FGF-BP2, as shown before, suggests a secreted protein with a M_r of about 22 kDa without the putative signal peptide.

Surprisingly these data reveal that in the supernatant of the FGF-BP2 transfected SW-13 cells predominantly a 38 kDa protein is present that is reactive to anti-His/Myc tag monoclonal antibodies and anti-FGF-BP2 peptide polyclonal antibodies. In vitro coupled transcription/translation reactions of the expression plasmid containing the open reading frame of FGF-BP2 also yielded a radioactive band of similar molecular weight (Q. Nguyen and A. Wellstein, unpublished data). Based on the apparent molecular weight difference, one can assume that that this is the dimeric form of FGF-BP2. Interestingly, this association of FGF-BP2 monomers is covalently linked and not mediated through cysteine residues. In the future the biochemical nature of this dimer is to investigate and furthermore it is to determine if the dimeric form of FGF-BP2 is essential for FGF-2 interaction and for its tumor promoting properties.

5.3 FGF-BP2 induced Tumor Growth in Athymic Nude Mice

For further discussion of the results of this study it seems necessary to review additional data on FGF-BP2, also obtained in the meantime by Q. Nguyen. The FGF-BP2 transfected human adrenal cell carcinoma cell line SW-13 that was used for the soft agar assay was also utilized for an animal study with athymic nude mice. Besides the fact that wild type SW-13 cells do not form colonies in soft agar, it was also shown that they do not form tumors in athymic nude mice, unless they are supplemented with exogenous FGFs or transfected with FGF genes with secreted signal peptides (Wellstein et al., 1990). It was previously demonstrated that overexpression of the FGF-BP1 gene in the SW-13 cell line does not only induce these cells to grow colonies in soft agar, but also to form highly vascularized tumors in athymic nude mice (Czubayko et al. 1994). In a similar experiment FGF-BP2- and empty vector- transfected SW-13 cells were injected subcutaneously in female athymic nude mice, with three animals per treatment. The animals were observed for at least eight weeks for tumor formation. The tumor sizes were estimated from the product of the perpendicular diameters of the tumors and after the mice were sacrificed, the tumors were extracted for total RNA for FGF-BP2 expression

analysis. The results from this experiment revealed that tumors had grown in all three animals injected with FGF-BP2 transfected cells while animals injected with mock transfected cells were tumor free, indicating that FGF-BP2 supports tumor growth *in vivo*. Northern Blotting analysis showed that cells in all excised tumors expressed high mRNA levels of FGF-BP2. Once again the thesis of secreted FGF-BP2 solubilizing bFGF deposited by the tumor cells and/or locally stored FGFs in the stromal tissue seems verified in these tumor studies *in vivo*, demonstrated for the FGF-BP2 transfected SW-13 cells in this case.

This data again seems to be consistent with the concept that secreted FGF-BP2, just like it is proposed for FGF-BP1, can recover bFGF from its immobilized state on the cell surface and thus serve as an extracellular carrier molecule to its receptor. But still the alternative approach of FGF-BP2 preventing the binding of bFGF to matix proteoglycans can not be ruled out. However, in any case it appears that also FGF-BP2 can bolster autocrine and paracrine activities of bFGF *in vitro* and *in vivo*.

5.4 FGF-BP2 Expression in Normal Tissue and in Tumor Cell Lines

The above data support the notion that expression of FGF-BP2 in SW-13 cells can render their inactive bFGF into an autocrine- and paracrine-acting factor that supports tumor growth and angiogenesis. To assess the significance of FGF-BP2 expression for human physiology or pathology, FGF-BP2 mRNA in normal adult tissues and tumor cell lines was assayed by Northern Blotting analysis. The analysis of a ClonTech Multiple Tissue Northern Blot and a ClonTech Human RNA Master Blot probed with the FGF-BP2 cDNA fragment revealed a noticeable expression pattern of this novel FGF-binding protein in normal human tissue as presented in Results 4.7.1, Figure 9 and 10. In the ClonTech Multiple Tissue Northern Blot (Figure 9) the strong expression of the FGF-BP2 mRNA message in peripheral leukocytes, heart, spleen and lung was striking. Interestingly a signal detected for liver appeared predominantly at a higher M_r , that was not present in other tissues. Instead of the 1.3 kb FGF-BP2 message that is present in the

other positive samples a distinct 1.8 kb message is visible. This peculiar mRNA signal at a higher M_r, which was accordingly also detected in the liver carcinoma HepG2 cell line, may be due to alternative splicing. A possible thesis is that the 1.8 kb species is a product of a differential transcription start site or a poly-(A) termination site.

