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Demonstration *in vivo* that stromelysin-3 functions through its proteolytic activity

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Abstract

Stromelysin-3 (ST3), a matrix metalloproteinase (MMP) expressed in aggressive carcinomas, has been shown to promote tumor development in different *in vivo* experimental models. However, the inability of its mature form to degrade extracellular matrix components casts doubt on whether ST3 functions *in vivo* as a protease. In this study, we evaluated whether the ST3 tumor-promoting effect could be ascribed to its proteolytic activity and whether this putative protease could be targeted with MMP inhibitors. Catalytically inactive mutant cDNA of human (h) ST3 or mouse (m) ST3 were generated and transfected into MCF7 cells. When injected into nude mice in the presence of matrigel, the mutant-bearing cells did not exhibit the enhanced tumorigenicity elicited by MCF7 cells transfected with wild-type ST3 cDNA. In a second approach, TIMP2 overproduction in MCF7 cells expressing hST3 was induced by retroviral infection. The co-expression of ST3 and TIMP2 failed to enhance the tumorigenicity of MCF7 cells. Notably, matrigel depleted of low-molecular-weight proteins and growth factors failed to promote the tumorigenicity of ST3-expressing MCF7 cells. These findings provide the first *in vivo* evidence that ST3 is indeed a protease that can modulate cancer progression by remodeling extracellular matrix and probably by inducing it to release the necessary microenvironmental factors. Thus, ST3 represents an interesting target for specific MMP inhibition.

Keywords: metalloproteases; tumor growth; stromelysin-3; MMP inhibitors

Introduction

Matrix metalloproteinases (MMP or matrixins) constitute a group of Zn^{++}-binding endopeptidases sharing functional and structural features. Because of their abilities to degrade most of the extracellular matrix components, they are thought to play a major role in tissue remodeling observed in physiological and pathological processes: embryonic development, wound healing, bone growth and resorption, organ involution and cancer progression (Noël *et al.,* 1997; DeClerck *et al.,* 1998; Rudolph-Owen and Matrisian, 1998). The activities of these enzymes are tightly controlled by physiological inhibitors, the tissue inhibitors of metalloproteinases or TIMP (Rudolph-Owen and Matrisian, 1998).

Stromelysin-3 (ST3 or MMP11), originally identified through its overexpression in primary breast cancers, was classified as a member of the MMP family on the basis of sequence homologies (Basset *et al,* 1990). In breast cancers, high levels of ST3 mRNA or protein have been associated with poor clinical outcome (Chenard *et al,* 1996; Tetu *et al,* 1998; Ahmad *et al,* 1998). ST3 expression is restricted to the stromal fibroblast adjacent to cancer cells (Rouyer *et al,* 1994), thus suggesting that breast cancer cells produce diffusible factor(s) inducing fibroblasts to synthesize proteases. In this context, Ahmad *et al* (1997) reported the ability of human mammary adenocarcinoma cells to directly activate the ST3 promoter. The role of ST3 in cancer progression is supported by our experimental evidence that: (1) down-regulation of ST3 expression in transformed fibroblastic 3T3 cells reduced their tumorigenic properties; (2) induction of ST3 expression in human breast adenocarcinoma MCF7 cells facilitated their tumor take in nude mice, and (3) tumor development was limited in ST3-deficient mice (Noël *et al,* 1996; Masson *et al,* 1998).

Although ST3 shares structural characteristics with other MMP, it presents several particular features. First, the ST3 prodomain contains an additional recognition site for convertase-like enzymes, such as furin. Consequently, unlike other MMP, the ST3 proenzyme is processed intracellularly and released as a mature enzyme (Pei and Weiss, 1995; Santavicca *et al,* 1996). Second, despite its ability to cleave alpha 1-proteinase inhibitor (α1PI), the putative mature form of ST3 does not degrade classic MMP substrates, such as the extracellular matrix components, gelatin or casein (Murphy *et al,* 1993; Pei *et al,* 1994; Noël *et al.,* 1995). *In vitro,* C-terminally

truncated forms of murine (m) ST3, but not of human (h) ST3 display weak enzymatic activities against laminin, type IV collagen or casein (Murphy *et al.,* 1993; Noël *et al.,* 1995). Third, ST3 contains an amino-acid substitution in the highly conserved MMP 'Met-turn' which may alter its enzymatic activity (Noël *et al.,* 1995). These unusual functional properties of ST3 raise the possibility that this protein does not function *in vivo* as an MMP.

Therefore, the aims of the present study were to determine whether the contribution of ST3 to tumor implantation results from its proteolytic activity and, in light of these findings, to evaluate the potential therapeutic relevance of developing ST3-specific inhibitors. The first approach consisted of generating a catalytically inactive enzyme. To do so, we replaced the active site glutamate of h- or mST3 with alanine by site-directed mutagenesis according to the method of Crabbe *et al.* (1994). These cDNA constructs were stably transfected into MCF7 cells. The second approach was intended to induce the local production of an MMP inhibitor (TIMP): MCF7 cells expressing ST3 (Noël *et al.,* 1998) were infected with a retroviral vector bearing TIMP2. In both types of experiments, the tumorigenicities of different clones were evaluated by subcutaneous (s.c.) injection into nude mice. We present evidence that *in vivo* ST3 contributes to cancer progression via its proteolytic activity, thereby raising the possibility to use specific ST3 inhibitors to develop therapeutic anticancer strategies.

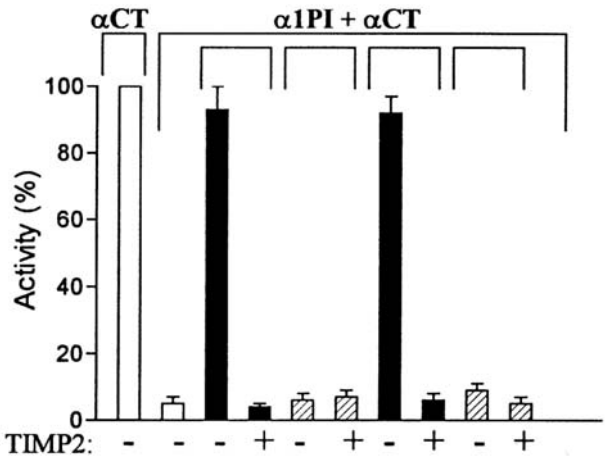
Results

Mutation of the glutamate active site of h- and mST3

Mutant catalytic domains (CD) had no proteolytic activity in vitro

To determine the importance of the glutamate located in the Zn-binding site for the *in vitro* enzymatic activity of ST3, site-directed mutagenesis of the catalytic domain (CD) was carried out to substitute Glu216 with Ala in hST3 (hCD216A) and Glu220 with Ala in mST3 (mCD220A). Wild-type hCD or mCD and mutant hCD216A or mCD220A were then expressed in *E. coli* using plasmid pET-3b, as previously described (Noël *et al.,* 1995). According to our previous data, refolding of wild-type hE and mEST3 from *E. coli* urea extracts yielded enzymes active against α1PI (Figure 1). However, mutated CD failed to degrade α1PI.

