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PRINCIPAL INVESTIGATOR: Robert Shiu, Ph.D.

CONTRACTING ORGANIZATION: University of Manitoba
Winnipeg, Manitoba, CANADA R3E-OW3

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INTRODUCTION:

Posttranslational processing is a major mechanism by which the biological activities of many proteins, including protein hormones and cellular growth regulatory proteins are modified. A new class of enzymes that perform protein processing are the Proprotein Convertases. Proprotein convertases, by virtue of their unique property of modifying the biological activities of growth-promoting and growth-suppressing cellular regulatory proteins, are therefore strategically involved in the neoplastic process. We have completed a preliminary survey of human breast cancer cell lines as well as 30 primary human breast tumor specimens and 10 normal breast tissue samples; this study has revealed an remarkable increase in the expression of several proprotein convertases (PC1, furin and PACE4, but not PC2) in breast cancer cells. These data suggested that aberrant expression of proprotein convertases may be a hallmark of human breast cancers. A direct linkage between proprotein convertase activity and breast cancer phenotype has been strengthened by the recent finding that the human breast cancer susceptibility gene product and tumor-suppressor, BRCA1, is a member of the granin family of proteins and it may be a natural target of proprotein convertases. **This IDEA proposal seeks to test the hypothesis that elevated expression of proprotein convertases is one mechanism by which breast cancer cells acquire their growth advantage, and that a perturbation (enhancement) of convertase expression in the developing mammary gland of transgenic mice will predispose the mammary gland towards neoplastic transformation.** Two specific objectives will be sought: [1] To determine in human breast cancer cell lines if altered expression of proprotein convertases affect cell growth and posttranslational processing of BRCA1. This will be accomplished by generating a series of MCF-7 human breast cancer cell lines with stable integration of sense or anti-sense convertase cDNAs (PC1 or furin) expression vectors. The growth *in vitro* and *in vivo* in athymic nude of these "transfected" cell lines having over-expression or reduced-expression of convertase will be compared to that of wild type MCF-7 cells. Also, the production of BRCA1 and its processed peptides in "transfected" and wildtype cells will be compared by using BRCA1 specific antibodies. [2] To elucidate the consequences of over-expression of convertases targeted to the mammary glands of transgenic mice in affecting Brca1 (the mouse homolog) processing and cellular transformation. We will target PC1 or furin to the mammary gland using the MMTV promoter. We will monitor for abnormal changes in the mammary glands at various developmental and functional stages. We will measure the profile of Brca1 (the mouse BRCA1 homolog) protein patterns in the same tissues. If over-expression of a convertase alone does not produce discernable abnormality in the transgenic mouse mammary glands, we will produce "double" transgenic mice between MMTV-convertase transgenic mice with MMTV-myc transgenic mice. The "double" transgenic mice will be used to test the hypothesis that convertases facilitate the oncogenic potentials of cellular proto-oncogenes such as c-myc towards breast tumorigenesis. The parameters to be studied in the "double" transgenic mice will be the same as those proposed for the single MMTV-convertase transgenic mice.

BODY:

Tasks 1 to 5: To determine in human breast cancer cell lines if altered expression of proprotein convertases affect breast cancer cell growth.

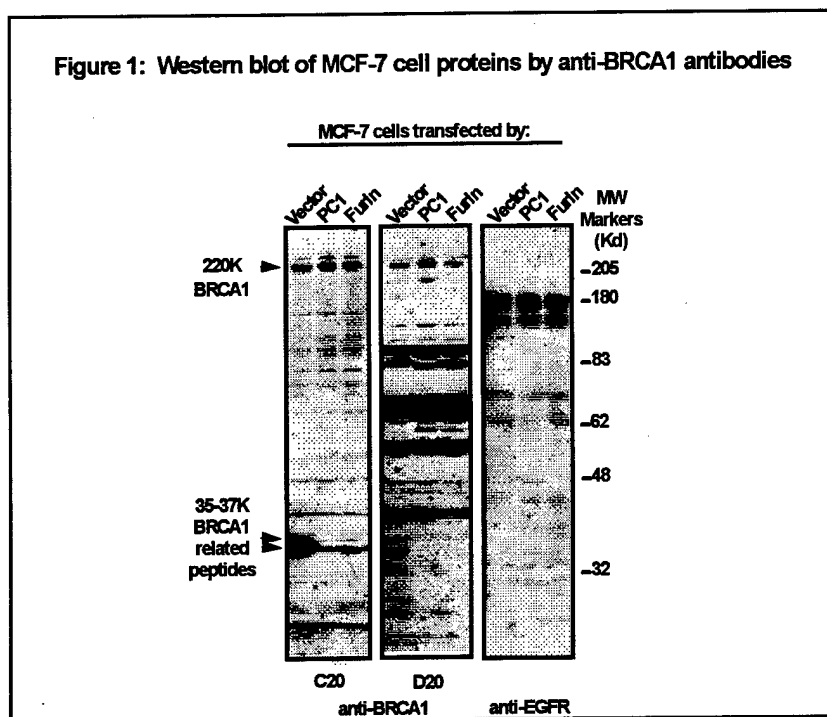
In the first 12-month period, we have already completed most of the studies outlined in Tasks

1 to 5. Two pro-protein convertase cDNAs--PC1 and furin--were stably transfected into human breast cancer cell line MCF-7 to study the altered breast cancer growth behaviour both *in vitro* and *in vivo*. The PC1 and furin over-expressing lines possessed an altered morphology when grown in culture. In the presence of fetal bovine serum, the convertase-over-expressing cells and the wildtype control cells grew with a similar rate. However, when a serum-free defined medium was used, the convertase-transfected cells grew slower than the vector transfected, controlled cells. The cell population doubling times of PC1- and furin-transfected cells were 1.5 times on plastic and 2 times on Matrigel than control cells. When grown *in vivo* in athymic nude mice supplemented with a high dose (5 mg/pellet) of estradiol, there was no significant difference between the growth rates of the convertase-transfected and control MCF-7 tumors. However, when the estradiol dose was reduced to 1.7 mg/pellet, the tumours from PC1 or furin transfected MCF-7 cells grew slower (growth rate 100-200 mm³/week) when compared with tumours of controlled MCF-7 (growth rate 585 mm³/week). It appears that the over-expression of proprotein convertases has rendered the breast cancer cells more dependent on estrogen for growth. The response to Tamoxifen treatment was also different, with the regression rate (70-130 mm³/week) of tumours from PC1 or furin transfected MCF-7 significantly less than their control MCF-7 tumours (547 mm³/week). Thus, the convertase-transfected cells have become more anti-estrogen resistant. The results of this study therefore show that an over-expression of proprotein convertases can profoundly influence the growth properties of breast cancer cells *in vitro* and *in vivo*, most notably in their sensitivity to estrogen and anti-estrogen responses. **This work is detailed in a manuscript (Cheng et al, 1998, appendix).**

Task 6: To determine in human breast cancer cells if altered expression of proprotein convertases affect the posttranslational processing of BRCA1.

A series of experiments have been conducted to examine the profiles of proteins and peptide that are immunoreactive to two anti-BRCA1 antibodies. In this study, the consequence of the over-expression of proprotein convertases PC1 and furin on the posttranslational processing of the endogenous BRCA1 protein was assessed by Western immunoblotting of total cellular proteins. The profiles of BRCA-1 immunoreactive peptides in wildtype, PC1-transfected and furin-transfected MCF-7 cells were compared, using two rabbit polyclonal antibodies (C20 is generated against the carboxyl terminal 20 amino acid residues, and D20, the amino terminal 20 amino acids). Our preliminary results are summarized in Figure 1 on page 7. Both C20 and D20 antibodies detected not only the 220K BRCA1 protein but also other minor and major protein species, and this is especially true for D20 (Figure 1, left and middle panels). All the signals disappeared when the antibodies were absorbed with the corresponding 20-residue peptide (data not shown), indicating the signals were specific.

