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13. ABSTRACT *(Maximum 200 words)*

Seprase/FAPα, a cell surface serine-type gelatinase with M_r 170, is thought to be involved in tumor invasion and metastasis. However, I have shown previously that overexpression of Seprase/FAPα in human SW-13 and MCF-7 epithelial carcinoma cells, has no effect on their Matrigel invasion, matrix adhesion and proliferation properties. Mammalian, bacteria and baculovirus expression systems have all been used to express and purify the molecule for further characterization, however, with little success. Ribozyme constructs targeting Seprase/FAPα were made, and despite their *in vitro* activities, none of which was effective in depleting endogenous levels of Seprase/FAPα in 1205LU and MDA-MB-436 cells. During the course of this project, a novel secreted fibroblast growth factor binding protein (FGF-BP2) was also characterized. I have demonstrated that FGF-BP2 has tumor promoting activity in both *in vitro* and *in vivo*. Whereas, FGF-BP2 is absent in normal breast tissues, abundant messages were induced in some breast tumor tissues, and most melanoma samples. Thus, in contrast to the apparent bystander role of Seprase/FAPα, preliminary evidences suggest that FGF-BP2 may play a role in cancer growth and metastasis.

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INTRODUCTION

The dissolution of basement membrane matrix by proteolytic enzymes is an essential step for the invasion/metastasis process of neoplastic cells. Seprase is thought to be involved in tumor invasion on the basis of its expression in highly metastatic melanoma cell lines and its localization in specialized tumor cell membrane structures called "invadopodia" (1,2). Seprase, a 170 kDa cell surface, serine-type gelatinase, is composed of two identical 97 kDa subunits which do not exhibit gelatinase activity in the monomeric form (3). Recently, it has also been shown that the Seprase subunit is identical to FAPα (Fibroblast Activation Proteinα) whose expression is selectively induced *in vivo* in stromal fibroblasts of various human carcinomas (breast, ovarian, colorectal, bladder and lung), and activated fibroblasts of healing wounds (3,4,5).

Previously, using the RT-PCR approach, I isolated a clone containing the entire ORF (open reading frame) of human Seprase/FAPα (4). To define its potential role in breast cancer, the molecule was overexpressed in two human epithelial carcinoma cell lines (SW-13 and MCF-7), and the characteristics of the transfected cells were examined. Surprisingly, Seprase/FAPα has not shown any effect on cellular proliferation in soft agar assay, motility and invasion in Matrigel, and adhesion on various extracellular matrix molecules in these transfected cells (4). It appears that Seprase/FAPα is probably not a rate-limiting molecule, and perhaps only a bystander in cancer. In parallel to the overexpression studies, various strategies have been employed to express and purify Seprase/FAPα for its biochemical characterization. In addition, efforts to suppress the endogenous Seprase/FAPα level in MDA-MB-436 breast cancer cells by ribozyme constructs have been carried out. These recent works will be presented and discussed. During the course of this project, I also had an opportunity to work on a novel, secreted fibroblast growth factor-binding protein (FGF-BP2) through a collaboration with Dr. Anton Wellstein (Lombardi Cancer Center) and Dr. Yanggu Shi (Human Genome Sciences, Inc.). The data obtained from preliminary studies which will also be included in this annual report suggest that FGF-BP2 may play an important role in breast cancer and melanoma growth and metastasis. A brief introduction of FGF-binding proteins is given below.

Growth and metastatic spread of tumor cells are directly correlated to tumor angiogenesis (6). Acidic and basic fibroblast growth factors (FGF-1 and FGF-2, respectively) which are prototypic members of the fibroblast growth factor family are potent angiogenic factors, and therefore they are thought to be involved in tumor growth and metastasis. FGF-1 and FGF-2 are widely expressed and distributed in the extracellular matrix of embryonic, adult and tumor tissues (7). However, these growth factors are immobilized in a latent state in the extracellular matrix by binding to matrix heparan sulphate proteoglycans. One established mechanism that can release active FGFs from the extracellular storage site is the digestion of heparan sulphate chains and proteoglycan protein cores by extracellular heparanases and proteases, respectively (8). An alternative mechanism involving a secreted FGF-binding protein (FGF-BP1) was also recently proposed (9, 10).