Figure 1: *Quantification of ST3 catalytic domain (CD) activity. Quantification of catalytic activity was based on the protein's capacity to inactivate α1PI, an inhibitor of αCT. Both wild-type hCD and mCD were able to inactivate α1PI leading to αCT activity. These activities were inhibited by TIMP2. Because the mutated forms of ST3 CD, hCD216A or mCD220A, could not cleave α1PI, αCT activity was inhibited.* □ *:no ST3;* ■ *: wild-type;* ▨ *: mutant ST3*



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Overexpression of full-length hST3 mutants in MCF7 cells failed to promote tumorigenicity

To determine the effect of this glutamate mutation on *in vivo* ST3 function, human breast adenocarcinoma MCF7 cells were stably transfected with pCMVhE216A vector containing the full-length coding region of mutant hST3. Neomycin-resistant clones were selected by Western blot analysis for expression of hE216A (Figure 2a). Three clones were found to express pro- and mature-mutant hST3 in their conditioned medium (MCF7hE216A/13, -hE216A/18 and -h216A/24). The pattern and levels of ST3 expression of these clones were similar to those of cells transfected with wild-type hST3 cDNA (MCF7hST3/9) (Noël *et al.,* 1996) (Figure 2a). They were stable in culture with neomycin. We compared the *in vitro* and *in vivo* properties of the site-directed mutant transfectants with those of wild-type ST3 transfectant, and cells transfected with the control vector alone (MCF7pCMV/2). *In vitro* proliferation rates and morphological features were similar for all the clones.

The *in vivo* tumorigenicity of these clones was evaluated by injecting 5 x 10^4 cells s.c. into nude mice. In all *in vivo* assays, cells were mixed with matrigel which is required for MCF7 cell implantation (Noël *et al.,* 1992). Inoculation of MCF7 cells expressing wild-type hST3 (MCF7hST3/9) enhanced the tumor incidence ($P<$ 0.0005) (Figure 3a) and increased the tumor volume ($P<0.005$) (Figure 3b). Indeed, 16 days after injection, MCF7hST3/9 cells generated tumors in all recipients, while no tumor was observed after injection of control cells (Figure 3a). Differences in tumor incidences were observed within the first month after injection but disappeared shortly thereafter. Consistent with our previous report (Noël *et al.,* 1996), ST3 expression by MCF7 cells shortened the tumor-free period. The tumor incidence (Figure 3a) and tumor volumes (Figure 3b) of the three mutant clones, MCF7hE216A/13, -hE216A/18 and -hE216A/ 24, were the same as those observed for control MCF7 cells (P>0.1). Thus, in contrast to mutated inactive ST3, the presence of functional ST3 clearly promoted tumor development.

Figure 2: *Western blots of MCF7 cells expressing exogenous hST3 (a) or mST3 (b). Conditioned media were prepared as described under Materials and methods. The positions of pro-and/or mature ST3 are indicated on the left, (a) Lane 1: control cells; lane 2: pCMVhST3 transfected cells; lanes 3-5: pCMVhE216A transfected cells, (b) Lane 1: pCMVmST3 transfected cells; lanes 2-4: pCMV mE220A transfected cells*

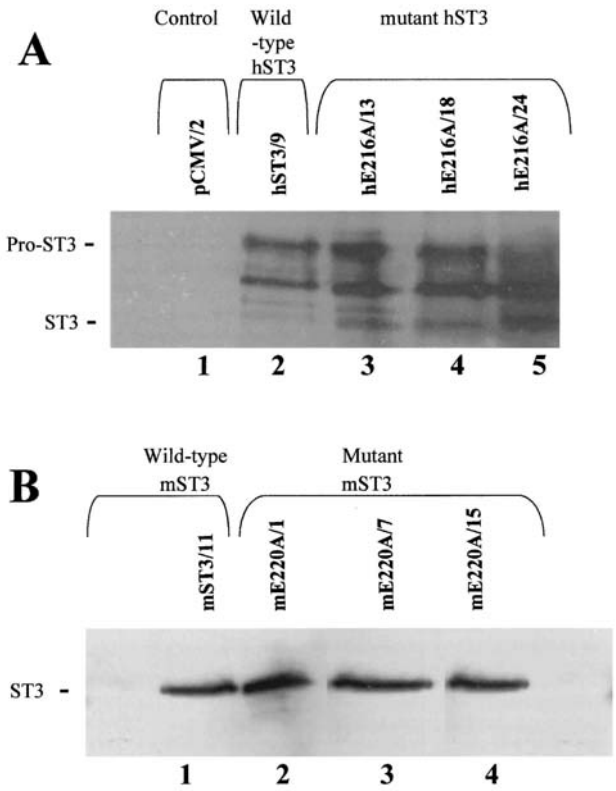
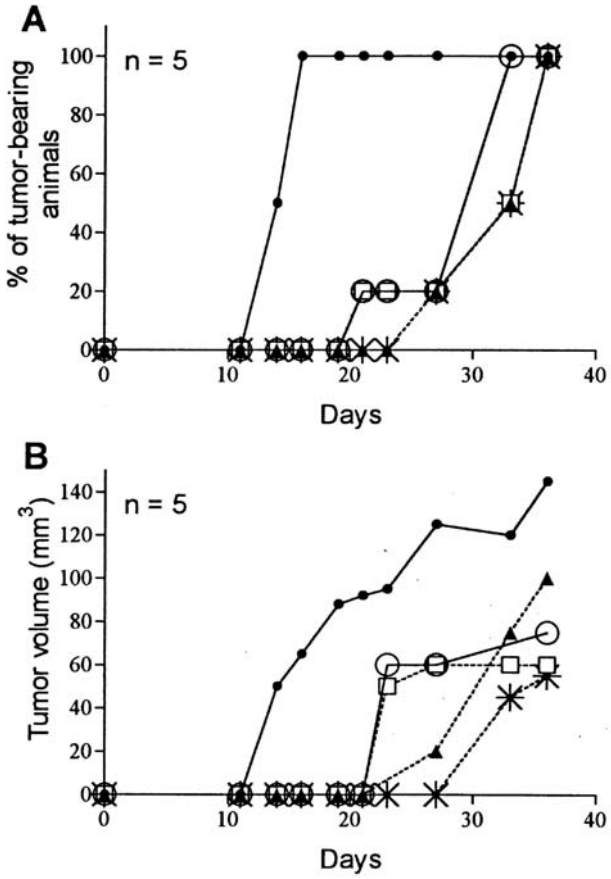