When comparing the profiles of the immunoreactive proteins in proprotein convertase (PC1 and furin) transfected and vector transfected (control) MCF-7 cells, this was no discernable difference for the 220K BRCA1. However, there appears to be a substantial reduction of a protein doublet of 35-37K, detected by both C20 and D20 antibodies, in both the PC1 and furin transfected MCF-7 cells. At present, the identity of these 35-37K proteins is not known. Since it is known (Wilson et



al, 1996) that the C20 antibodies also recognize the ~190K epidermal growth factor receptor (EGFR), we performed a Western blot using anti-EGFR antibodies. Unlike another breast cancer cell line MDA-MB-468 that expresses high levels of EGFR (Thomas et al, 1996), the MCF-7 cells express low levels of EGFR which was detected by anti-EGFR antibodies (Figure 1, right panel) but not by C20 antibodies (left panel). Importantly, the 35-37K proteins detected by C20, and to a lesser degree by D20 (middle panel), are not detected by anti-EGFR

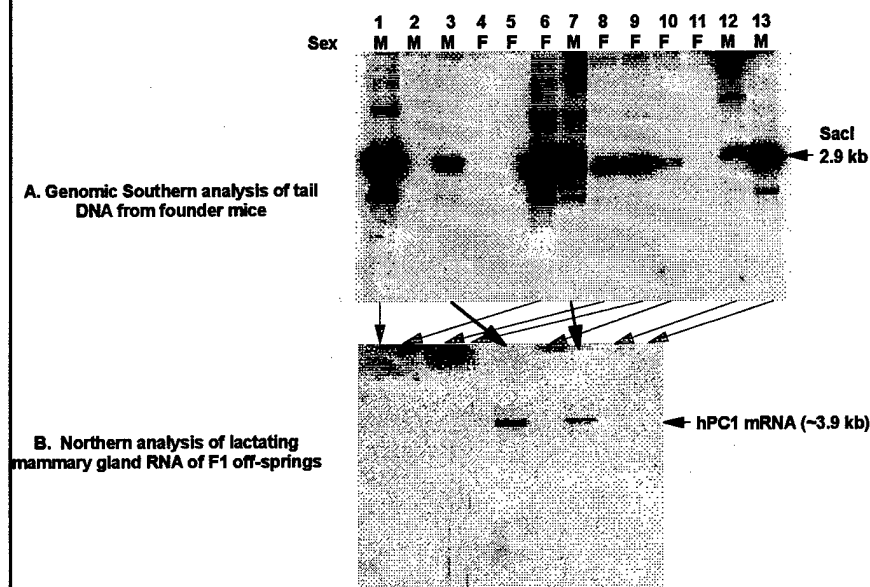
antibodies, indicating these latter protein doublets are related to BRCA1 but not to EGFR. Further characterization of BRCA1 and related proteins are underway.

Tasks 7-9: To Construct MMTV-hPC1 and MMTV-hfurin targeting vectors and to generate heterozygous transgenic mice with the targeted expression of human PC1 and human furin in the mammary glands.

In order to study the role of proprotein convertases in mammary gland development and tumorigenesis, transgenic mice bearing a MMTV-hPC1 or MMTV-hfurin transgene will be studied. We have successfully produced the MMTV-hPC1 targeting vector, and introduced by pronucleus injection into ova of the CD1 mice. The first round of injection, followed by intrauterine replacement, has resulted in 69 live-births. Of these, 9 mice were transgenic for MMTV-hPC1 as determined by Southern hybridization analysis of tail genomic DNA digested with SacI, yielding a diagnostic DNA fragment of 2.9 kb (Figure 2A on page 8). Many of the founder mice carry multiple copies of the transgene. Each of the nine founders were bred with wildtype CD1 mice to produce F1 generation of heterozygotic mice. One female F1 mouse from each progeny was tested for transgene expression in the mammary gland, by Northern hybridization. Figure 2B shows that the off-springs of founder mice #3 and #7 expressed the hPC1 transgene that produces a predicted mRNA transcript of approximately 3.9 kb. The two positive transgenic mouse lines (#3 and #7) are currently being bred to homozygosity and then used for future studies on the biological effects of proprotein convertase over-expression on mammary gland development and tumorigenesis.

In the next year, we will continue to generate more transgenic mouse lines for MMTV-hPC1. Also, we will produce new transgenic mouse lines harboring a second transgene, MMTV-hfurin. After assessing the effects of individual transgenes on the mammary gland in the second year, the third year will include the assessment of the interaction of the two transgenes (PC1 and furin) in

Figure 2: Integration of MMTV-hPC1 transgene and expression of hPC1 mRNA in mammary glands of transgenic mice



mammary gland biology by generating and studying bi-transgenic mice. Finally, we will study the interactions of proprotein convertase with a cellular oncogene such as *c-myc* and *wnt1*, again by generating bi-transgenic mice (e.g., MMTV-PC1 x MMTV-*wnt1*) and even tri-transgenic mice (MMTV-PC1 x MMTV-furin x MMTV-*wnt1*). The ultimate goal is to create a transgenic mouse model that resembles human breast cancer, and which will be used to evaluate the role that the new class of

enzymes (proprotein convertases) play in mammary gland development and tumorigenesis.

CONCLUSIONS:

Proprotein convertases are members of a new class of endoproteolytic enzymes that are believed to play important roles in human neoplasia. Based on our previous detection of proprotein convertases in human breast tumors, the present study is designed to study the biological significance of these enzymes in breast cancer. Proprotein convertase gene transfections into MCF-7 human breast cancer cells led to profound changes in the breast cancer cells. MCF-7 cells that over-expressed proprotein convertases have become more dependent on estrogen for growth both *in vitro* and *in vivo* as tumors grown in athymic mice. As well, convertase-transfected breast cancer cells become more resistant to the anti-estrogen Tamoxifen. In addition, preliminary experiments have suggested that the profiles of BRCA1 related peptides may be altered in the convertase-over-expressing breast cancer cells. To further study the role of proprotein convertases in mammary gland development and tumorigenesis, transgenic mice bearing a convertase transgene targeted to the mammary gland have been generated. Characterization of these novel transgenic mice with respect to breast development and tumorigenesis is in progress.

REFERENCES:

- (1) Min Cheng, N. Xu, N. Seidah**, M. Chrétien*** and Robert P.C. Shiu (1998) Elevated Expression of Proprotein Convertases Altered Breast Cancer Cell Growth In Response to Estrogen and Tamoxifen (manuscript submitted for publication, Appendix I)
- (2) Wilson, C.A., Payton, M.N., Pekar, S.K., Zhang, K., Pacifici, R.E., Gudas, J.L., Thukral, S., Calzone, F.J., Reese, D.M., and Slamon, D.J. (1996) BRCA1 protein products: antibody specificity. *Nature Genetics* 13: 264-265.