FGF-BP1 is a 17 kDa, secreted heparin-binding protein that binds to FGF-1 and FGF-2 in a non-covalent, reversible manner (11). FGF-BP1 mRNA is expressed at high levels in squamous cell carcinoma (SSC) tissues, SSC cell lines of different origin, and in some colon cancer cell lines (9,10). Overexpression of FGF-BP1 in a non-tumorigenic human cell line SW-13 results in the release of active FGF-2 in cell culture media, and induces these cells to grow colonies in soft agar and highly vascularized tumors in athymic nude mice (9). Abrogation of endogenous FGF-BP1 levels of human

SSC (ME-180) and colon carcinoma (LS174T) cell lines by specific targeting of ribozymes results in the reduction of biologically active FGF-2 released from cultured cells (10). Furthermore, the growth and angiogenesis of xenograft tumors in mice was decreased in parallel with the reduction of FGF-BP1. Thus, these studies suggest that FGF-BP1 can mobilize and activate latent FGF-2 stored in the extracellular matrix, and being utilized as an angiogenic switch molecule in some human tumors. Recently, a human cDNA clone containing an ORF for a novel protein (FGF-BP2) that has a low amino acid sequence similarity to FGF-BP1 was isolated, and its gene structure and potential role in tumor growth and metastasis are currently investigated.

BODY

I. Expression of recombinant Seprase/FAPα.

Thus far, gelatin is the only known substrate for Seprase/FAPα. To determine other potential substrates and the substrate specificity of the enzyme, there is a need to purify sufficient amounts of enzyme for its biochemical characterization. To circumvent difficulties normally associated with the purification of membrane-bound proteins, efforts have been made to construct and express secreted Seprase/FAPα dimers that possess gelatinolytic activity (4). Various strategies were used, including: A partial truncation of the signal/anchor domain, an insertion of the RNRQKR sequence (cleaved by furin-like family of intracellular processing endoproteases) immediately after the signal/anchor domain, and a swap of the signal domain with that of secreted stromelysin-3. However, I found that an intact signal/anchor domain of Seprase/FAPα is essential for its mRNA stability and/or the formation of dimers which are recognized by mAb F19 (4).

Since secreted Seprase/FAPα molecules can not be expressed, the baculovirus expression system (Gibco BRL) was chosen for its potential high level of heterologous gene expression. Using the polymerase chain reaction overlap extension method (12), the ORF of Seprase/FAPα without its starting codon was constructed and cloned in-frame into a pFASTBAC HT donor plasmid. Following the protocol suggested by the manufacturer, the recombinant bacmid DNA was selected and used to transfect Sf9 insect cells with Cellfectin reagent. Harvested recombinant baculovirus particles from the transfection were subsequently used to infect fresh insect cells at a Multiplicity of Infection of 5.0. Cells were harvested after 48-72 h post-infection and assayed for Seprase/FAPα expression using Western blotting analysis with mAb F19.

Fig.1 shows the presence of a mAb F19 reactive band of Mr 170 kDa in lysates of cells infected with baculoviruses containing full-length Seprase/FAPα ORF, whereas no band was detected in lysates of control non-infected insect cells. Insect cells were also infected with baculoviruses containing a mutated Seprase/FAPα construct lacking a partial signal/anchoring domain (FAP_{Δ} 4-12). Consistent with my previous findings (4), Seprase/FAPα expression was not detected in either serum-free cultured media (Fig. 1) or cell lysates (not shown) of insect cells infected with baculoviruses containing the mutated construct. Since the pFASTBAC HT plasmid contains a His tag encoding region at the 5' end of the polycloning site, Seprase/FAPα dimers expressed by insect cells are bound to Ni-coupled resins and eluted with 100 mM imidazole. An elution profile of Seprase/FAPα monitored by Western blotting analysis is shown in Fig. 1. However, a lot of contaminating proteins are still present in the final enzyme preparation (not shown). The limiting factor is probably due to the low expression level of Seprase/FAPα in infected insect cells, which is not significantly better than that of transfected SW-13 cells. Attempts to make a Seprase/FAPα fusion protein with glutathione-S-transferase in bacteria using the pGEX-4T-1 vector (Pharmacia Biotech) were also carried out, but without any success (results not shown).