Figure 3: *Analysis of MCF7 cells expressing inactive hST3. Cells mixed with matrigel were injected s.c. into nude mice. Tumor incidence (a) and tumor volume (b) were evaluated as described under Materials and methods. pCMV/2 (○): control MCF7 cells; hST3/9 (●): MCF7 cells expressing wild-type hST3; hE216A/13 (A), hE216A/18 (□) and hE216A/24 (*): three MCF7 cell clones expressing inactive hST3 (dashed lines), n indicates the number of mice injected with each clone*



Overexpression of full-length mST3 mutants in MCF7 cells failed to promote tumorigenicity

Using the same procedure as that described above, three MCF7 clones stably transfected with pCMVmE220A vector and expressing inactive mutant mST3 (MCF7mE220A/l; -mE220A/7 and -mE220A/ 15) were obtained (Figure 2b). Their *in vitro* proliferation rates were similar to those of control MCF7 pCMV/2 cells and MCF7mST3/11 cells transfected with wild-type mST3 cDNA.

As previously reported (Noël *et al.,* 1996), mST3 expression shortened the tumor-free period ($P<0.0005$) (Figure 4a) and accelerated tumor growth ($P<0.005$) (Figure 4b). In contrast, the three clones expressing inactive mutant mST3 failed to enhance MCF7 cell tumorigenicity as assessed by tumor incidence and tumor volume ($P>0.1$).

Because these observations demonstrated that the tumor-promoting effect of both h- and mST3 required an intact CD for the enzyme's proteolytic activity, we tested whether the ST3 effect on MCF7 cell tumorigenicity could be inhibited by the physiological MMP inhibitor, TIMP2.

Targeting of hST3 with hTIMP2

MCF7hST3/9 cells expressing wild-type hST3 or control MCF7pCMV/2 were infected with viral vectors bearing pBabeTIMP2. Three clones co-expressing stable levels of hST3 and high levels of TIMP2 (between 1 and 3 ng/μg of DNA) (MCF7hST3TIMP2/7, -hST3TIMP2/16 and -hST3TIMP2/17) were selected. ST3 expression by these clones was similar to that of the MCF7hST3/9 cells (Figure 5). Both pro- and mature ST3

forms were detected together with some degradation products including a 28-kDa form (Noël *et al.,* 1995). Two control clones producing similar amounts of TIMP2, but not hST3, were also selected (MCF7pCMVTIMP2/2 and -pCMVTIMP2/11).

Figure 4: *Analysis of MCF7 cells expressing inactive mST3. Cells mixed with matrigel were injected s.c. into nude mice. Tumor incidence (a) and tumor volume (b) were evaluated as described under Materials and methods. pCMV/2 (○): control MCF7 cells; mST3/11 (●): MCF7 cells expressing wild-type mST3; mE220A/l (▲), mE220A/7 (□) and E220A/15 (*): three MCF7 cell clones expressing inactive mST3 (dashed lines), n indicates the number of mice injected with each clone*

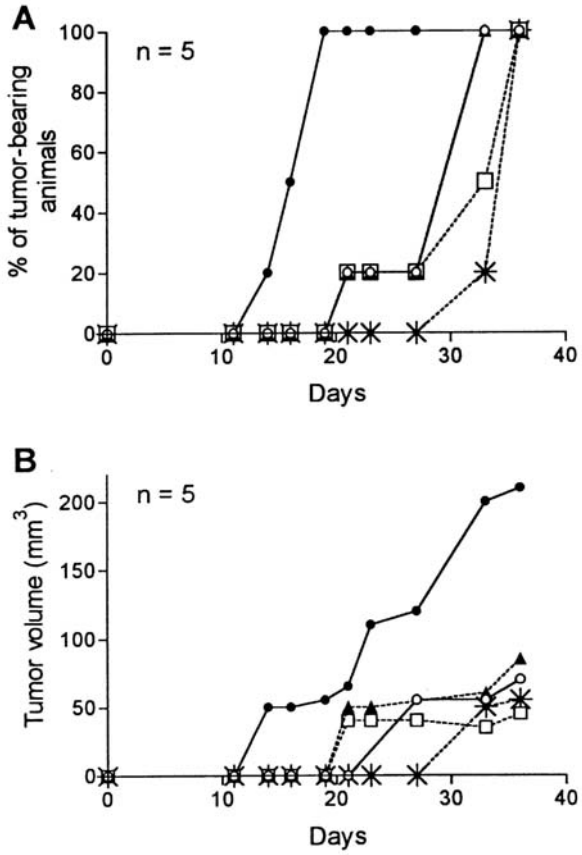
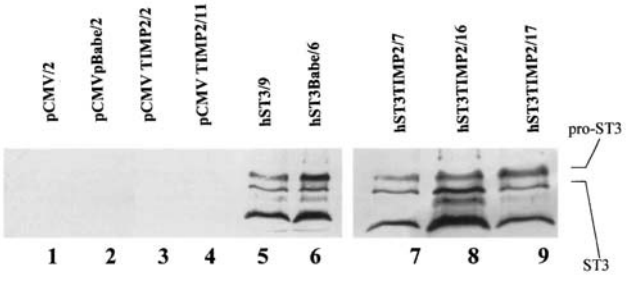


Figure 5: *Western blot of MCF7 cells expressing exogenous hST3 and induced to over-express TIMP2 or not. Conditioned media were prepared as described under Materials and methods and Western blots were incubated with monoclonal 5ST-4C10 which recognizes the ST3 CD. The positions of pro- and mature ST3 are indicated on the right*



To obtain control cells negative for TIMP2 expression, MCF7hST3/9 cells and control MCF7pCMV/2 cells were infected with the control viral vector (pBabe) and gave rise to MCF7 hST3pBabe/6 cells and MCF7pCMVpBabe/2 cells, both lacking TIMP2 expression, and respectively expressing hST3 or not (Figure 5). The *in vitro* growth rates of all the clones were evaluated and no obvious difference was observed (data not shown).