The fact that the strongest FGF-BP2 mRNA signal was observed in peripheral leukocytes confirms the findings of Ogawa et al.(Ogawa et al., 2001, Introduction 1.5) who found a protein named Ksp37, which is identical to FGF-BP2, as a novel serum protein produced by cytotoxic lymphocytes. From all peripheral blood leukocytes the authors found FGF-BP2 expression limited to Th1-type CD4+ T cells, effector CD8+ T cells, γδ T cells and CD16+ NK cells. Besides a number of FGF-BP2 negative tumor cell lines (see below) obviously no other normal adult tissue was analyzed in this study and therefore the authors draw the conclusion that FGF-BP2 is tightly regulated and selectively produced by cytotoxic lymphocytes. However, from the findings in this study it appears to be correct that FGF-BP2 mRNA has a distinct and tight expression pattern within tumor cell lines, where it is almost exclusively expressed by melanoma cell lines, besides a different species in the HepG2 liver carcinoma cell line (Results 4.7.3, Figure 13), but it is obviously incorrect that FGF-BP2 is selectively produced by cytotoxic lymphocytes.

Furthermore the examined RNA Blot, the ClonTech RNA Master Dot Blot exposed findings that are corresponding to what was seen within the Multiple Tissue Northern Blot. Again an intense positive staining for peripheral leukocytes and spleen was visible, but also for spinal cord, occipital lobe and trachea, besides many other tissues with a weaker signal (Figure 10). The significance of the widespread FGF-BP2 message in these tissues of whatever origin is nonetheless unknown at the moment.

In contrast the expression pattern of the FGF-BP2 mRNA in tumor cell lines does not show such a wide-ranging expression pattern at all. In fact the FGF-BP2 mRNA expression from all 37 cell lines of various tumor types is limited to all of the melanoma cell lines and the liver carcinoma cell line HepG2. Correspondingly to these findings Ogawa et al also failed to detect the FGF-BP2 message in any tumor cell line examined in their study. The cell lines scanned in their survey included Jurkat, Molt-4, MT-2, TL-Mor, CCRF-CEM, Daudi, LCL-Nag, U937, K562, HEL, HeLa and also HepG2. It is not

reported whether a somewhat different signal was detected in the liver carcinoma cell line or not.

Basically it is striking that while the FGF-BP2 mRNA signal is present frequently in normal adult tissue in contrast it shows a restrictive and distinct expression under pathological conditions. Moreover most interesting is the fact that FGF-BP2 is expressed in all examined melanoma cell lines while no message was detectable in normal human neonatal melanocytes, indicating that the expression of the gene is initiated during premalignant or malignant stages in the process of skin carcinogenesis. It is well established that bFGF is a potent mitogen and required for human melanocytes in cell culture (Coleman and Lugo, 1998). Matching to what this study revealed for FGF-BP2 it was previously shown that in contrast to normal human melanocytes which do not express bFGF, abundant bFGF messages are detected in all melanoma tumors and most cell lines derived from metastatic melanomas (Reed et al., 1994, albino et al., 1991). In addition, inhibition of bFGF or its receptor FGFR-1 synthesis in melanoma cell lines by antisense oligonucleotides leads to the suppression of tumor growth *in vitro* and *in vivo* (Becker et al., 1998, Wang et al., 1997). These studies suggest that bFGF and its activated signal pathway play a crucial role in the development of melanoma. Together with this evidence and the here displayed analogous expression pattern of FGF-BP2 one can suggest that FGF-BP2 is a tumor promoting agent by solubilizing matrix bound bFGF and therefore it is likely that FGF-BP2 may play an important role in melanoma progression and skin carcinogenesis.

5.5 Comparison of FGF-BP1 and FGF-BP2 Expression in Normal Tissue and in Tumor Cell Lines.

The expression of FGF-BP1 under physiological and pathological conditions has been evaluated previously (Czubayko et al., 1994, Tuveson, 1998). Nevertheless in this study the two commercially available blots that where probed with the FGF-BP2 cDNA probe were all the same again probed with the FGF-BP1 cDNA fragment, in order to have