II. Depletion of endogenous Seprase/FAPα in MDA-436 cells.

Previously, three hammerhead ribozyme constructs targeting Seprase/FAPα at nucleotide residues 703, 892 and 2079 (Rbz1, Rbz2 and Rbz3, respectively) were made. Despite their specific *in vitro* cleavage activities, none of the constructs were effective in suppressing the endogenous Seprase/FAPα level of 1205LU melanoma cells (4). Subsequently, two new ribozymes targeting

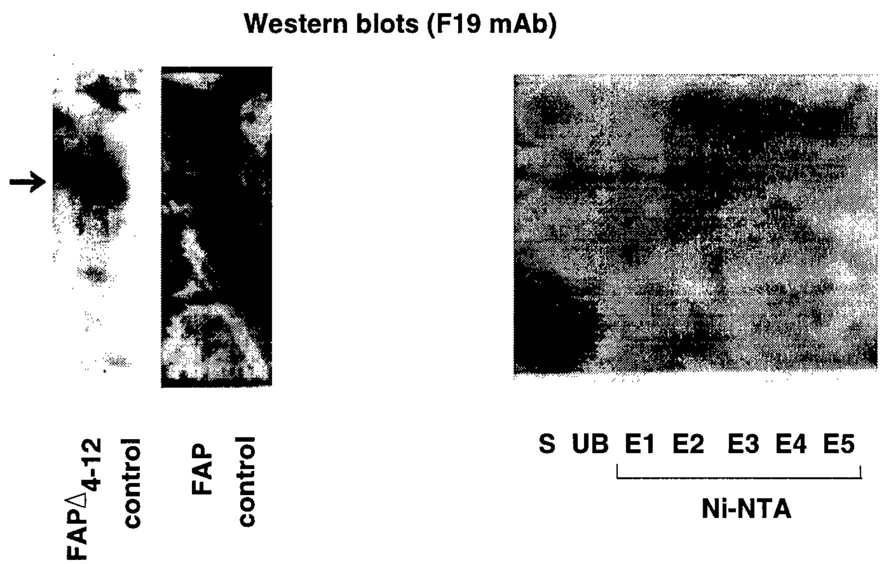

Fig. 1. Seprase/FAP alpha expression in Sf9 Cells and its elution from a Ni-coupled column.

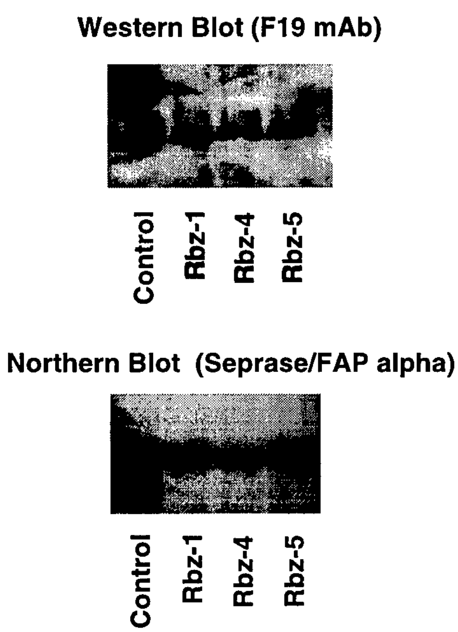

Fig. 2. Ribozymes targeting FAP alpha expression in MDA-436 cells.

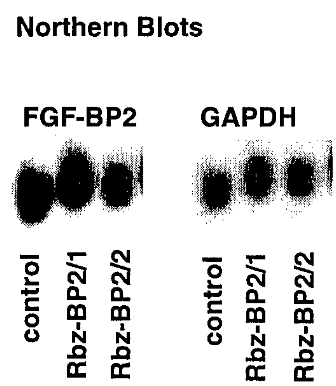

Fig. 3. Ribozymes targeting FGF-BP2 expression in SK-MEL-5 melanoma cells.

Seprase/FAPα at residues 283 and 675 were constructed (Rbz 4 and Rbz 5, respectively). These and Rbz1 constructs were transfected separately into invasive breast cancer MDA-436 cells, and their *in vivo* cleavage activities were determined by both Western and Northern blotting analysis. As shown in Fig. 2, the endogenous levels of Seprase/FAPα in ribozyme transfected cells are similar to that of parental cell line at both mRNA and protein levels. Thus far, I am unable to deplete endogenous Seprase/FAPα levels of melanoma and breast cancer cells using the ribozyme approach. In contrast, I also constructed two hammerhead ribozymes targeting FGF-BP2 at nucleotide residues 223 and 267 (Rbz-BP2/1 and Rbz-BP2/2, respectively), and the Rbz-BP2/2 is effective in decreasing the basal FGF-BP2 message level of SK-MEL-5 melanoma cells by about 50% (Fig. 3). The GAPDH (glyceraldehyde-3-phosphate dehydrogenase) levels of ribozyme transfected and parental melanoma control cells are similar. Thus, it appears that the targeted Seprase/FAPα cleavage sites may not be accessible for the ribozymes due to the message's secondary structure and possible occupation at the sites by cytoplasmic proteins.