In agreement with our previous observations (Noël *et al.,* 1996), hST3 expression shortened the tumor-free period ($P<0.0005$) (Figure 6a) and stimulated tumor growth ($P<0.005$) (Figure 6b). TIMP2 production by MCF7hST3TIMP2/7, -hST3TIMP2/16 and -hST3TIMP2/17 abolished the additional tumor-promoting effect of hST3, thereby prolonging the tumor-free period (Figure 6a) and slowing tumor growth (Figure 6b). Under these conditions, the incidence and growth of tumors were similar to those observed after injection of MCF7pCMVTIMP2/2 and -pCMVTIMP2/11 control cells ($P>0.1$) (Figure 6).

These findings strongly support our hypothesis that ST3 promotes MCF7 cell tumorigenicity via its proteolytic activity.

Effect of depleted matrigel on the tumor-promoting effect of hST3

Matrigel contains low-molecular-weight proteins, such as growth factors and cytokines, that could be released by the proteolytic action of hST3. Therefore, we compared the tumorigenicities of MCF7 cells expressing hST3 (MCF7hST3/9) or not (MCF7pCMV/2) and mixed with matrigel or cytokine-depleted matrigel. Depleted matrigel did not affect the tumorigenicity of control MCF7 cells (Figure 7). While injection cells of complete matrigel shortened the tumor-free period ($P<0.0005$) and promoted tumor growth of MCF7hST3/9 ($P<0.005$), these effects were abolished in the presence of depleted matrigel.

Histological examination of tumors

Tumors that developed after injection of control MCF7 cells, wild-type ST3 transfectants or site-directed mutant ST3 transfectants were studied by light microscopy. In each case, cells were organized into nodules of malignant cells surrounded by a few stromal cells (Figure 8). No histological differences were observed between tumors, regardless of the ST3 and/or TIMP2 expression levels.

Discussion

ST3 is expressed in most invasive carcinomas (Rouyer *et al.,* 1994) and high levels of expression indicate a poor prognosis (Chenard *et al.,* 1996; Ahmad *et al.,* 1998; Tetu *et al.,* 1998). *In vivo* experimental data demonstrated that ST3 is involved in the early steps of tumor development (Noël *et al.,* 1996). This function of host-derived ST3 was further substantiated by the less successful tumor implantation observed in ST3-deficient mice (Masson *et al.,* 1998). Despite this accumulating body of evidence supporting a role for this MMP in cancer progression, the mechanism of ST3 action remains to be elucidated. The inability of mature ST3 forms to degrade *in vitro* classical extracellular matrix substrates cleaved by other MMP (Murphy *et al.,* 1993; Pei *et al.,* 1994; Noël *et al.,* 1995) raised doubts as to the ability of ST3 to function *in vivo* as a protease. Therefore, the present study was undertaken to determine whether the *in vivo* ST3 effect requires this enzyme's proteolytic action.

With this aim, inactive mutants of h- and mST3 were obtained by replacing the active-site glutamate with alanine (Crabbe *et al.,* 1994). Substitution of the active site Glu by either Ala in gelatinase A (E375A) (Crabbe *et al.,* 1994) or by Gln (E200Q) in interstitial collagenase (Windsor *et al.,* 1994) has been shown to generate completely inactive enzymes. As assessed by using the recombinant CD of both h- and mST3 (Noël *et al.,* 1995), this mutation yielded enzymes inactive against α1PI. Transfection of MCF7 cells with inactive full-length h- and mST3 gave rise to different clones expressing the proteinase at levels similar to that of the previously described MCF7hST3/9 clone (Noël *et al.,* 1996). The *in vivo* properties of the different mutant transfectants were similar to those of control MCF7pCMV/2 cells. Therefore, the ST3 tumor-promoting effect can be completely inhibited by inactivation of the ST3 catalytic site.

In addition, the ability of a physiological MMP inhibitor to block *in vivo* effects of ST3 was evaluated by inducing TIMP2 production in MCF7 cells expressing hST3 or not. The TIMP2 overproduction induced by retroviral-mediated gene transfer did not influence cell growth *in vitro.* Because MCF7 cells do not produce MMP (Noël *et al.,* 1994, 1998), similar tumor development was observed after injection of control MCF7 cells expressing TIMP2 (MCF7pCMVTIMP2) or not (MCF7pCMV). In contrast, TIMP2 production by MCF7 cells expressing hST3 (MCF7hST3TIMP2) inhibited the ST3 tumor-promoting effect by prolonging the tumor-free

period and by slowing tumor growth. Taken together, these results indicate that TIMP2 prevents ST3-mediated tumor growth enhancement probably by inhibiting the catalytic action of this enzyme, since it did not affect tumor growth in the absence of the enzyme. Thus, by using two complementary approaches, site-directed mutagenesis and induction of TIMP2 production, we were able to demonstrate that the ST3 tumor-promoting effect was due to the enzyme's proteolytic activity and that this MMP can indeed be targeted by a physiological MMP inhibitor.

In vivo, ST3 is not produced by the tumor cells themselves, but by peritumoral fibroblasts (Basset *et al.,* 1990). It is now well documented that fibroblasts influence the tumorigenicity of different tumor types (Camps *et al.,* 1990; Gartner *et al.,* 1992; Price *et al.,* 1996; Noël *et al.,* 1998). Recently, we showed that this tumor-promoting effect of fibroblasts is attributable, at least in part, to their production of proteases and can be inhibited by a synthetic MMP inhibitor or by local TIMP2 production (Noël *et al.,* 1998). Among the stromal MMP, ST3 appears to be a primary candidate. Indeed, fibroblasts derived from ST3-deficient mice ($ST3^{-/-}$) were unable to promote MCF7 cell tumorigenicity, while co-injection of these tumor cells and fibroblasts issued from wild-type mice ($ST3^{+/+}$) led to increased tumor incidence and size (Masson *et al.,* 1998). Therefore, ST3 appears to be a stroma-derived factor that promotes tumor-cell implantation in a paracrine manner (Masson *et al.,* 1998).