comparable conditions for a comparison of the quantity of the detected messages for both proteins. Concerning the expression pattern of FGF-BP1 the results revealed a more or less consistent outcome with what was described before (Introduction 1.6, Czubayko et al., 1994, Tuveson, 1998). Again it was not possible to detect FGF-BP1 signals on a tissue Northern Blot (Results 4.7.2, Czubayko et al. 1994), though it was possible to detect the FGF-BP1 message on the RNA Dot Blot (Results 4.7.2, Figure 11). Similar to what was shown before (Introduction 1.6, Tuveson, 1998) high levels of FGF-BP1 mRNA were found in trachea, colon and uterus and a somewhat lower expression in various other tissues. In Figure 12 the distribution of FGF-BP1 and FGF-BP2 is assigned and also the quantity of their signal intensities is presented. On the whole it is obvious that the FGF-BP2 is expression is not only more intense than the expression of FGF-BP1 but also more distributed (Figures 9,10,11,12, Table 3). On the other hand the expression of FGF-BP1 seems highly regulated and tissue specific, at least under physiological conditions. The only tissue that expresses high levels of both FGF-BPs appears to be trachea, while the expression levels of the two FGF-BPs in other tissues are diverse in their intensity. But interestingly in some tissues, e.g. pituitary gland, adrenal gland and salivatory gland, the expression levels appear to be somewhat proportional to each other (Figure 12). This may indicate that in gland tissues the two FGF-BPs undergo similar regulation mechanisms. Apparently the expression of FGF-BP2 is particularly intense in tissues of the central nervous system as in a sample from the occipital lobe and from the spinal cord, and also in tissues connected to the immune system, as spleen. The strong expression in spleen, though may be due to an intense presence of leukocytes, namely TH-1 lymphocytes in this organ. In these tissues with prominent FGF-BP2 appearance the FGF-BP1 message is at its lowest. On the other hand in the tissues with strong expression of FGDF-BP1, as colon and uterus, the FGF-BP2 signal is notably weak. This again might be an indicator for a reciprocal expression of two proteins with similar or at least analogous function. Finally in the RNA Dot Blot it was possible to show an expression of FGF-BP1 in neuroectodermal-derived tissues, as in occipital lobe, although with a faint signal (Figure 11). So far it was believed, that the FGF-BP1 expression would be restricted to neuroectodermal tissues.

Concerning the expression patterns of the FGF-binding proteins within tumor cell lines we almost find a reverse setting. Under the pathological conditions in tumor cell lines this study was able to show that the FGF-BP2 mRNA signal is limited to all tested melanoma cell lines and possibly also to a liver carcinoma cell line. The FGF-BP1 expression, although as well highly regulated and tissue specific is positive in more cancer cell lines of different origins (Introduction 1.6, Table 1, Czubayko et al., 1994, Tuveson, 1998). The FGF-BP1 mRNA signal was tested positive in colonic adenocarcinoma cell lines and in tumor samples from colon cancer patients (Tuveson 1998), as well as in breast carcinoma cell lines and tumor tissues. Most importantly though many squamous cell carcinoma cell lines derived from different origins show a positive signal for FGF-BP1 expression. These squamous cell carcinomas include tumors of the lung, bladder, cervix and also skin. While the functional significance of FGF-BP1 expression in breast-, colon- and cervix carcinoma has been discussed elsewhere, with the new evidence of this study concerning FGF-BP2 the role of the FGF binding proteins in skin carcinogenesis shall be evaluated again (Discussion 5.6).

5.6 Skin Carcinogenesis and Expression of FGF-BP2 in Human Melanoma Tissue

The role of FGF-BP1 in skin carcinogenesis has been discussed extensively in the introduction to this study (Introduction 1.7, Kurtz et al., 1997, Aigner et al, in review), where it was reviewed that both in the mouse as well as in the human, FGF-BP1 is thought to play a crucial role in the early stages of skin squamous cell carcinoma. Kurtz et al. were able to show that in the developing skin of the mouse FGF-BP1 expression starts at embryonic day 9, reaches peak levels perinatally and is downregulated during postnatal development. Furthermore FGF-BP1 expression in the adult skin is dramatically increased early stages of carcinogen-induced transformation by TPA *in vivo* and by ras-activation *in vitro* (Kurtz et al., 1997). Using *in situ* hybridization techniques, the authors were able to show precisely the site of FGF-BP1 expression in the skin, namely in staged mouse embryos and TPA induced skin carcinoma samples. Later,

Aigner et al. were able to show similar findings in human skin cancer and wound healing, also using in situ hybridization to localize the FGF-BP1 activation precisely (Aigner et al., submitted).