**ELEVATED EXPRESSION OF PROPROTEIN CONVERTASES ALTERED BREAST
CANCER CELL GROWTH IN RESPONSE TO ESTROGEN AND TAMOXIFEN**

Min Cheng, N. Xu, N. Seidah**, M. Chrétien*** and Robert P.C. Shiu

University of Manitoba
Department of Physiology
Faculty of Medicine
Winnipeg, Manitoba CANADA R3E 0W3

**Clinical Research Institute of Montreal
Montreal, Quebec, CANADA

***Loeb Research Institute
Ottawa Civic Hospital
Ottawa, Ontario, CANADA

ABSTRACT

Two pro-protein convertase cDNAs--PC1 and furin--were stably transfected into human breast cancer cell line MCF-7. The PC1 and furin over-expressing cells possessed an altered morphology when grown in culture on a plastic substratum. In the presence of fetal bovine serum, the convertase-over-expressing cells and the vector-transfected, wild type control cells grew with a similar rate. However, when a serum-free defined medium was used, the convertase-transfected cells grew slower than the vector transfected, control cells. The cell population doubling times of PC1- and furin-transfected cells were 1.5 times on plastic and 2 times on Matrigel than that of control cells. When grown *in vivo* in athymic nude mice supplemented with a high dose (5 mg/pellet) of estradiol, there was no significant difference between the growth rates of the convertase-transfected and control MCF-7 tumors. However, when the estradiol dose was reduced to 1.7 mg/pellet, the tumours from PC1 or furin transfected MCF-7 cells grew slower (growth rate 100-200 mm³/week) when compared with tumours of control MCF-7 (growth rate 585 mm³/week). It appears that the over-expression of proprotein convertases has rendered the breast cancer cells more dependent on estrogen for growth. The response to Tamoxifen treatment was also different, with the regression rate (70-130 mm³/week) of tumours from PC1 or furin transfected MCF-7 significantly less than their control MCF-7 tumours (547 mm³/week). Thus, the convertase-transfected cells have become more resistant to anti-estrogen. The results of this study therefore show that an over-expression of proprotein convertases can profoundly influence the growth properties of breast cancer cells *in vitro* and *in vivo*, most notably in their sensitivity to estrogen and anti-estrogen.

INTRODUCTION

It is believed that the abnormal expression of autocrine or paracrine growth factors, together with oncogenes, play a major role in human breast cancer progression (Dubik and Shiu, 1988; Dickson and Lippman, 1995; Musgrove *et al.*, 1991). Growth factors such as EGF, TGF α , TGF β , IGF-I and -II and somatostatin, and receptors such as IGF receptor type I, Neu and integrin have been shown to be generated from their inactive precursors by the actions of proprotein convertases (PCs) or contain potential PC processing sites (Mbikay *et al.*, 1993). Pro-protein convertases (PCs) are a family of serine proteinases of the subtilisin/kexin type. To date seven known mammalian convertases have been named PC1/PC3, PC2, PC4, PC5/PC6, PC7/PC8/LPC/SPC7, furin/PACE and PACE4 (Seidah *et al.*, 1998). Six human PCs (except PC4) were cloned. Their tissue and cellular localization, structural and evolutionary characteristics, cleavage specificities and potential physiological substrates have been researched and reported (Seidah, 1998). Thus, an altered expression of PCs could profoundly influence the growth characteristics of breast and other cancers by changing the production or availability of biologically active growth regulators. Consistently with this hypothesis is the finding that elevated expression of convertase members in human lung (Schalken *et al.*, 1987; Mbikay *et al.*, 1997) and breast cancers (Scopsi *et al.*, 1995; Cheng *et al.*, 1997) has been observed. Also PC7 has been identified at a chromosome translocation breakpoint in a human lymphoma (Meerabux *et al.*, 1996). The over-expression of PACE4 via gene transfection in mouse squamous cell carcinoma resulted in enhanced tumor cell invasiveness (Hubbard *et al.*, 1997). Finally, the discoveries that the breast cancer susceptibility gene products, BRCA1 and BRCA2, contain numerous potential PC cleavage sites (Jensen *et al.*, 1996; Steeg, 1996) are highly suggestive of an important role of PCs in human breast tumorigenesis. Taken together, the above findings

have provided a compelling argument for an important role of the PC family of genes in human cancer development and progression. As a continual study to understand the role of PCs in the regulation of human breast cancer, we have studied the consequences of deregulated expression of PCs on the growth behaviour of breast cancer cells. Here we report that MCF-7 cells overexpressing PC1 or furin possess an altered cell shape and growth behaviour *in vitro* and *in vivo* in athymic nude mice in response to estrogen and the antiestrogen, tamoxifen.

MATERIALS AND METHODS

Gene transfection, cell lines and cell culture

Full length cDNAs for mouse PC1, (mPC1), and human furin (hfurin) cloned into the expression vector pRc/CMV and pcDNA3 respectively, were transfected into MCF-7 human breast cancer cells using calcium phosphate (Mammalian Transfection kit and protocol, Stratagene). MCF-7 cells were also transfected with pcDNA3 vector alone and these clones were used as control cells. Cell culture conditions were described as before (Cheng *et al.*, 1997). Neomycin-resistant clones were selected in 1 mg/ml geneticin in culture medium. MCF-7 clones were analyzed by Southern for DNA integration, Northern analysis for mRNA expression, and immunoprecipitation or Western blotting for protein production.

Southern blot analysis

Genomic DNA was isolated from MCF-7 cell clones as described (Hogan *et al.*, 1986). Ten μg of DNA was digested with the appropriate restriction endonucleases: PstI for mPC1-transfected, and BamHI + BglII for hfurin-transfected cells. The DNA digests were loaded on 0.8% agarose gels with 1X TBE buffer (0.9M Tris borate, 0.002M EDTA), and run overnight at 20-30 volts. Gels were then denatured in a 0.5M NaOH with 1.5M NaCl solution for 30 min twice with gentle shaking and neutralized in 1M $\text{NH}_4\text{Ac}/20$ mM NaOH solution for another 30 min twice. The DNA was then transferred to nitrocellulose filters (NitroPure membrane, Micron Separations Inc., Westboro, MA) as described by Maniatis *et al.* (1982). Hybridization with ^{32}P -labelled mPC1 or hfurin cDNA was carried using conditions similar to those for Northern hybridization (see below).

Northern analysis

The procedures of RNA isolation from MCF-7 cells were described previously (Cheng

et al., 1997). Thirty five μg total RNA was denatured in 33% (v/v) formamide and 2.2M formaldehyde, size-fractionated on a 1% (w/v) agarose-2.2M formaldehyde denaturing gel, and then transferred onto nitrocellulose membranes as described by Maniatis *et al.* (1982). Membranes were backed for 2h at 80°C, and then prehybridized in 50% (v/v) deionized formamide, 5x Denhardt's solution (1x Denhardt's = 0.02% (w/v) each of BSA, Ficoll and polyvinylpyrrolidone), 5xSSPE (1xSSPE = 0.18M NaCl, 0.01M NaH_2PO_4 , and 1mM EDTA), 250 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA, and 0.1% sodium dodecyl sulfate (SDS) at 42°C for 4 hrs. For hybridization, a fresh aliquot of the same solution was used. Hybridization was performed in the presence of nick-translated ^{32}P -labelled mPC1 or hFurin cDNA at 42°C for 16-18h. Blots were then washed twice in 2xSSC (1xSSC = 0.15M NaCl and 0.015M sodium citrate) - 0.1% SDS for 15 min at room temperature, followed by one wash in 1xSSC - 0.1% SDS for 10 min at 42°C, 0.1 SSC - 0.1% SDS for 5 min at 42°C, and twice for 10 min at 65°C. All blots were exposed to x-ray film at -70°C with intensifying screens for 7-14 days.