Figure 6: *Effect of local human TIMP2 production on MCF7 cell tumor development. Cells mixed with matrigel were injected s.c. into nude mice. Tumor incidence (a) and tumor volume (b) were determined as described under Materials and methods. pCMV/2 (○) or pCMVpBabe/2 (▽): control cells; pCMVTIMP2/2 (●) and pCMVTIMP2/11 (*): control MCF7 cell clones expressing TIMP2; hST3pBabe/6 (Δ) and hST3/9 (▲): MCF7 cells expressing hST3; hST3TIMP2/7 (□), hST3TIMP2/16 (▼), hST3TIMP2/17 (dashed lines): MCF7 cells expressing both hST3 and TIMP2. n indicates the number of mice injected with each clone*

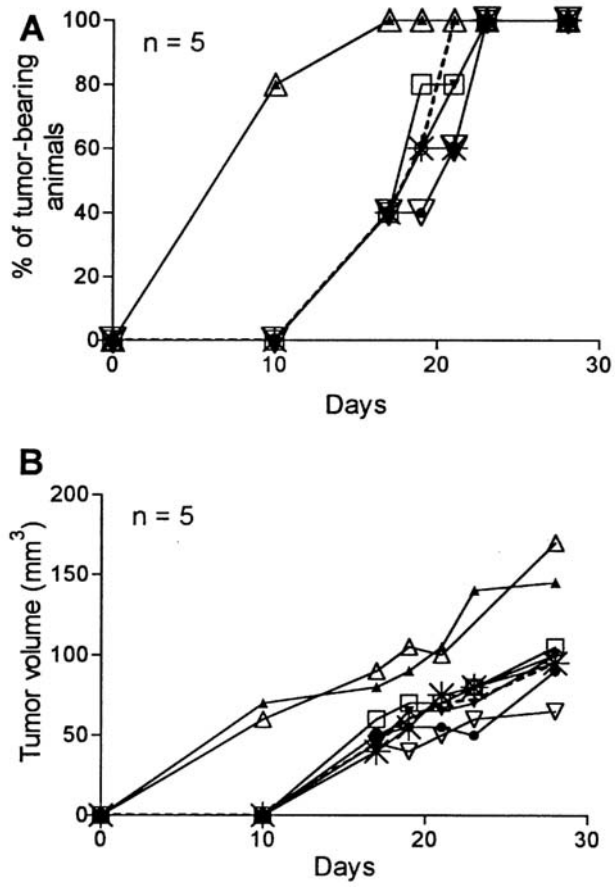


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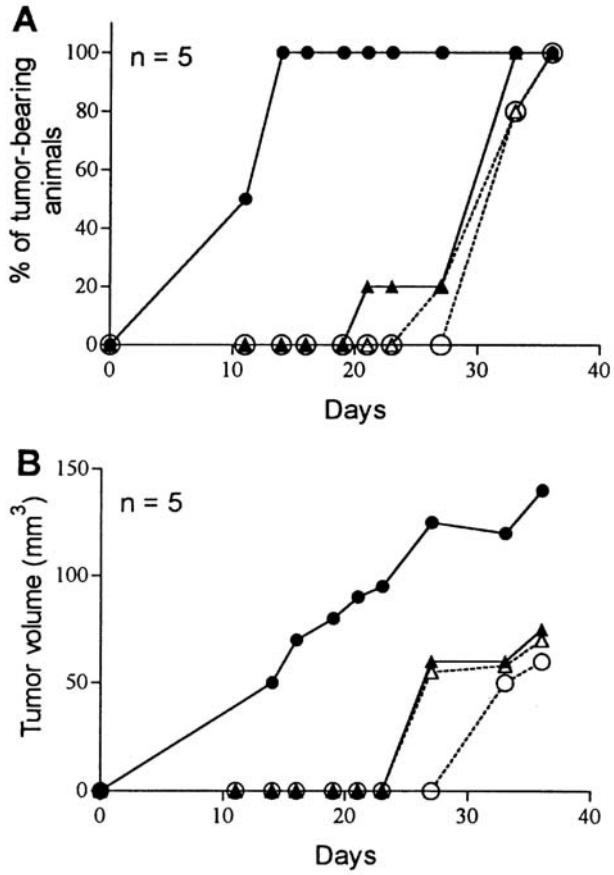
Figure 7: *Analysis of the effect of extracellular matrix-associated factors on the hST3 tumor-promoting effect. MCF7 cells were mixed with low-molecular-weight protein-depleted matrigel (open symbols and dashed lines) or intact matrigel (closed symbols) and injected s.c. into nude mice. Tumor incidence (a) and tumor volume (b) were determined as described under Materials and methods. pCMV/2 (Δ,▲): control cells, MCF7 hST3/9 (○,●): MCF7 expressing hST3. n indicates the number of mice injected with each clone*