After detection of restricted FGF-BP2 expression in melanoma tumor cell lines, FGF-BP2 expression *in situ* was analyzed Dr. Rafael Cabal-Manzano examined several melanoma tumor samples by *in situ* hybridization for their expression of FGF-BP2. In that study cryostat sections of representative melanoma tissues, obtained from the Lombardi Cancer Center Histopathology and Tissue shared Resource Core Facility, were hybridized with a sense and antisense digoxigenin labeled riboprobe of the FGF-BP2 *BamH1-Xba1* fragment containing the complete coding region. The signal was detected using alkaline phosphatase conjugated-sheep-anti-digoxigenin antibodies with BCIP/NBT (bromchlor-indolyl-phosphate/nitroblue tetrazolium) as a substrate. FGF-BP2 and mock transfected SW-13 cells were used for positive and negative control, respectively. The FGF-BP2 signal was detected predominantly in the cytoplasm of FGF-BP2 transfected cells with the antisense riboprobe, whereas no signal was detected in FGF-BP2 transfected cells probed with the sense riboprobe, or empty vector transfectants with either riboprobe, indicating the specifity of the FGF-BP2 probe (R. Cabal-Manzano and A. Wellstein unpublished data). In the melanoma tumor tissues, abundant expression of the FGF-BP2 signal were observed. An intense FGF-BP2 signal was detected mainly in melanoma cells using the antisense riboprobe (R. Cabal-Manzano and A. Wellstein unpublished data). This data, showing the FGF-BP2 molecule to be localized *in situ* of human skin cancer, strongly strengthens the hypothesis that FGF-BP2 may play a role in the development of melanoma growth and metastasis.

5.7 The *in vitro* Regulation of FGF-BP2 by Fetal Bovine Serum, EGF and TPA

As discussed in the introduction of this thesis the expression of FGF-BP1 is known to be regulated by several drugs and growth factors. These agents include mitogenic substances as the phorbol ester TPA, the growth factor EGF and also fetal bovine serum (FBS) that

have an inductive effect on the FGF-BP1 expression (Introduction 1.8, Harris et al., 2001, Harris et al, 2000(a) Harris et al, 2000(b)), and on the other hand the agent all-*trans* retinoic acid (tRA) has been shown to down-regulate the FGF-BP1 mRNA (Introduction 1.9, Liaudet-Coopman and Wellstein, 1996, Liaudet-Coopman et al., 1997). Whereas in these former studies the regulative mechanisms of FBS and tRA where shown for a SCC cell line of the cervix, the upregulation of the FGF-BP1 mRNA by TPA was detected also in skin carcinogenesis, as described above.

Taking into consideration, the so far detected analogies of FGF-BP2 and FGF-BP1 it was only reasonable to evaluate whether similar regulative mechanisms would be noticeable for the novel binding protein for FGF. For this purpose two melanoma cell lines (1205LU and SK-MEL5) both expressing sufficient levels of the FGF-BP2 mRNA and also the FGF-BP2 transfected adrenal cell carcinoma cell line SW-13 were chosen. The SW-13 cell line, overexpressing the FGF-BP2 signal was chosen to evaluate possible posttranscriptional regulation mechanisms. The Northern Blotting experiments in this study did not reveal a significant and/or consistent mechanism for either the regulation of the FGF-BP2 mRNA signal by EGF or TPA (data not shown) or by Fetal Bovine Serum (Results 4.8.1, Figure 14). In numerous experiments with the three agents it was impossible to bare any remarkable change of expression of the protein's mRNA signal, as detected previously for FGF-BP1, where a fourfold induction of the FGF-BP1 signal by EGF and also by FBS was displayed. The experiments were carried out under various conditions, analogous and different to the settings with FGF-BP1. They were completed in the presence and absence of FBS and in combination with EGF, as well as at different time points of harvesting and also with any reasonable concentration of the drug or growth factor. However, this study failed to expose any distinct *in vitro* regulation of the FGF-BP2 mRNA by any of the known inductive substances for FGF-BP1. Particularly remarkable was the fact that that the phorbol esther TPA did not show any effect on the FGF-BP2 expression signal, since its previously described inductive function was exceptionally evident in skin carcinogenesis. In addition it was checked if bFGF itself would have an effect of the expression of its binding protein. Again no significant or consistent result could be obtained (data not shown).