Immunoprecipitation

After washing three times in cysteine-free Dulbecco's modified Eagle's medium with 2 mM L-glutamine, penicillin 100 u/ml and 3.5 g/liter glucose, cells were labeled with 100 $\mu\text{Ci}/\text{ml}$ L-cysteine ^{35}S (ICN, 800 Ci/mmol) for 24 hours at 37°C, 5% CO_2 incubator. The medium was collected and centrifuged at 800 rpm for 5 minutes, to remove cellular debris. The medium was dialyzed for 24 hours in a Spectrapor membrane tubing (M.W. cut off: 3500, spectrum Medical Industries, Inc) and lyophilized. Lyophilized conditioned medium was dissolved in 50 to 100 μl of immunoprecipitation buffer [IPB consisting of 50 mM Tris pH 7.5, 100 mM NaCl, sodium deoxycholate 0.5%, SDS 0.1%, NP40 0.5% and 100 K.I.U./ml Trasylol (Aprotinin)]. Cells were washed three times with cold phosphate-buffered saline (PBS) and removed from the dish

with a rubber policeman. After centrifuges at 800 rpm, for 5 minutes, the cell pellets were stored at -20°C . The cell pellet was aspirated through a 21 gauge needle in 50-100 μl of IPB and centrifuged at 35,000 rpm, 4°C for 10 minutes to remove cellular debris. In order to immunoprecipitate mPC1 protein, 1 μl rabbit anti-PC1 serum (Basak *et al.*, 1995) directed against the C-terminal peptide sequence was added to 10-25 μl of either cell lysate or medium in a total volume of 50 μl . Normal rabbit serum was used as control. After incubation at 4°C overnight, 10 μl of washed Pansorbin slurry (fixed *S. aureus* containing Protein A, from Calbiochem) was added. Immunoprecipitates were washed successively with 1 ml IPB, 1 ml IPB plus 3M urea, and 1 ml IPB. Washed immunoprecipitates were solubilized in SDS-PAGE sample buffer (25 mM phosphate buffer, pH 7.0, 2% SDS, 10% (v/v) glycerol, 5% 2-mercaptoethanol, trace of bromophenol blue) at 100°C for 5 minutes, the bacterial pellets were removed by centrifuged at 35,000 rpm, 4°C , 5 minutes. Samples were subjected to SDS-PAGE (3% stacking gel; 15% resolving gel), and gels were soaked for 30 min in staining solution (7% acetic acid, 20% methanol, 0.05% Commassie Blue), followed by destaining for 30 minutes (7% acetic acid, 10% methanol). Fluorography was done by soaking the gel in DMSO for 1.5 hours (dimethyl sulfoxide), 20% (2,5-diphenyloxazole) PPO in DMSO for 3.5 hours, and finally rehydrated in ddH₂O. The impregnated gel was dried under vacuum and autoradiographed on Kodak X-Omat AR X-ray film.

Western blot analysis

Cultured cells were washed with chilled PBS, scraped, centrifuged at 1000g for 5 minutes at 4°C and cell pellets were resuspended in 200 μl of 50 mM Tris-HCL, 20 mM EDTA, 5% SDS, 5 mM β -glycerophosphate, 1 mM AEBSF [4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (Boehringer Mannheim)] and 70 K.I.U./ml aprotinin. Protein concentrations were

determined by the Bio-Rad (Hercules, CA) protein assay kit as described by the manufacturer. Fifty micrograms of each protein was separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose (Bio-Rad). To detect hfurin proprotein, a polyclonal rabbit anti-furin antiserum (Basak *et al.*, 1995) was used as the first antibody at 1:500 dilution. The second antibody used was horseradish peroxidase conjugated goat anti-rabbit IgG at 1:10,000 dilution. Visualization was accomplished using the Supersignal[®] detection system (Pierce, USA) according to the manufacturer's instructions.

Cell growth *in vitro*

Cells were plated in 24 well plates at 1.25×10^4 cells/well in 5% FBS medium and allowed to attach for overnight. The culture medium was then changed to serum-free medium supplemented with human transferrin (10 $\mu\text{g/ml}$) and bovine serum albumin (200 $\mu\text{g/ml}$) (Karey and Sirbasku, 1988). Two types of cell culture surfaces were used: plastic and plastic coated with the basement membrane Matrigel matrix (Becton Dickinson Labware). To make matrigel coated culture surface, the Matrigel matrix was diluted with serum free medium at 1:3 ratio. For 24-well plates, 0.25 ml of this diluted Matrigel solution was used for coating according to manufacturer's protocol. After the prescribed culture period (up to 8 days), the cells were detached with trypsin (for cells on the plastic surface) or dispase (for cells on matrigel matrix) and counted a Coulter particle counter (Model ZBi, Coulter Electronics, Hialeah, FL). For each determination, the average cell number of 4 wells was used to compute the cell doubling time. The cell doubling time was defined as the time (days) required for the cell number to double during the logarithmic growth phase.

Cell growth *in vivo*

Six- to seven-week old female balb/c or CD-1 strain athymic nude mice were obtained

from Charles River Canada, St. Constant, Québec. Animals were kept inside a laminar-flow air filtration system, and food and water were supplied *ab libitum*. The mice were kept under standard conditions for 5-7 days before implementation of estradiol pellets. Two doses of 90-day release estradiol pellets were used: 5 mg/pellet (to achieve a blood level of >900 pg/ml) and the 1.7 mg/p (to achieve a blood level of 500-600 pg/ml). Placebo pellets were used in control animals. The estradiol, placebo and Tamoxifen (see later) were obtained from Innovative Research of America, Sarasota, Florida. Each pellet was implanted subcutaneously in the dorsal midline, caudal to the neck, through a small stem incision which was sealed by Vetbond™ tissue adhesive (3M Animal Care Products, St. Paul, MN).

Breast cancer cell clones were trypsinized and centrifuged at 500 rpm for 5 min and resuspended at $2-5 \times 10^6$ cells/50 μ l culture medium. The cells were then injected subcutaneously in the flanks of the animals, one cell type on each flank. Each experimental group had 4 mice. For each experimental group, there was a correspondent group with placebo pellet implanted. Tumor volumes were monitored every 7 days by caliper measurement of the 3 dimensions (a,b,c) and were calculated using the formula for a ellipsoid ($v=4/3\pi abc/2$). After 6 weeks, average tumor volumes were approximately 1100-4600 mm³ (depending on the cell type), the estrogen pellets were removed and Tamoxifen (TAM) pellets (5.0 mg/pellet, to achieve a blood level of 2-2.5 ng/ml) were implanted. Tumour volumes were measured and calculated as before. For percentage of tumour regression, the last tumour volume before the removal of the estradiol pellets was used as the initial tumour volume during TAM treatment, and divided by the subsequent tumour volume during the subsequent 5 weeks.

Statistical analysis

Statistics (linear regression analysis) was performed by Sigma Stat (Jandel Corp.).

RESULTS

Transfection and expression of pro-protein convertases mPC1 and hfurin in MCF-7 human breast cancer cells

MCF-7 cell clones surviving G418 selection were first analyzed by Southern hybridization of genomic DNA for the integration of an intact transfected pro-protein convertase cDNA (mPC1 or hfurin). Fig. 1A shows the expected 2.0 and 0.4 Kb DNA diagnostic fragments hybridized with the ³²P-labelled mPC1 cDNA probe in clone 6. A G418 resistant clone 7 that was transfected with the vector pcDNA3, was referred to as wild type (wt) MCF-7, was included for comparison. Clone 6 also expressed high levels of mPC1 mRNA (Fig. 1B) compared with the control Clone 7. The endogenous hPC1 mRNA was very low relative to the transfected mPC1 mRNA and was not detectable under the Northern hybridization conditions used. ³⁵S-cysteine labelled cell lysate and medium of Clone 6 were subjected to immunoprecipitation using anti-PC1 antibodies. Fig. 1C shows that in the culture medium the predominant protein was an approximately 85 kDa mPC1 species specifically recognized by the antibodies. This same protein was immunoprecipitated from cell lysate (not shown).