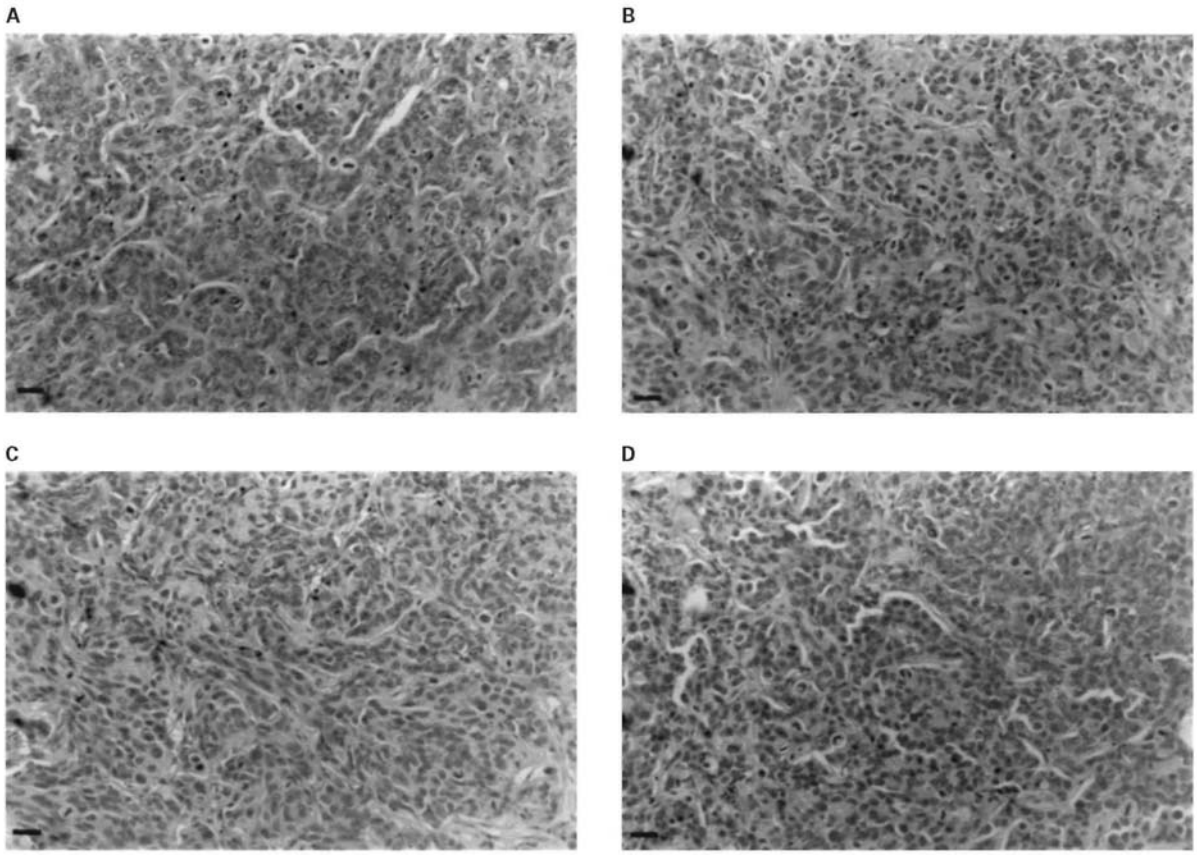
The mechanism of ST3 action is still unknown. As described previously, ST3 did not influence either cell proliferation, or cell invasion. Since no specific substrate has been identified for ST3, one can postulate that this protein is involved in the activation of other proteases, in the shedding of cell surface molecules or receptors, or in the activation of growth factors. Arguments supporting a particular role for ST3 among the MMP family members include its unusual activation process (Pei and Weiss, 1995; Santavicca *et al.,* 1996), its gene expression regulation (Anglard *et al.,* 1995; Guérin *et al.,* 1997), and its unusual proteolytic activity (Pei *et al.,* 1994; Noël *et al.,* 1995). The unusual ST3 enzymatic properties (Murphy *et al.,* 1993; Pei *et al.,* 1994; Noël *et al.,* 1995) are further substantiated by the development of synthetic substrates for ST3 (Kannan *et al.,* 1999). ST3 efficiently cleaves substrates harboring unusually long side chains, thus suggesting that this MMP may hydrolyze yet unidentified substrate(s) containing amino acids not commonly recognized by the other members of this family of enzymes. In this context, it should be noted that, in our model, the tumor-promoting effect of ST3 was observed when ST3-expressing cells were injected with complete matrigel but not with depleted matrigel. These data indicate that ST3 action is dependent upon extracellular matrix-associated factors. They are in accordance with the recent observations that the fibroblast contribution to tumor promotion could be associated with their ability to remodel extracellular matrix and probably to the release or activation of growth factors present in matrigel (Masson *et al.,* 1998; Noël *et al.,* 1998).

Recently, insulin-like growth factor binding protein-1 (IGFBP-1) was identified as a potential physiological substrate for hST3 (Manes *et al.,* 1997). However, hST3 shares IGFBP-1 proteolytic activity with other MMP, such as gelatinase A, stromelysin-1 and matrilysin (Manes *et al.,* 1997). Western blots showing similar amounts

of IGFBP-1 in both normal and depleted matrigel (data not shown) indicated the lack of tumor-promoting effect in the presence of the latter.

Regardless of the exact mechanism of ST3 action, our results demonstrate that the *in vivo* ST3 effect on tumor development requires a functional ST3 CD, and extracellular matrix and its associated factors. ST3 probably releases or activates growth factors or cytokines stored in the extracellular matrix. Therefore, ST3 is indeed a unique MMP that cannot be substituted for by other MMP, its potential role as a target of specific MMP inhibitor(s) in therapeutic anticancer strategies requires further investigation.

Figure 8: *Histological examination of tumors derived from control MCF7pCMV/3 (a), MCF7hST3/9 (b), mutant MCF7hE216A/ 13 (c) and MCF7hST3TIMP2/7 (d). Tumor sections were stained with hematoxylin and eosin. (Bar = 90 μm)*



Materials and methods

Construction of h- and mST3 mutants

The cDNA containing the full-length coding region of h- and mST3 cloned in pBluescript SK^+ vector were modified by site-directed mutagenesis for substitution of Glu216 by Ala 216 in the human enzyme (primer 5-CAGGTGGCAGCC-CATGCATTTGGCCACGTGCTG-3' was designed to create an *Nsi*I restriction site, in bold), and for substitution of Glu220 by Ala220 in the mouse enzyme (primer 5-CAAGTGGCGGCTCATGCATTTGGCCATGTTCTG-3' was synthesized to create an *Nsi*I restriction site, in bold). The human (hE216A) or murine (mE220A) constructs were digested with *Spe*I and *Xho*I and ligated into the pCMV vector (Noël *et al.,* 1996)) to obtain pCMVhE216A or pCMVmE220A, respectively. All recombinant plasmids were sequenced with an automated sequencer (Epicenter Technologies, Madison, WI, USA) to verify that no additional mutation was introduced during plasmid construction.

Expression and production of mutated recombinant ST3 CD

After mutagenesis, cDNA fragments encoding the CD of human (residues Phe98 to Gly272) or murine (residues Phel02 to Ser276) ST3 were amplified by polymerase chain reaction using as template plasmids pCMVhE216A or pCMVmE220A, respectively. Primers 1(5'-TAACTTTAA-GAAGGAGATATACATATGTTCGTGCTTTCTGGCGG-3') and 2(5'-GCAGCCGGATCCGGTCTAGACCTCGAGT-CACTAGCCCAGGGC-3') for hST3 and primers (5'-TAACTTTAAGAAGGAGAT ATACATATGTTCGTCCT-GTCAGGAGA-3') and 4(5'-GCAGCCGGATCCGGTCTA-G AC CTCGAGT CACTAGC TCA AAGTT GGG GCGC-3') for mST3 were used for the reaction. Amplified products were then cloned into the pET-3b vector which was used for recombinant ST3 expression in isopropyl β-D-thiogalactopyr-anoside induced log-phase bacterial cultures [*Escherichia coli* (BL21(DE)pLysS cells] (Noël *et al.,* 1995).