III. Analysis of FGF-BP2 gene.

Sequence similarity searches revealed that the newly isolated FGF-BP2 cDNA sequence is located on one of the six unordered genomic DNA pieces derived from a BAC clone for human chromosome 4 whose genome sequencing is in progress. Although the promoter region of FGF-BP2 has not been determined, on the basis of its cDNA sequence and the most 5' expressed sequence tag containing a fragment of FGF-BP2 coding sequence, it appears that the gene is composed of 2 exons with an intervening intron of 1.8 kb in length as depicted in Fig. 4. The ORF of FGF-BP2 resides entirely in exon 1 while exon 2 contains most of the 3' untranslated region of the gene. Interestingly, the FGF-BP2 gene is about 22 kb 5' upstream of the FGF-BP1 gene, and both genes are in the same transcriptional orientation.

The full length FGF-BP2 cDNA consists of 1120 base pairs, excluding the poly-(A) tail. There is no homology between FGF-BP1 and FGF-BP2 cDNA sequences. The deduced amino acid sequence of FGF-BP2 contains 223 amino acids with a Mr of 24,570 kDa, including a putative 19 amino acid signal peptide. Overall, FGF-BP2 has 21% amino acid identity, and 41% homology with respect to FGF-BP1. In contrast, human FGF-BP1 shows 63% amino acid identity and 74% homology to mouse FGF-BP1 (13).

IV. Biological activity of FGF-BP2 in stable SW-13 transfected cells.

To address the potential role of FGF-BP2 in tumor growth, a human adrenal carcinoma SW-13 cell line was chosen as a model cell system. Wild type SW-13 cells express high levels of FGF-2, but lack both FGF-BP1 and FGF-BP2 expression. Moreover, these cells do not form colonies in soft agar, nor do they form tumors in athymic nude mice unless they are supplemented with exogenous FGFs or transfected with FGF genes with secreted signal peptides (14). I transfected SW-13 cells with a eukaryotic pCR3.1 vector containing an ORF of FGF-BP2 or with an empty vector for a negative control, and stably transfected cells were selected after G418 treatment. Transfected cells were plated on agarose dishes. In the absence or low concentrations of exogenous FGF-2 (0.05 - 0.10 ng/ml), there was at least twice as many colonies formed by FGF-BP2 transfectants relative to the mock controls as shown in Fig. 5. This observed difference is greatly diminished at high concentrations of exogenous FGF-2 (> 0.25 ng/ml). Similar observations were made when

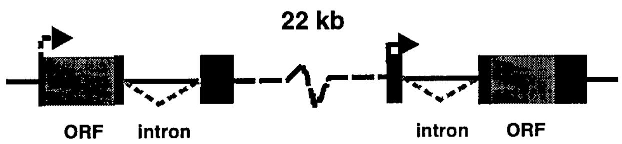

Fig. 4. Gene structures of FGF-BP1 and FGF-BP2.

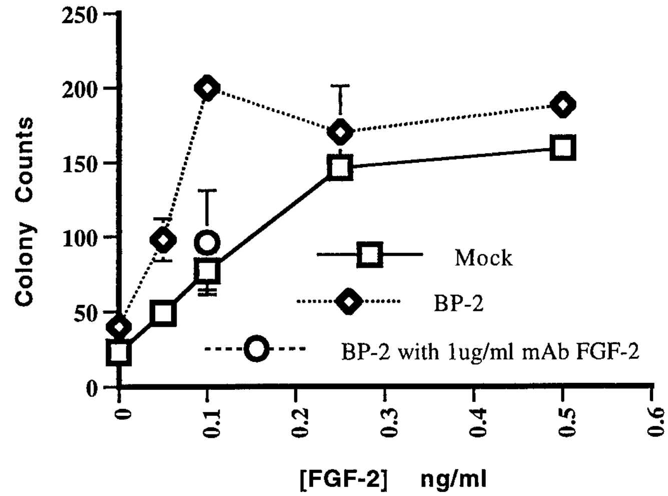

Fig. 5. Colony formation in soft agar assay.