MCF7 clones transfected with hfurin cDNA cloned into the pcDNA3 expression vector were similarly analyzed for hfurin integration and expression. Fig. 2A shows the expected 1.4 Kb diagnostic genomic DNA fragment hybridized to ³²P-hfurin cDNA in a G418 resistant clone 33. Clone 33 also expressed the expected 3.5 Kb hfurin mRNA (Fig. 2B); the endogenous furin transcript was very low in abundance and was not detectable using the conditions of X-ray film exposure. When total cellular proteins were subjected to Western blot analysis, a prominent ~ 90 kDa furin protein was detected by the anti-hfurin antibodies (Fig. 2C) in clone 33 but not in the vector transfected wild type (clone 7) MCF-7 cells.

Morphology of mPC1- and hFurin-overexpressing MCF-7 cells

The wild type MCF-7 cells growing on plastic culture ware showed a typical epithelial-like features - flat and polygonal. PC1 or furin transfected cells, however, demonstrated an altered morphology, in that they are more retractile and spindly, and possessed more prominent cellular processes (Fig. 3). On Matrigel coated substratum, however, there was no obvious morphological difference among these three kinds of cells (Fig. 3). All three cell types grew in clusters which expanded in size as cell proliferation proceeded.

Effect of over-expression of proprotein convertases on MCF-7 proliferation *in vitro*

The proliferation of the PC1- and furin- over-expressing MCF-7 clones (6 and 33, respectively) was compared to that of wild type control MCF-7 cells (clone 7) on plastic substratum and on an extracellular matrix (Matrigel) substratum, in the presence or absence of fetal bovine serum. When grown in the presence of 5 to 10% fetal bovine serum, there was no significant difference in the growth rates between clone 6, clone 33 and wild type (clone 7) cells when grown on either substratum (data not shown). When a serum-free medium [supplemented with transferrin and bovine serum albumin (Karey and Sirbasku, 1988)] was used, a significant reduction (1.5-fold) in the growth rate of PC1- and furin-transfected cells was observed when compared to wild type MCF-7 cells (Table 1) grown on plastic. However, the difference in the proliferation rates between the convertase-overexpressing cells and the wild type cells was most dramatic when cells were grown on extracellular-matrix (Matrigel)-coated substratum (Table 1 and Fig. 4). Table 1 summarized the growth rates, expressed in doubling times, of the three cell types grown on plastic and Matrigel in serum-free medium. During log phase growth on plastic, the doubling time of convertase-transfected clones 6 and 33 was approximately 1.5 times (3.9-4.2 days vs. 2.6 days) longer than that of control wild type cells. The corresponding values

for Matrigel was 2.0 times (3.2-3.8 days vs. 1.8 days). Thus, the over-expression of PC1 or furin significantly reduced the growth of the human breast cancer cells MCF-7 *in vitro* in serum-free medium.

Growth study of PC1 and furin transfected MCF-7 *in vivo* in athymic nude mice

17 β -estradiol is essential for MCF-7 cell growth *in vivo* (Shafie and Grantham, 1981). In the first experiment, 5.0 mg estradiol pellets (blood level >900 pg/ml), were implanted subcutaneously into the nude mice. Under the influence of this supraphysiological concentration of estradiol, there was no significant difference in the growth rates between the tumours of wild type control cells (867 ± 304 mm³/weeks) and PC1-transfected cells (458 ± 114 mm³/week) (Fig. 5). When lower dose (1.7 mg) estradiol pellets (blood level 500 pg/ml) were used, the wild type MCF-7 tumours grew at a rate (585 ± 150 mm³/week) similar to that in high dose estradiol, but the PC1-transfected MCF-7 tumours grew much slower (93 ± 14 mm³/week) (Fig. 6). Consequently there was a highly significant reduction ($P < 0.05$) in the growth rate of PC1 over-expressing MCF-7 tumours. Thus, the experiments of Figures 5 and 6 suggest that the PC1 transfected cells were more sensitive to estradiol concentrations than wild type cells. In a third experiment, the growth in nude mice of the PC1-transfected MCF-7 (clone 6) was compared to that of furin-transfected cells (clone 33). As shown in Fig. 7, there was no difference between the two MCF-7 clones. Therefore, both PC1- and furin-transfected cells grew slower *in vivo* in nude mice in the presence of the lower dose estradiol. In all the mice implanted with placebo pellets, no tumour growth was observed (not shown).

The sensitivity of the established tumours to the anti-estrogen, Tamoxifen, was also evaluated. After 6 weeks of growth, the estradiol pellets were removed and Tamoxifen (5.0 mg/pellet) (blood level 2-2.5 ng/ml) pellets were implanted. Fig. 8 and 9 show that the control

MCF-7 tumours regressed faster than PC1- or furin-transfected tumours. The control tumours decreased with a rate of 547 mm³/week, while PC1- and furin-transfected tumours regressed at the rates of 71-113 mm³/week and 134 mm³/week, respectively.

DISCUSSION

Recent studies, including our own (Cheng *et al.*, 1997; Schalken *et al.*, 1987; Mbikay *et al.*, 1997; Scopsi *et al.*, 1995), have indicated that cancer cells in general possess an elevated expression of proprotein convertases when compared to their normal counterparts. Such observations have led to the hypothesis that an elevated expression of proprotein convertases is associated with, and may contribute to, the development and/or progression of human cancers. To gain further insight into the biological functions of proprotein convertases in human breast cancer, we have generated via gene transfection MCF-7 human breast cancer cell lines that over-express each of the two proprotein convertases, PC1 and furin. These two enzymes were chosen based on our previous finding (Cheng *et al.*, 1997) that they are expressed in human breast cancer cell lines and primary human breast tumors. PC1 was chosen as a representative member of proprotein convertase that is localized to secretory granules and is responsible for the cleavage of proteins secreted by the regulated secretory pathway. Furin was chosen to represent a membrane-anchored convertase enzyme that is localized to the Golgi apparatus and plasma membrane, and cleaves proteins as they pass through the trans-Golgi enroute and secreted by the constitute secretory pathway (Molloy *et al.*, 1994; Steiner *et al.*, 1992).

In the transfected MCF-7 cell clones, the over-expression of PC1 and furin was confirmed by Northern detection of mRNAs and proteins by specific antibodies. In this (Fig. 1 and 2) and our previous study (Cheng *et al.*, 1997), the expression of the endogenous convertases, although easily detectable by RT-PCR, was not readily discernable by Northern analysis or immunoblotting or immunoprecipitation. Thus, the levels of endogenous convertase expression in MCF-7 cells are minimal when compared to the elevated expression of the convertases in the transfected MCF-7 cell lines harboring the corresponding transgenes (CMV-

mPC1 or CMV-hfurin).