The CD of human wild-type (hCD) or mutant ST3 (hCD216A) or those of murine wild-type (mCD) or mutant ST3 (mCD220A) were prepared from bacterial inclusion bodies denatured with 8 M urea and purified through a Q-Sepharose anion-exchange column (Pharmacia Biotec Inc., Uppsala, Sweden). Monomeric proteins renatured by dialysis against progressively decreasing concentrations of urea were separated from aggregates by size-exclusion chromatography, using a gel-filtration column (Kannan *et ai,* 1999).

Enzymatic activity determination of mutated recombinant ST3 CD

ST3 activity was quantified using an indirect activity assay based on the ST3 ability to inactivate α1PI, an inhibitor of α-chymotrypsin (αCT). Briefly, the activity of αCT (1 μg) against a synthetic substrate (N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide, Sigma Chemical Co, St. Louis, MO, USA) was evaluated in the presence of α1PI (1 μg) preincubated or not with ST3 hCD or hCD216A (1 μg), or mCD or mC220A (500 ng). The change of the substrate from colorless to yellow indicated the presence of ST3 activity, which was quantified at 405 nM using a Beckman DU640 spectrophotometer (Kannan *et al.,* 1999). ST3 activity was inhibited by TIMP2 prepared as previously described (Noël *et al.,* 1995).

Cell culture

Human breast adenocarcinoma MCF7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, glutamine (292 mg/ml), sodium bicarbonate (2.1 mg/ml) and penicillin-streptomycin (100 U/ ml).

To prepare conditioned medium, subconfiuent cells in T-flasks (75 cm^2, Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) were washed twice, then cultured in 5 ml of serum-free medium. After 48 h, the medium was collected, centrifuged at 1000 r.p.m. for 10 min and frozen until use. Cells were collected and DNA content was measured fiuorimetrically according to Labarca and Paigen (1980).

For cell proliferation assays, 10^4 cells were seeded in 24-well polystyrene plates (Costar, Cambridge, MA, USA) and maintained in culture for 7 days. DNA content was evaluated every 2 days.

Preparation of stably transfected cells

MCF7 cells were transfected with pCMVhE216A or pCMVmE220A constructs (10 µg) using a BioRad electro-poration apparatus at 400 V and 125 µF. Transfectants were selected with the neomycin analog G418 (400 µg/ml; Gibco BRL, Gaithersburg, MD, USA) (Noël *et al.,* 1996). Individual clones were collected and maintained separately.

Retroviral infection of MCF7 cells

The pBabe control vector, pBabeTIMP2 vector and virus-producing cells were obtained as previously described (Noël *et ai,* 1998). For retroviral infection, 10^6 MCF7 cells stably transfected with pCMVhST3 vector and expressing hST3 (clone MCF7hST3/9) or control MCF7 cells transfected with the control pCMV vector (clone MCF7pCMV/l) (Noël *et ai,* 1996) were seeded in 10-cm dishes (Falcon). After 24 h, cells were washed and cultured with virus-containing medium [from 1 to 5 x 10^s colony-forming units (CFU/ml)] supplemented with Polybrene (8 μg/ml), [as previously described (Noël *et al.,* 1998)]. Cells were then selected with puromycin (0.5 μg/ml; Serva Feinbiochemica, Heidelberg, Germany) through the puromycin-resistance gene of the pBabe vector and with neomycin analog G418 (400 μg/ml) through the pCMV vector. The selection medium was changed

every 3 days until drug-resistant colonies appeared (3 weeks). Individual clones were then collected and cultured separately.

Quantification of TIMP2 production

The amount of TIMP2 was measured by enzyme-linked immunosorbent assay (ELISA) in medium conditioned by infected cells, as previously described (Noël *et al.,* 1998). Results are expressed as a function of cell DNA content.

Western blotting

Conditioned media were first dialyzed overnight against 50 mM ammonium hydrogen-carbonate and then lyophilized. Proteins resuspended in 50 mM Tris-HCl (pH 7.4), 100 mM NaCl were subjected to sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Immunoblot analysis was performed by using monoclonal antibody 5ST-4C10 raised against the ST3 CD and the Enhanced Chemiluminescence Detection kit (Dupont NEN, Boston, MA, USA), as previously described (Noël *et ai,* 1995).

Matrigel preparations

We produced our own preparations of matrigel by extraction of extracellular matrix proteins from murine Engelbreth Holm Swarm (EHS) tumor cells as described by Taub *et ai* (1990). To deplete matrigel of low-molecular-weight proteins ('depleted matrigel'), ice-cold matrigel was precipitated with 20% ammonium sulfate (Taub *et ai,* 1990), centrifuged, the pellet was resuspended in the same buffer and recentrifuged. The final pellet was solubilized by dialysis against 50 mM Tris-HCl (pH 7.4), 150 mM NaCl.

In vivo tumorigenicity assay

Subconfiuent MCF7 clones were trypsinized, washed twice and harvested by centrifugation at 1000 *g* for 5 min. Cells suspended in cold serum-free medium were mixed with an equal volume of cold matrigel (10 mg/ml). A total volume of 0.4 ml containing 5 x 10^4 cells was injected s.c. into 6-8-week-old female nude mice (Iffa-Credo, L'Arbresle, France), pre-implanted with 60-day release estradiol pellets (Innovative Research of America, Sarasota, FL, USA). Tumorigenicity defined as the capacity of cells to induce tumor formation. Two parameters were evaluated: the tumor incidence (percentage of tumor-bearing animals) and the tumor-volume. The larger (a) and smaller (b) diameters of tumors were measured every 2 days and served for tumor-volume calculation according to the formula: a x b^2 x 4 (Noël *et al.,* 1992). Tumors smaller than 50 mm^3 were not taken into account because of technical imprecisions of the measurements. Interindividual variations of tumor volumes within each group were always less than 20%.