From these data I conclude that besides the discovered similarity of the two FGF binding proteins, the mechanisms for their regulation in vitro must be somehow diverse, at least in these different cell lines. Since there is no cell line detected that expresses both FGF-BP1 und FGF-BP2 a direct comparison and conclusions from that are impossible. From what is known about the in vitro regulation of FGF-BP1 by FBS, TPA and EGF one can presume that none of the known activation pathways for FGF-BP1 in the ME-180 squamous cell carcinoma cell line, as there are PKC, ERK kinase (MEK) and p38 MAP kinase, is activated by FGF-BP2 in a similar way for a transcriptional regulation of the gene in the melanoma cell lines. However, it is important to know that these two analogous binding proteins undergo different regulative pathways. Whatever mechanisms there are to up-regulate the FGF-BP2 expression remains unclear. Nevertheless, we know that there must be an initiation for the expression of the protein, at least in melanocytes that progress into melanoma cells and from this conclusion one can hypothesize that the initiation process of factors that are able to activate latent FGFs seems to be even more complex as known so far. Taking the potential relevance of FGF-BP2 for melanoma progression into account it seems to be important to find an answer to the question what the regulative mechanisms for this novel protein are, if not the ones that affect FGF-BP1.

5.10 The *in vitro* Regulation of FGF-BP2 by all-*trans* Retinoic Acid

Finally a potential downregulation of the gene expression of FGF-BP2 by retinoic acid was evaluated. All-*trans* retinoic acid (tRA) has been shown to inhibit growth and aberrant reverse differentiation of squamous cell carcinomas and, furthermore, FGF-BP1 has been found to be a target for down-regulation by retinoids (Introduction 1.9, Liaudet-Coopman and Wellstein, 1996, Liaudet-Coopman et al., 1997). In fact Liaudet-Coopman et al. found six different human squamous cell carcinoma cell lines to be down-regulated by tRA by 39-89% within 24 hours. The authors were able to prove that retinoic acid down-regulates FGF-BP1 mRNA in a time- and dose-dependent manner, as well as well as that the effect of tRA was reversible and FGF-BP1 mRNA returned to control levels

within 24 hours after removal of tRA (Introduction 1.9). Liaudet-Coopman et al. were able to present that the mRNA down-regulation of FGF-BP1 by tRA was due to a combination of transcriptional and post-transcriptional mechanisms. In a following study of Liaudet-Coopman et al., the authors revealed that tRA treatment not only reduces FGF-BP1 expression *in vitro*, but also *in vivo*, as demonstrated in cervical squamous cell carcinoma xenografts in athymic nude mice (Liaudet-Coopman et al., 1997). It was shown that *in vivo* tRA treatment not only reduced the FGF-BP expression in the xenografts but also inhibited angiogenesis, induced apoptosis of the tumor cells and lead to a decrease of the tumor growth rate.

Taking these details into account it was reasonable to evaluate whether similar findings would come to pass for FGF-BP2. Consequently, as described above (Results 4.8.2) a series of experiments was carried out to assess a potential regulative effect of the drug. Corresponding to the settings from the FGF-BP1 tRA study (Liaudet-Coopman and Wellstein, 1996) both melanoma cell lines and also the FGF-BP2 transfected SW-13 cell line were analyzed in various experimental settings, as most of them are described in the results. However, this study could not reproduce the effect of tRA on FGF-BP1 for FGF-BP2. Neither various time-course trials (Results 4.8.2, Figures 15,16, 17), nor a dose-response experiment (Results 4.8.2 Figure 19) with tRA achieved to reveal any significant and/or consistent changes for the mRNA expression signal of FGF-BP2 in the melanoma cell lines 1205LU or SK-MEL5. Also, the FGF-BP2 transfected SW-13 cell line did not show any response in its FGF-BP2 signal after treatment with retinoic acid (Results 4.8.2, Figure 18). This evidence stresses the observation that FGF-BP2 not only shows no transcriptional down-regulation by retinoic acid, but that also no posttranscriptional decrease of the FGF-BP2 RNA seems to be present. These facts, as determined by Northern Blotting analysis, clearly disclose that FGF-BP2 does not seem to be a target for down-regulation by retinoic acid, at least in none of the so far known tumor cell lines that express its mRNA. This data, together with the findings from the FBS, TPA and EGF- Northern Blotting experiments, make it clear that the FGF-BP2 mRNA regulation is different from what is known about FGF-BP1. So far, we now understand that FGF-BP2 is not influenced by the regulators of FGF-BP1, but we do not

know if, and by what it may be regulated. However, again it seems valuable to comprehend that these two FGF binding proteins experience a difference in their regulation by retinoic acid. From the fact that FGF-BP2 is not influenced by retinoic acid and therefore able to fulfill its potential role as an activator of immobilized FGFs one can conclude, that this might be a reason for non-responding of skin carcinomas to treatment with retinoic acid.