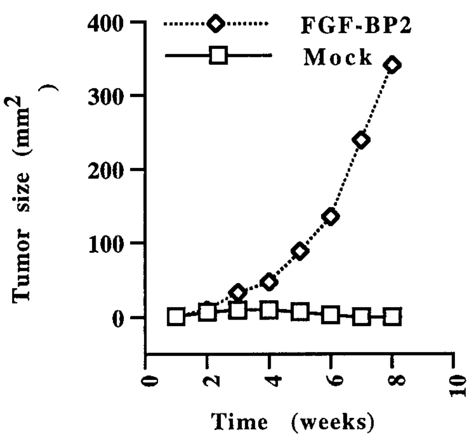

Fig. 6. Tumor growth in athymic nude mice.

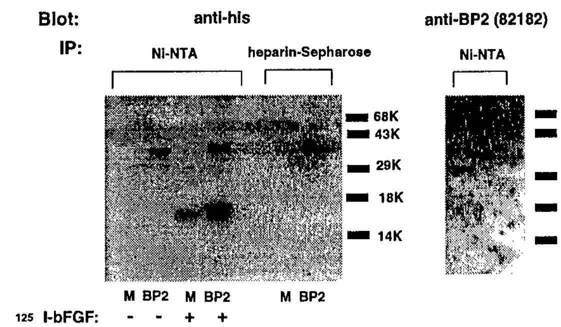

Fig. 7. Interaction of FGF-BP2 with FGF-2 and heparin.

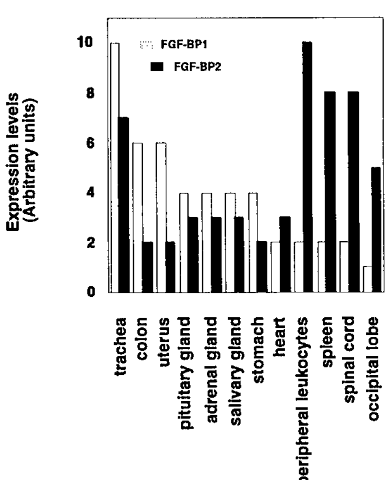

Fig. 8. Relative mRNA expression levels of FGF-BPs in normal adult tissues.

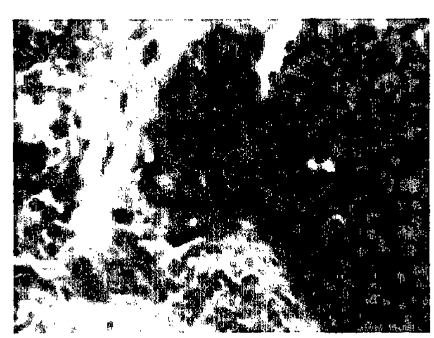

Fig. 9. Localization of FGF-BP2 messages in human melanoma tissues.

exogenous FGF-1 was used instead of FGF-2 (data not shown). Treatment with an anti-FGF-2 antibody effectively reduced colony formation of FGF-BP2 transfectants to the same level of untreated mock transfected cells, indicating that free FGF-2 is the driving force for colony formation. These data indicate that FGF-BP2 promotes colony formation, and enhances the availability of active FGF-2 at low concentrations to cellular receptors. Mock and FGF-BP2 transfected SW-13 cells were injected subcutaneously into athymic nude mice, and tumor growth was monitored over an 8 week period. Tumors were formed in all 3 animals injected with FGF-BP2 transfected cells, whereas animals injected with mock control cells were tumor free, indicating that FGF-BP2 supports tumor growth *in vivo* (Fig. 6). Moreover, all excised tumors contain high levels of FGF-BP2 messages.

V. Initial characterization of recombinant human FGF-BP2.

I have also constructed and expressed FGF-BP2 with a Myc/His tag at the carboxyl terminus of the molecule. Proteins in 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 36 kDa was only observed for samples of FGF-BP2/Myc/His transfected cells (Fig. 7). A similar reactive band was detected when the blot was probed with a polyclonal antibody raised against a FGF-BP2 peptide. It appears that the recombinant FGF-BP2 is secreted into the supernatant as a dimer. Further biochemical characterization for this dimeric molecule will be conducted. 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 16 kDa was detected in samples of FGF-BP2/Myc/His transfectants whereas a faint radioactive band was seen for the mock control samples (Fig. 7). This data demonstrate that FGF-BP2 can interact with FGF-2. An immunoreactive band of M_r 36 kDa was also detected for FGF-BP2/Myc/His samples eluted from heparin-Sepharose beads, indicating that FGF-BP2 is a heparin binding protein (Fig. 7).