The first noticeable phenotypic change in the MCF-7 clones that over-expressed PC1 or furin was that of an altered cell shape. The transfected MCF-7 cells have a retracted, elongated and spindle morphology, with elongated cell processes (Fig. 3) when grown on plastic substratum. It is not known at present the mechanisms responsible for this altered morphology, however, it is possible that the excess production of PC1 and furin may have altered the processing, and therefore functions, of important cell adhesion proteins that may include integrins, laminin, fibronectin and collagens. The fact that all seven members of the proprotein convertases contain a consensus integrin-binding sequence, arginine-glycine-aspartic acid (RGD), suggests an interaction between convertases and integrins, and that convertases may play a role in the processing of integrins. Indeed, furin has been shown to cleave integrin pro-alpha 3 and 6 subunits and is believed to be involved in the endoproteolytic processing of integrins (Lehmann *et al.*, 1996). Thus, an altered cell adhesion and morphology seen with our convertase-transfected cells on plastic substratum may be a consequence of changes in integrin function. However, when the convertase-transfected cells were grown on the complete extracellular matrix, Matrigel, there was no discernable changes in their adhesion and morphology (Fig. 3). Both wild type and transfected MCF-7 cells grew as clumps that expanded in size. Thus, it appears that exogenously supplemented extracellular matrix proteins could compensate for the loss of functions of endogenous adhesion molecules.

In addition to morphological changes, the most striking effect of convertase over-expression was on the sensitivity of the breast cancer cells to hormones on cell proliferation. When cell growth was assessed in medium supplemented with fetal bovine serum that contained a full-supplement of steroids and other growth mitogens, there was no discernable difference in

the rate of growth of wild type and convertase-transfected MCF-7 cells (data not shown). However, when assessed in serum-free medium supplemented with transferrin and bovine serum albumin and the absence of steroid hormones, there was a striking difference in the growth rate of the convertase-transfected breast cancer cells as compared to that of the wild type control cell. However, instead of having accelerated cell growth as one might predict, the convertase transfected cells actually grew much slower, and this difference can be seen whether the cells were grown on plastic substratum or Matrigel (Fig 4 and Table 1).

Since there was no estrogen or other hormones used in the serum-free medium, it was suspected that the convertase-transfected cells had become more dependent on hormones for growth. We therefore conducted a series of studies in which the growth *in vivo* of the breast cancer cells were assessed in athymic nude mice. As expected, neither the wild type control MCF-7 nor the convertase-transfected clones grew in athymic mice without estradiol supplement. When supplemented with estradiol pellets at high, supraphysiological concentrations (5 mg/pellet), both wild type and PC1-transfected MCF-7 tumors grew at a similar rate (Fig. 5). When the estradiol dosage was reduced to 1.7 mg/pellet, however, there was a striking and highly significance difference in the growth of PC1-transfected and wild type MCF-7 tumors, in that the PC1-transfected cells grew at a rate 1/6th that of wild type cells (Fig. 6). The growth rate of the Furin-transfected MCF-7 tumors was also slow, similar to that of PC1-transfected MCF-7 tumors (Fig. 7). Thus, it appears that the over-expression of proprotein convertases has rendered the breast cancer cells more estrogen-dependent. The results of this *in vivo* experiment is consistent with that of the *in vitro* growth assay, in that both bioassays have revealed a higher dependency on hormonal factors on growth for MCF-7 cells with elevated expression of convertases.

The athymic mice study also revealed that the convertase-transfected MCF-7, once established in the presence of estradiol supplement, regressed at a rate that was 1/7th that of wild type MCF-7 tumors in the presence of the antiestrogen Tamoxifen (Fig. 8 and 9). Thus, the over-expression of convertase has also rendered the breast cancer cells more Tamoxifen-resistant.

It is presently unclear as to the mechanisms by which the over-expression of proprotein convertases leads to reduced estrogen sensitivity and increased Tamoxifen resistance in MCF-7 cells. It was not due to an alteration of estrogen receptor levels because the estrogen receptor contents, as measured by ligand binding (McGuire, 1973), of the convertase-transfected MCF-7 were unchanged as compared to that of wild type control cells (data not shown). Since estrogen-induced cell proliferation and Tamoxifen-induced tumor regression both involve estrogen receptor-mediated induction of gene and cellular functions, and in the absence of altered estrogen receptor contents, it appears that the over-expression of convertases was likely to affect the activities of co-factors required for estrogen receptor activities (Paik *et al.*, 1994). Alternatively, the over-expression of convertases may have negatively impacted on signal transduction pathways that cross activate estrogen receptor signals or events downstream to estrogen receptor activation of gene expression.

In conclusion, the results of the present study show that the over-expression of proprotein convertases can profoundly influence the growth behaviour of human breast cancer cells, most notably in their responsiveness to estrogen and anti-estrogen actions. It appears that proprotein convertases may be potentially useful markers for hormone-dependent breast cancer and for anti-estrogen resistance.

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TABLE 1. Comparison of cell growth (doubling time in days) *in vitro* in serum-free medium.

Cell type	Plastics	Matrigel
Wild type (clone 7)	2.58 ±0.31	1.77 ±0.03
PC1 (clone 6)	*3.87±0.22	*3.18±0.26
furin (clone 33)	*4.18±0.37	*3.83±0.73

Number of independent experiments performed (n=3)

Numbers are mean ± S.E.M. (n=3)

* denotes significant difference from Wild type (clone 7); P < 0.05

Table 2a. Cell growth *in vivo* in athymic nude mice

Tumour	Growth rate mm³/wk
PC1 tumours	93.3±14.5
Wild type tumours	*585±149.9

Numbers are mean ± S.E.M. (n=3)

* P < 0.05

PC1 tumors refer to tumors of MCF-7 cells transfected with the CMV-PC1 vector

Vector tumors refer to tumors of MCF-7 cells transfected with the CMV vector only

Table 2b. Cell growth *in vivo* athymic nude mice

Tumour	Growth rates (slope) mm³/wk
PC1 tumours	230.6±31
furin tumours	212.5±23.2

Numbers are mean ± S.E.M. (n=3)

Furin tumors refer to tumors of MCF-7 cells transfected with the CMV-furin vector

TABLE 3a. Tumor regression *in vivo* after Tamoxifen treatment

Tumour	Regression Rate mm³/week
PC1 tumours	-71±11.2
Wild type tumours	*-547.06±122.1

Numbers are mean ± S.E.M. (n=3)

*P<0.05

TABLE 3b. Tumor regression *in vivo* after Tamoxifen treatment

Tumour	Regression Rate mm³/week
PC1 tumours	-112.8±6.35
furin tumours	-133.9±41

Numbers are mean ± S.E.M. (n=3)

LEGENDS TO FIGURES

Fig. 1 Detection of mPC1 integration and expression in transfected MCF-7 cells.

A. Southern analysis was performed with 10 μg of PstI digested genomic DNA from clone 6 and clone 7 (vector transfected wild type control). ^{32}P -labelled mPC1 cDNA was used as a probe. The expected 2 Kb and 0.4 Kb bands were present in clone 6 but not in clone 7. **B.** Northern analysis was performed with 30 μg total RNA from clone 6 and wild type clone 7 cells. ^{32}P -labelled mPC1 cDNA was used as the probe. A 2.5 Kb transcript was observed in clone 6 but not in clone 7. **C.** Immunoprecipitation was carried out by incubating ^{35}S -cysteine labelled proteins derived from the conditioned medium of clone 6 using either rabbit anti-PC1 antiserum (AS) or normal rabbit serum (NS) as control. A 85 kDa band was precipitated with anti-PC1 antiserum in the clone 6 conditioned medium.

Fig. 2 Detection of hfurin integration and expression in transfected MCF-7 cells.