5.8 Tumorigenic Activity of FGF-BP2 transfected SW-13 Cells in Soft Agar Assays

The biological activity of FGF-BP1 transfected SW-13 cells in soft agar assay, indicating the tumorigenic potential of the binding protein, has been a well studied experimental model in the past. Previously Czubayko et al. (Czubayko et al.,1994) were able to show that SW-13 FGF-BP1 transfected cells grow large colonies in soft agar compared with cells transfected with the empty vector. To test whether endogenous bFGF was stimulating the SW-13 FGF-BP1 transfected cells through an autocrine mechanism, a neutralizing monoclonal antibody specific for bFGF was included in the assay. Treatment with the α-bFGF antibody at a concentration of 1 µg/ml blocked colony formation of the FGF-BP1 transfected SW-13 cells almost completely, indicating that indeed bFGF was the driving force of autocrine growth after FGF-BP1 transfection.

Ogawa et al., who characterized FGF-BP2 as a novel serum protein produced by cytotoxic lymphocytes were the first to publish the sequence similarity of FGF-BP1 and FGF-BP2 (Introduction 1.5; Ogawa et al., 2001). In their study they also tried to find functional similarities to FGF-BP1 but failed to do so (Discussion 5.2, 5.3). Ogawa et al. claim that in their study FGF-BP2 had no effect on the exogenous bFGF-dependent or on the spontaneous colony growth of SW-13 cells in soft agar.

However, in the present work soft agar assays with FGF-BP2 transfectants were carried out similar to the FGF-BP1 experiments by Czubayko et al. In contrast to the findings of Ogawa et al., the data shown in Results 4.9 and Figure 20 revealed doubled colony formation by FGF-BP2 transfectants compared to the empty vector transfected cells.

However both transfected cell populations were stimulated to form more colonies in the presence of exogenous bFGF, especially at high exogenous bFGF concentrations the difference observed for the two cell populations was greatly diminished. This observation may be due to the fact that FGF-BP2 enhances the availability of active bFGF at low concentrations to cellular receptors, whereas at high concentrations the receptors are potentially saturated. Addition of an α-bFGF antibody resulted in reduced colony formation in FGF-BP2 transfectants to the same level as that of the untreated empty vector transfected cells, indicating that free bFGF is the controlling force for colony formation in the FGF-BP2 model. The observation that exogenous aFGF has the same effect on the colony formation as bFGF, although at relatively high aFGF concentrations, matches the observations of Esch et al., who were previously able to show that bFGF is biologically more potent than aFGF in the absence of heparin (Esch et al., 1985).

This demonstrates that expression of this novel secreted binding protein activates one group of very potent angiogenesis factors, namely aFGF and bFGF, just like it is known for FGF-BP1. Both FGFs are typically not exported by cells into their medium, consistent with a lack of a secretory signal peptide (Burgess and Maciag, 1989). All in all one can say that similarly to the function previously ascribed to FGF-BP1 (Introduction 1.4, Figure 2, Czubayko et al., 1994, Czubayko et al., 1997, Rak and Kerbel, 1997), these data from the soft agar assays suggest that FGF-BP2 may be able to solubilize and activate endogenous matrix bound bFGF and allows it to reach its cellular receptors in the SW-13 cell model system.

This conclusion is based on the fact that the neutralizing α-bFGF antibody added to the cells in soft agar can inhibit the spontaneous colony formation of FGF-BP2 transfected SW-13 cells (Figure 20). Conceivably bFGF reaches the extracellular compartment, where it stimulates its receptors and can also be bound and neutralized by the added α-bFGF antibody. In addition it was also possible show that FGF-BP2 is likely to be effective in presenting low concentrations of both exogenous aFGF and bFGF to their receptors to induce colony formation.