V. Constitutive expression of FGF-BP2 mRNA.

Analysis of a ClonTech RNA Master Dot Blot containing poly-(A) RNA samples of 50 human tissues using both FGF-BP1 and FGF-BP2 probes revealed distinct expression patterns for these binding proteins. Their expression patterns in selected tissues are shown in Fig. 8, with high levels of FGF-BP2 message are found in peripheral blood leukocytes, spleen and spinal cord. I have also examined FGF-BP2 mRNA levels in various human tumor cell lines. Thus far, I only found FGF-BP2 message present in all human melanoma cell lines tested (1205LU, SK-MEL-5, SK-MEL-24, SK-MEL-31), but not in normal human neonatal melanocytes (data not shown). Furthermore, together with Dr. Rafael Cabal-Manzano (Lombardi Cancer Center), we carried out *in situ* hybridization experiments for a few human melanoma samples. Strong FGF-BP2 signals were detected in melanoma cells with the anti-sense riboprobe (Fig. 9). It is well established that FGF-2 and its activated signal pathway play a crucial role in the development of melanoma (15, 16). With the expression of FGF-BP2 in all tested melanoma cell lines and melanoma tissues, coupled with its tumor promoting activity presumably mediated through the solubilization of matrix-bound FGF-2, these evidences suggest that the molecule may also be involved in melanoma progression.

VI. FGF-BP2 messages in human breast cancer tissues.

In a preliminary study, FGF-BP2 mRNA levels in a few human breast cancer tissues were examined. Due to limited amounts of tissue available in biopsy samples, FGF-BP2 messages were determined by Southern blotting analysis. cDNA samples were generated by RT-PCR (reverse transcription-polymerase chain reactions) using oligo $(dT)_{12-18}$, and FGF-BP2 specific oligonucleotide primers with extracted total RNAs as templates. In 2 out of 6 breast cancer samples analyzed, strong FGF-BP2 message signals were observed (Fig. 10). In contrast, there was no FGF-BP2 signal detected on a Northern blot containing total RNAs extracted from 15 different human breast cancer cell lines (MCF-7, MDA-231, MDA-435, SK-BR-3, BT-20, etc...). To determine the cellular source of FGF-BP2 messages in human breast cancer tissues, *in situ* hybridization was also carried out for a few tumor samples (4 cases). Surprisingly, abundant FGF-BP2 messages are localized primarily in breast tumor cells (Fig. 11). There was no FGF-BP2 message detected in 2 normal breast samples (not shown). Thus, it appears that FGF-BP2 messages are specifically induced *in vivo* in tumor tissues by unknown factors, and that the molecule may play a role in breast cancer.

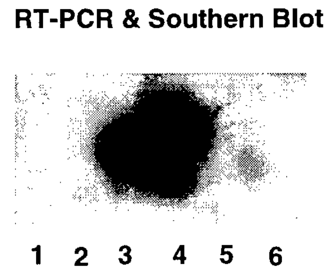

Fig. 10. FGF-BP2 message levels in human breast tumor tissues.