A. Southern analysis was performed with 10 μg genomic DNA from hfurin transfected MCF-7 clone 33 and wild type control cells (clone 7) digested with BamHI and BglII and using ^{32}P -labelled hfurin cDNA as a probe. The presence of the transfected hfurin cDNA generated the expected 1.4 Kb band in clone 33. The endogenous hfurin gene has yielded two larger bands common to both clone 33 and wild type clone 7 cells. **B.** Northern analysis was carried out with 30 μg total RNA from clone 33 and wild type clone 7. The expected 3.5 Kb transcript was found in clone 33. Due to the low level of expression of the endogenous furin gene, the furin transcript was not detected in clone 7 by Northern blot using total RNA. **C.** Western blot was performed with 50 μg protein of hfurin-MCF-7 clone 33 and wild type MCF-7. A 90 kDa protein band

was detected by anti-hfurin antibodies (1:500 dilution) in the clone 33 cells, but not in the wild type clone 7 MCF-7 cells.

Fig. 3 Morphology of mPC1- and hfurin-transfected MCF-7. The appearance of mPC1- (clone 6) and hfurin- (clone 33) overexpressing MCF-7 clones was compared to that of wild type MCF-7 clone 7 cells. The wild type cells growing on plastic culture showed typical epithelial-like cell features - flat and polygonal. Clone 6 and clone 33 cells are more retractile and spindly, possessing more prominent cellular processes. Cells grown on Matrigel did not show obvious morphological changes.

Fig. 4 Effect of overexpression of mPC1 and hfurin on MCF-7 proliferation *in vitro* on Matrigel-coated substratum. Each point represents the mean of quadruplicates \pm SEM.

Fig. 5 Growth study of mPC1-transfected MCF-7 and wild type MCF-7 cells in athymic nude mice receiving 5 mg estradiol pellets. Two million mPC1-MCF-7 clone 6 and wild type MCF-7 clone 7 cells were injected subcutaneously in the opposite flanks of 7-week-old female balb/c athymic nude mice. The tumour size was measured once a week and calculated as described in "Materials and Methods". Each point represents the mean of triplicate tumour volumes (\pm SEM).

Fig. 6 Growth study of mPC1- transfected MCF-7 and wild type MCF-7 cells in athymic nude mice receiving 1.7 mg estradiol pellets. Two million mPC MCF-7 clone 6 and wild type MCF-7 clone 7 were injected subcutaneously in the opposite flanks of 7-week-old female balb/c athymic nude mice. The tumours size was measured each week and calculated. All values represent the mean of triplicate determinations \pm SEM. When not visible, the standard error bars fall within the symbols.

- Fig. 7** Growth study of mPC1-MCF-7 and hfurin-MCF-7 cells in athymic nude mice receiving 1.7 mg estradiol pellets. Five million transfected cells were injected subcutaneously in the flanks of 7-week-old female CD1 athymic nude mice. All values represent the mean of triplicate determinations \pm SEM.
- Fig. 8** Tumour regression *in vivo* during Tamoxifen (TAM) treatment. After estradiol pellets were removed, TAM pellets (5 mg/pellet) were implanted. The day of estradiol pellet removal was taken as week 0, and the tumour volume taken was considered 100%. Each point represents the mean (\pm SEM) of tumour regression (%) for mPC1-MCF-7 clone 6 and wild type clone 7.
- Fig. 9** Tumour regression *in vivo* during Tamoxifen (TAM) treatment. Determination of tumour regression was described in "Materials and Methods" and legends to Fig. 8. The tumour regression of mPC1-MCF-7 clone 6 and hfurin-MCF-7 clone 33 is compared.