Figure 21: Models of FGF-BP2 Functions

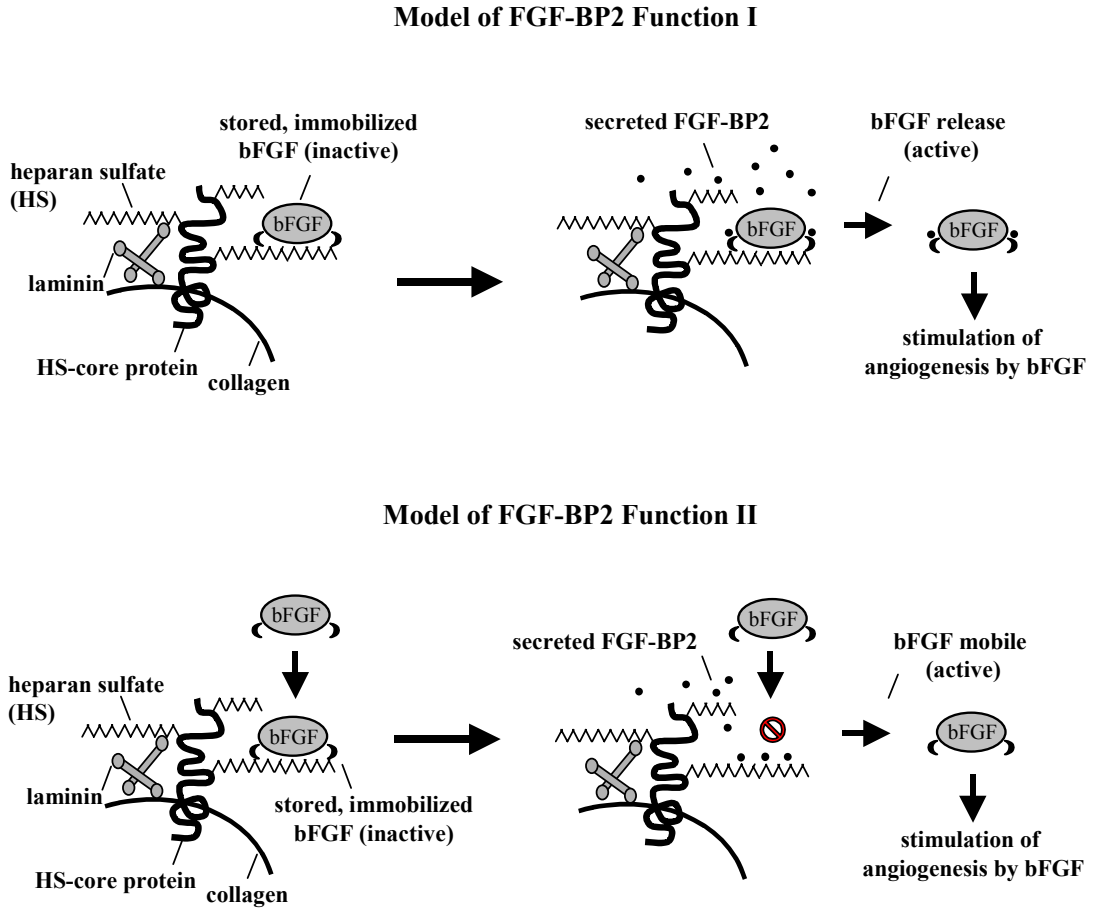

Figure 21: Models of FGF-BP2 functions. Model I displays the mechanism of bFGF activation by FGF-BP2 that has previously been established for FGF-BP1: secreted FGF-BP2 binds to immobilized bFGF, which is stored in the extracellular matrix. bFGF is released in a soluble and bioactive form, allowing it to reach its target cell receptor and stimulate angiogenesis. (Adapted from Rak J. and Kerbel, R.S. (1997) bFGF and tumor angiogenesis – back in the limelight? *Nature Med.* **3**, 1083-1084). Model II shows a possible alternative mechanism of bFGF activation by FGF-BP2: secreted FGF-BP2 prevents the binding of bFGF to matrix heparan sulfate proteoglycans and allows free bFGF to reach its target cell receptor and stimulate angiogenesis.

This data of FGF-BP2 and its biological activity is fully compatible with the established model of FGF-BP1 displacing bFGF bound to matrix heparan sulfate proteoglycans and presenting it to its receptors. However, it is further complicated by the ability of FGF-BP2 to bind to heparin-Sepharose (Q. Nguyen and A. Wellstein, unpublished data) and

therefore likely to interact with heparan sulfate proteoglycans. Since the interaction nature among bFGF, FGF-BP2 and heparan sulfate proteoglycans is not fully characterized, it is also possible that secreted FGF-BP2 prevents the binding of bFGF to matrix heparan sulfate proteoglycans in the first place and enables free bFGF to reach its receptors to mediate in vitro and in vivo its tumor promoting activities (Figure 21). This alternative mechanism of FGF-BP2 interaction with bFGF and the heparan sulfate proteoglycans was previously discussed for FGF-BP1, that not only binds to bFGF but also to heparan sulfate proteoglycans (Wu et al., 1991; Czubayko et al. 1994).On the other hand Aigner et al. were recently able to show that FGF-BP1 is more likely to bind to bFGF than to heparan sulfate proteoglycans (Aigner et al., 2001). However, for the novel FGF-binding protein it is at this point impossible to decide whether bFGF or FGF-BP2 first bind to cellular heparan sulfate proteoglycans, although it is likely that the mechanism will be similar to what was revealed for FGF-BP1. Further experiments with cells deficient in heparan sulfate may be able to answer the question definitely.