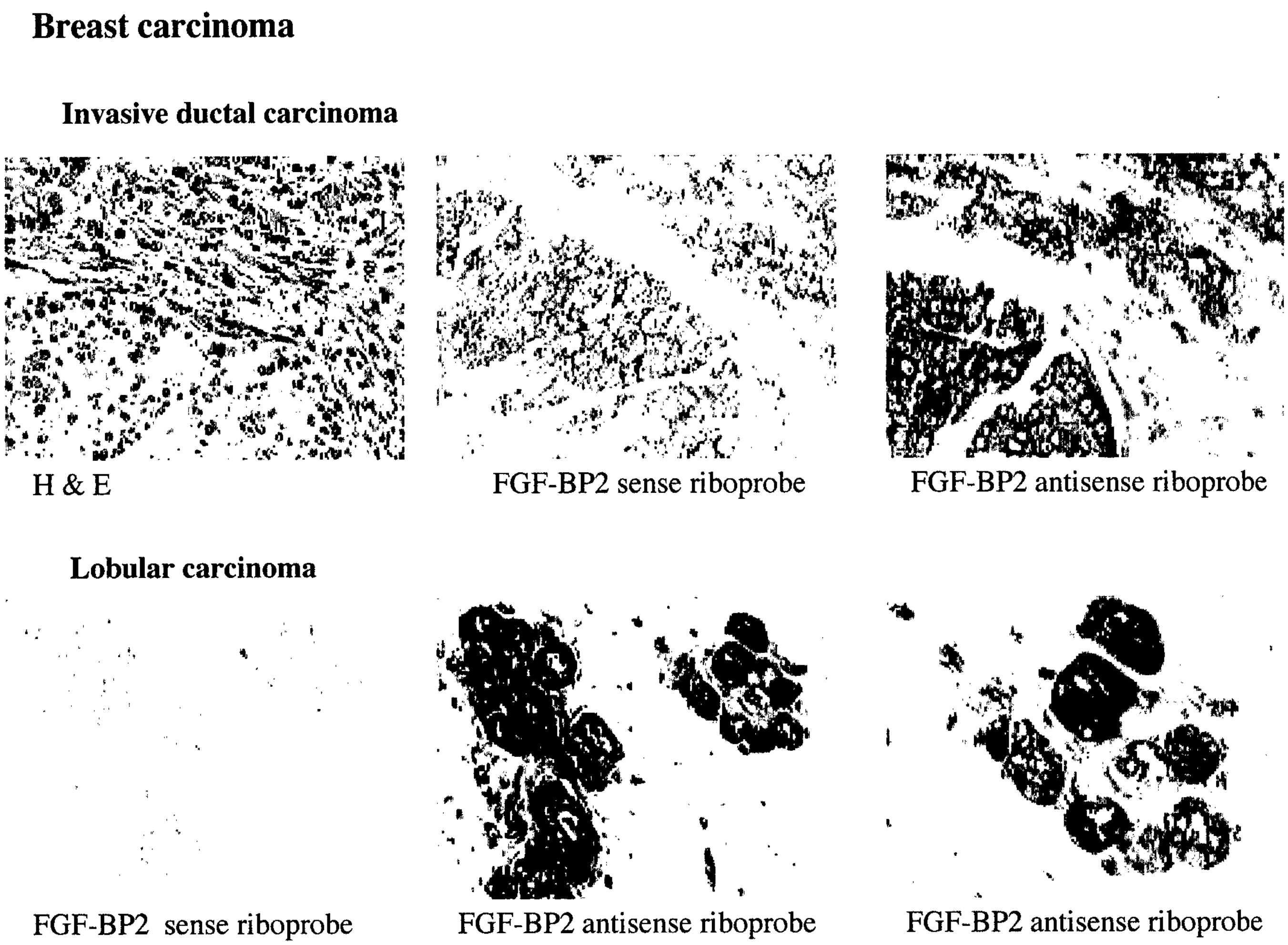

Fig. 11. Localization of FGF-BP2 messages in human breast cancer tissues.

CONCLUSIONS

A great deal of efforts has been put in expressing and purifying Seprase/FAPα molecules. Mammalian (SW-13 cells), bacteria (E.coli) and insect (Sf9) cell expression systems have all been used, but perhaps due to the nature of the molecule, I am still unable to purify sufficient amounts of Seprase/FAPα dimers for further biochemical studies. Several ribozyme constructs targeting Seprase/FAPα have been constructed, and despite their *in vitro* activities, none of which is effective in depleting the endogenous levels of Seprase/FAPα in 1205LU melanoma and MDA-436 breast cancer cells. Besides these technical difficulties, my functional data for Seprase/FAPα suggest that the molecule is probably an innocent bystander in breast cancer. Since the original isolation of Seprase/FAPα cDNA by Wolfgang J. Rettig's group in 1994 (17), no functional data has been reported for the molecule besides the use of ^{125}I-mAb F19 directed against Seprase/FAPα for imaging carcinoma lesions (18).