At the end of the *in vivo* assay, tumors were excised, fixed in 10% buffered formalin and embedded in paraffin, then 4-μm intervals thick sections were cut and stained with hematoxylin and eosin.

Statistical analysis

Tumor volumes were analysed using Student's *t-test (P* values <0.005 were considered to be significant). For tumor incidence, data were compared using the log rank test.

Abbreviations

α1PI: alpha 1-proteinase inhibitor; αCT: alpha-chymotryp-sin; CFU: colony-forming units; DMEM: Dulbecco's modified Eagle's medium; ELISA: enzyme-linked immunosorbent assay; h- and mCD: human and murine ST3 catalytic domains; h- and mE: full-length h- and mST3 mutants; IGFBP-1: insulin-like growth factor binding protein-1; MMP: matrix metalloproteinase; s.c: subcutaneous; SDS-PAGE: sodium dodecyl sulfate-polyacryla-mide gel electrophoresis; ST3: stromelysin-3; TIMP: tissue inhibitor of metalloprotease.

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References

Ahmad A, Marshall JF, Basset P, Anglard P and Hart IR. (1997). *Int. J. Cancer,* 73, 290-296.

Ahmad A, Hanby A, Dublin E, Poulsom R, Smith P, Barnes D, Rubens R, Anglard P and Hart I. (1998). *Am. J. Pathol.,* 152,721-728.

Anglard P, Melot T, Guérin E, Thomas G and Basset P. (1995). *J. Biol. Chem.,* 270, 20337-20344.

Basset P, Bellocq JP, Wolf C, Stoll I, Hutin P, Limacher JM, Podhajcer OL, Chenard MP, Rio MC and Chambon P. (1990). *Nature,* 348, 699-704.

Camps JL, Chang SM, Hsu TC, Freeman MR, Hong SJ, Zhau HE, von Eschenbach AC and Chung LWK. (1990). *Proc. Natl. Acad. Sci. USA,* 87, 75-79.

Chenard MP, O'Siorain L, Shering S, Rouyer N, Lutz Y, Wolf C, Basset P, Bellocq JP and Duffy M J. (1996). *Int. J. Cancer,* 69, 448-451.

Crabbe T, Zucker S, Cockett MI, Willenbrock F, Tickle S, O'Connell JS, Scothern JM, Murphy G and Docherty AJP. (1994). *Biochemistry,* 33, 6684-6690.

DeClerck YA, Imren S, Montgomery AMP, Mueller BM, Reisfeld RA and Laug WE. (1998). In: *Chemistry and Biology of Serpins.* Church D, *et al.* (eds) Plenum Press: New York, pp 89-97.

Gartner MFRM, Wilson EL and Dowdle EB. (1992). *Int. J. Cancer,* 51, 788-791.

Guérin E, Ludwig MG, Basset P and Anglard P. (1997). *J. Biol. Chem.,* 272*,* 11088-11095.

Kannan R, Ruff M, Kochins JG, Manly SP, Stoll I, El Fahime EM, Noël A, Foidart JM, Dive V, Rio MC and Basset P. (1999). *Protein Expr. Purif.,* 16, 76-83.

Labarca C and Paigen K. (1980). *Anal. Biochem.,* 102, 344-352.

Manes S, Mira E, Barbacid MM, Cipres A, Fernandez-Resa P, Buesa JM, Merida I, Aracil M, Marquez G and Martinez AC. (1997). *J. Biol. Chem.,* 272*,* 25706-25712.

Masson R, Lefèbvre O, Noël A, Chenard MP, Wendling C, Kebers F, Lemeur M, Dierich A, Foidart JM, Basset P and Rio MC. (1998). *J. Cell Biol,* 140, 1535-1541.

Murphy G, Segain JP, O'Shea M, Cockett M, Ioannou C, Lefèbvre O, Chambon P and Basset P. (1993). *J. Biol. Chem.,* 268, 15435-15441.

Noël A, Simon N, Raus J and Foidart JM. (1992). *Biochem. Pharmacol.,* 43, 1263-1267.

Noël A, Polette M, Lewalle JM, Munaut C, Emonard H, Birembaut P and Foidart JM. (1994). *Int. J. Cancer,* 56, 331-336.

Noël A, Santavicca M, Stoll I, L'Hoir C, Staub A, Murphy G, Rio MC and Basset P. (1995). *J. Biol. Chem., 110,* 22866-22872.

Noël A, Lefèbvre O, Maquoi E, Vanhoorde L, Chenard MP, Mareel M, Foidart JM, Basset P and Rio MC. (1996). *J. Clin. Invest.,* 97, 1924-1930.

Noël A, Gilles C, Bajou K, Devy L, Kebers F, Lewalle JM, Maquoi E, Munaut C, Remacle A and Foidart JM. (1997). *Invasion Metast.,* 17, 221 -239.

Noël A, Hajitou A, L'Hoir C, Maquoi E, Baramova E, Lewalle JM, Remacle A, Kebers F, Brown P, Calberg- Bacq CM and Foidart JM. (1998). *Int. J. Cancer,* 76, 267-273.

Pei D, Majmudar G and Weiss SJ. (1994). *J. Biol. Chem.,* 269, 25849-25855.

Pei D and Weiss S. (1995). *Nature,* 375, 244-247.

Price JE. (1996). *Breast Cancer Res. Treat.,* 39, 93-102.

Rouyer N, Wolf C, Chenard MP, Rio MC, Chambon P, Bellocq JP and Basset P. (1994). *Invasion Metast.,* 14, 269-275.

Rudolph-Owen LA and Matrisian LM. (1998). *J. Mammary Gland Biol. Neoplasia,* 3, 177-190.

Santavicca M, Noël A, Stoll I, Segain JP, Angliker H, Anglard P, Chrétien M, Seidah N and Basset P. (1996). *Biochem. J.,* 315,953-958.

Taub M, Wang Y, Szcesny TM and Kleinman HK. (1990). *Proc. Natl. Acad. Sci. USA,* 87, 4002-4006.

Tetu B, Brisson J, Lapointe H and Bernard P. (1998). *Hum. Pathol.,* 29, 979-985.

Windsor LJ, Bodden MK, Birkedal-Hansen B, Engler JA and Birkedal-Hansen H. (1994). *J. Biol. Chem.,* 269, 26201-26207.