6. ABSTRACT

Angiogenesis is one of the essential alterations in cell physiology that dictate malignant growth and metastasis and has long been a major focus in molecular cancer research. More than a dozen distinct proteins are currently known to induce proliferation of endothelial cells *in vitro* and/or angiogenesis *in vivo*. Some of the most effective and best-studied angiogenic factors are fibroblast growth factors (FGFs) that are potent stimulators of new blood vessel formation during tumor growth. However, some FGFs (aFGF and bFGF) are upon secretion immobilized in the extracellular matrix (ECM) and unable to reach their high affinity receptors. There are several possible mechanisms by which bFGF can be released from its matrix storage site and thus activated. One established mechanism is the action of an FGF binding protein (FGF-BP1). FGF-BP1 activates bFGF and is thus able to stimulate tumor cell proliferation and angiogenesis. Recently a human cDNA clone containing an open reading frame for a protein, which shows amino acid sequence similarity of 21% and homology of 41 % to FGF-BP1 was discovered. This novel molecule was named FGF-BP2.

In this study the FGF-BP2 expression profile under physiological conditions in normal human tissue and in the pathological setting of tumor cell lines was evaluated by Northern Blotting analysis. Following its *in vitro* gene regulation by various drugs and growth factors was studied. Finally the FGF-BP2 cDNA was cloned into an expression vector, the gene was overexpressed in the human adrenal carcinoma cell line SW-13 and then analyzed for its tumorigenic potential in soft agar growth assays.

This study revealed a widespread FGF-BP2 mRNA expression in normal human tissue. Among 36 tumor cell lines tested though, FGF-BP2 mRNA expression was limited to all of the melanoma cell lines. Interestingly, no FGF-BP2 mRNA expression was detected in normal human neonatal melanocytes. Furthermore it was evaluated if some of the well defined mechanisms for gene regulation that are known for FGF-BP1 also exist for FGF-BP2 as tested in two melanoma cell lines. However, in contrast to what is known for FGF-BP1 it was neither possible to show a significant up-regulation of the FGF-BP2 mRNA expression by Fetal Bovine

Serum, EGF and TPA, nor was any down-regulation inducible by all-*trans* retinoic acid. Finally it was shown that FGF-BP2 overexpression in the human adrenal carcinoma cell line SW-13 promotes a bFGF-dependent colony formation *in vitro*, indicating its tumorigenic potential.

This study demonstrates that the novel FGF-binding protein has not only structural but also functional similarities to FGF-BP1. Its biological activity, together with other data that are not presented here suggest that FGF-BP2 is like FGF-BP1 a tumor promoting agent that may solubilize matrix bound bFGF. However, FGF-BP2 shows a different expression pattern from FGF-BP1 and does not seem to follow similar mechanisms of transcriptional or posttranscriptional gene regulation. Most interesting though seems to be the function that FGF-BP2 might have in melanoma progression since it is known that bFGF and its activated signal pathway play a crucial role in the development of melanoma.

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8. ABBREVIATIONS

aFGF (FGF-1)	Acidic Fibroblast Growth factor
bFGF (FGF-2)	Basic Fibroblast Growth Factor
bp	base pair
BP	Binding Protein
BSA	bovine serum albumin
cDNA	complementary DNA
cpm	counts per minute
dCTP	desoxycytosin tri-phosphate
DEPC	diethylpyrocarbonate
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
ECM	Extracellular Matrix
EDTA	etylendiamine tetraacedic acid
EGF	Epidermal Growth Factor
FBS	Fetal BovineSerum
GAPDH	glyceraldehyd-3 phosphat dehydrogenase
HGF	Hepatocyte Growth Factor
IMEM	Improved Minimal Essential Medium
kb	kilo base
LB	Luria-Bertani
MOPS	3-(*N*-morpholino)propanesulfonic acid
ORF	open reading frame
PCR	polymerase chain reaction
PTN	Pleiotrophin

RNA	ribonucleic acid
SCC	Squamous Cell Carcinoma
SDS	sodium dodecyl sulfate
SSC	sodium chloride/sodium citrate
ssDNA	single stranded desoxyribonucleic acid
TBE	Tris-base/ boric acid/ EDTA
TGFα	Transforming Growth Factor Alpha
TPA	12-O-tetradecanoyl phorbol 13-Acetat
tRA	all-*trans*-retinoic acid
VEGF	Vascular Endothelial Cell Growth Factor