Due to my research interest in proteases/growth factors involved in tumor angiogenesis and invasion/metastasis processes, over the past year I also engaged in the characterization of a novel, secreted FGF-BP2. On the basis of the recently cloned FGF-BP2 cDNA sequence, genomic and expressed tag sequences available in the database through sequence similarity searches, its gene structure has been elucidated. I have expressed recombinant FGF-BP2, and have shown that it is a heparin-binding molecule and capable of interacting with FGF-2. I have also demonstrated that in a SW-13 adrenal carcinoma cell system, FGF-BP2 promotes tumor formation in *in vitro* and *in vivo*, presumably through the release of latent extracellular matrix bound FGF-1 and FGF-2. The expression pattern of FGF-BP2 messages in tissues and tumor cell lines is very distinct from that of a well characterized FGF-BP1 molecule which has the same tumor promoting activity (9,10). In addition, preliminary study revealed that abundant FGF-BP2 messages are localized in tumor cells of melanoma and breast tumor tissues.

In contrast to the bystander role played by Seprase/FAPα molecule, the evidences that I have obtained to date suggest that FGF-BP2 may play a significant role in tumor growth and metastasis of breast cancer and melanoma. Moreover, with a significant progress already made on the FGF-BP2 project, I therefore would like to pursue this project for the remainder of the Award. The original and revised statements of work are included in the Appendices.

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A. ORIGINAL STATEMENT OF WORK

Role of mp170 seprase in breast cancer

Task 1. Biochemical characterization of mp170.

A. Substrate specificity determination for secreted wild type mp170 [Months 1-18].
*Construct and express secreted form of mp170.
*Determine cleavage sites on potential substrates.
*Explore mp170 specificity on regions N- and C-terminal to natural cleavage sites using quenched fluorescent peptide substrates.

B. Define functional domains/residues of mp170 [Months 18-36].
*Construct deletion and substitution mutant cDNAs.
*Express the mutants and analyze the effects of mutation on mp170 activity and subunit dimerization.

Task 2. Effect of mp170 on breast tumor cells.

A. mp170 overexpression in MCF-7 cells [Months 1-6].
*Transfect cells with wild type mp170 cDNA and analyze stable transfectants for their growth and invasive properties.
*Transfect cells with some of the mutant cDNAs generated in Task 1B and examine their effects on tumor cell growth and invasion [Months 24-36].

B. mp170 inactivation in MDA-MB-436 cells [Months 6-24].
*Construct ribozymes targeting mp170.
*Establish ribozyme stably transfected MDA-MB-436 cells and analyze their growth and invasive properties.

Task 3. Effect of stromal mp170 on tumor growth/invasiveness [Months 36-48].

A. Generate mp170 inactivated WI-38 fibroblasts.

B. Evaluate the *in vitro* effect of stromal mp170 on the proliferation and invasiveness of tumor cells in co-culture systems.

C. Assess the influence of stromal mp170 on tumorigenicity and metastatic abilities of ML-20 and *lacZ* transduced MDA-MB-231 cells in nude mice.

B. REVISED STATEMENTS OF WORK

Role of a novel secreted FGF-binding protein (FGF-BP2) in breast cancer

Task 1. Characterize FGF-BP2 expression in human breast cancer tissues by *in situ* hybridization.

A detailed study on the mRNA expression patterns of FGF-BP2 in normal, hyperplastic, lobular (*in situ* and *invasive*) and ductal carcinoma (*in situ* and *invasive*) breast tissues. Depending on the availability of generated antibodies against FGF-BP2, parallel immunohistochemical studies on these samples will also be conducted.

Task 2. Characterize FGF-BP2 function in human breast cancer cells.

* Overexpress FGF-BP2 in MCF-7 breast cancer cells.
* Examine tumor promoting activity of FGF-BP2 transfected breast cancer cells in *in vitro* and *in vivo* assays.

C. KEY RESEARCH ACCOMPLISHMENTS

* The gene structure of FGF-BP2 is delineated on the basis of sequencing information available in the data base.

* FGF-BP2 has tumor promoting activity *in vitro* and *in vivo.* Its activity is presumably mediated through the solubilization of extracellular matrix bound FGFs.

* FGF-BP2 is a heparin-binding, secreted protein and it is capable of interacting with FGF-2.

* The expression pattern of FGF-BP2 is distinct from that of FGF-BP1. Abundant FGF-BP2 messages are found to be up-regulated in melanoma and breast tumor tissues.

D. LIST OF REPORTABLE OUTCOMES

1. **Individual Allocation Grant from American Cancer Society**. A $15,000, non-renewable grant was recently obtained for the period July 1999 to June 2000, to work on the FGF-BP2 project. The grant covers for reagent supplies used for the project.

2. **A manuscript based on the work of FGF-BP2 is under preparation**. When it is accepted in a peer-reviewed journal, a copy will be forwarded.