Fig. 1

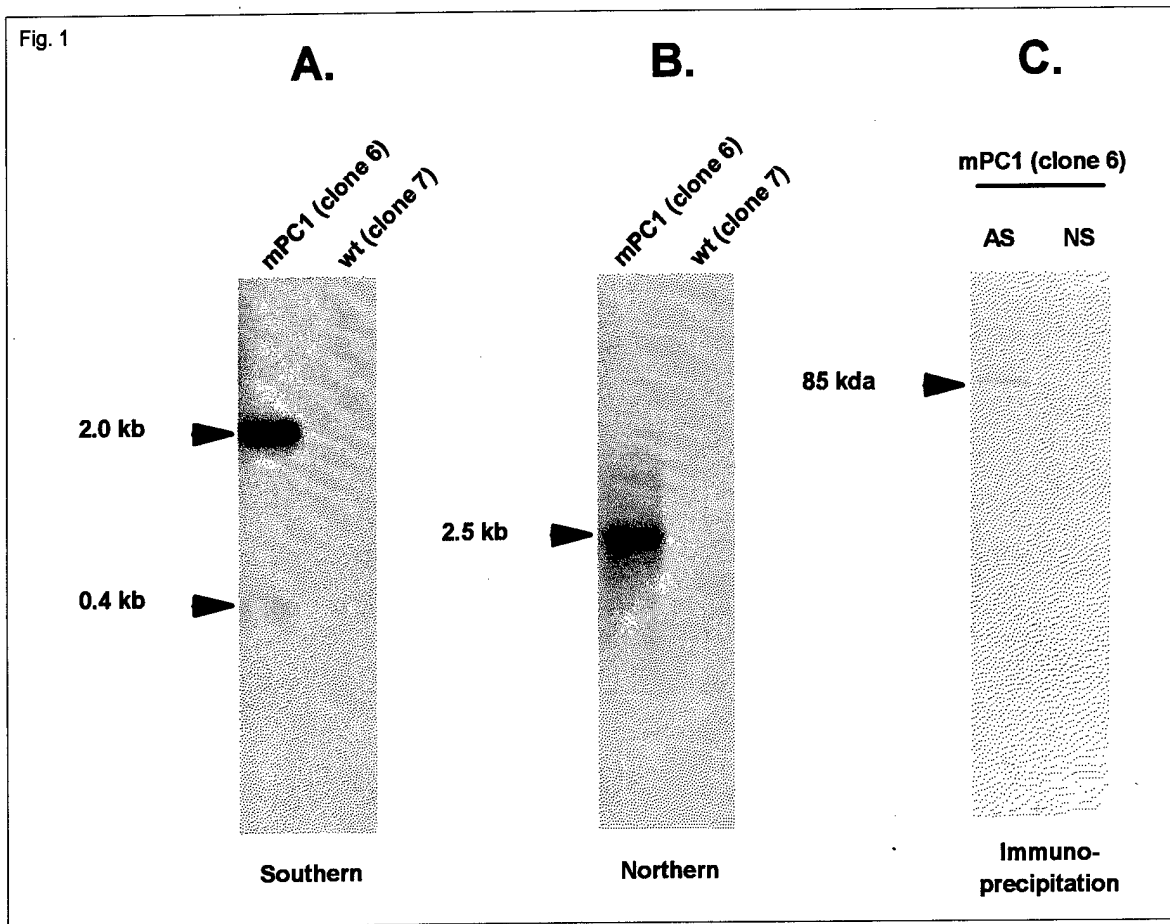


Fig. 2

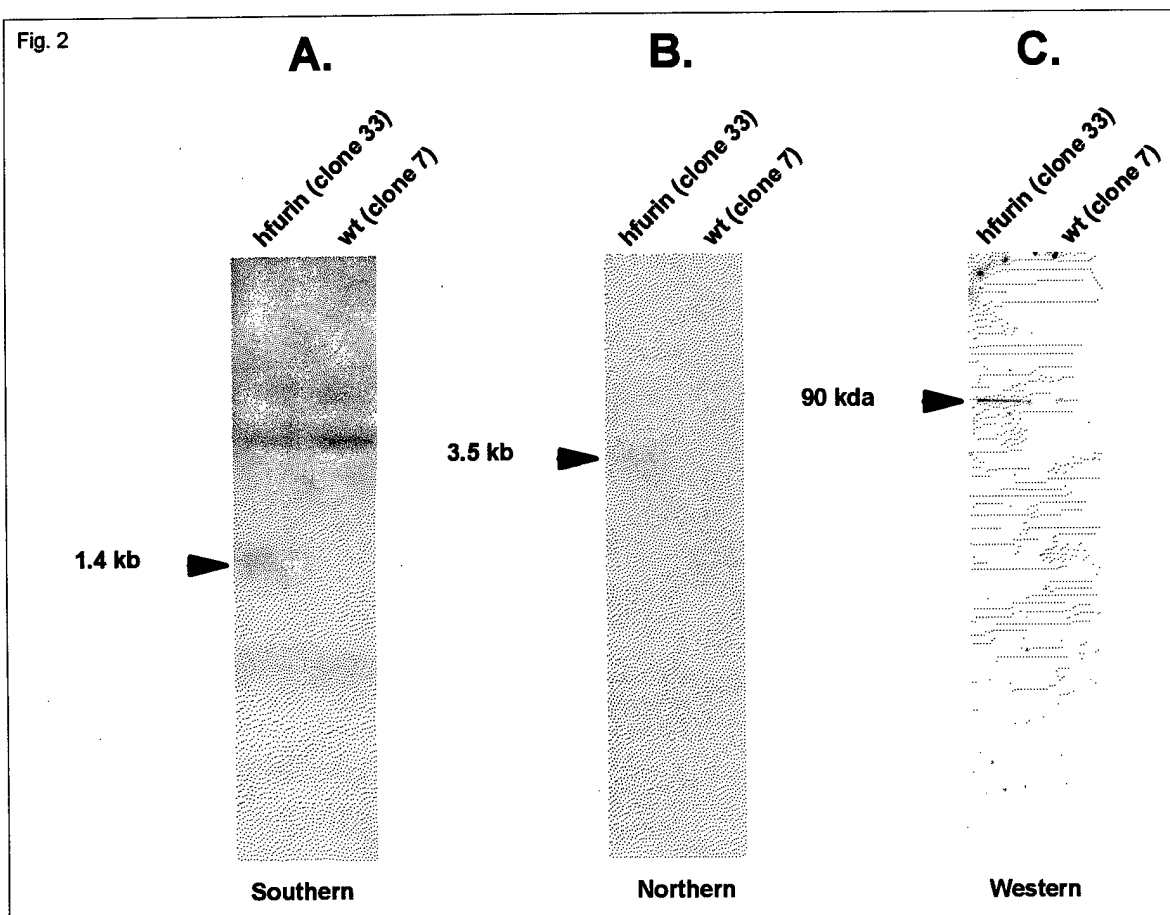


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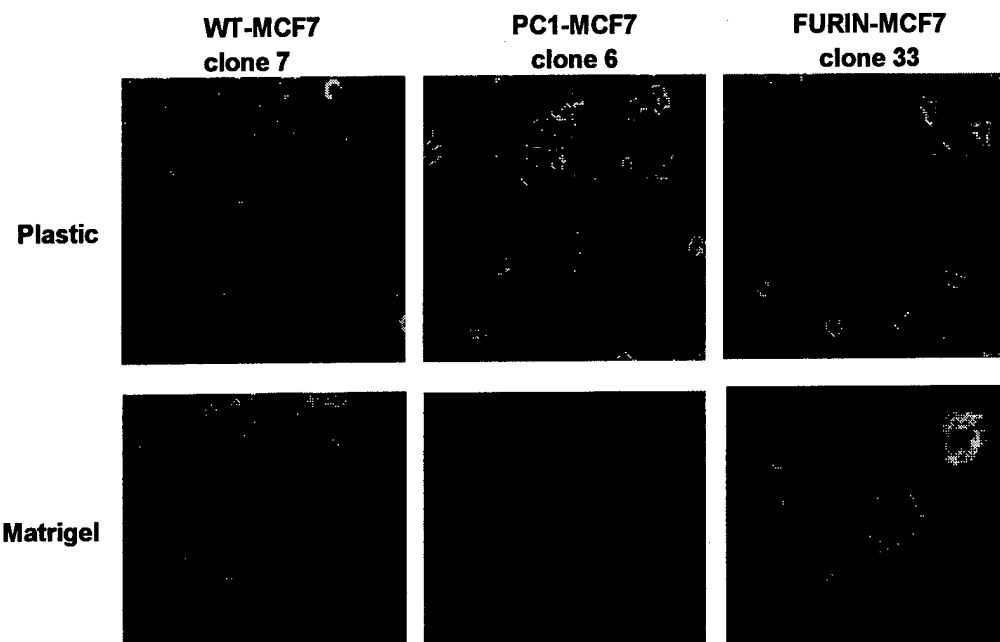


Fig.4

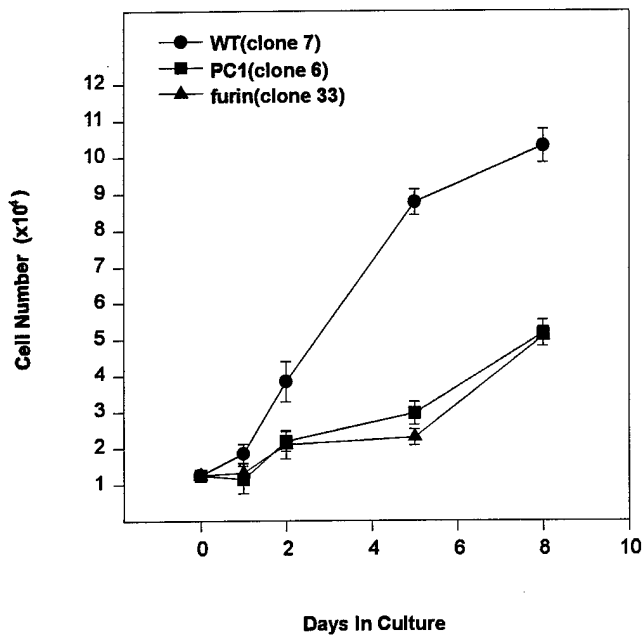


Fig.5

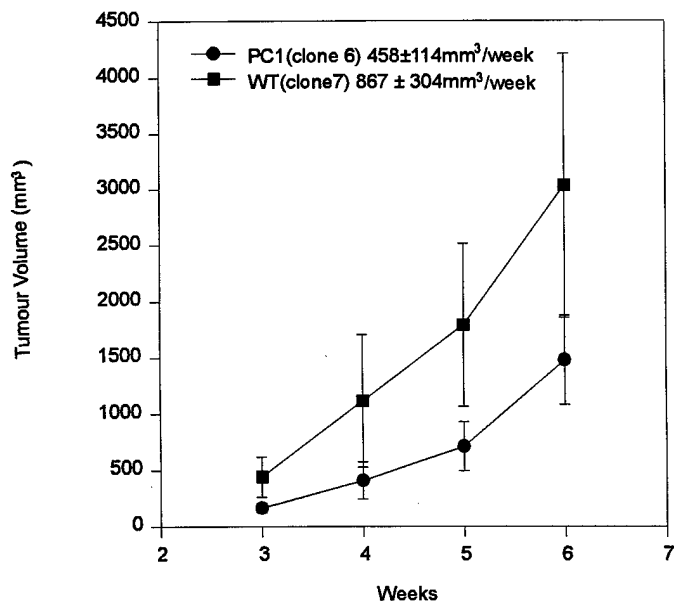


Fig.6

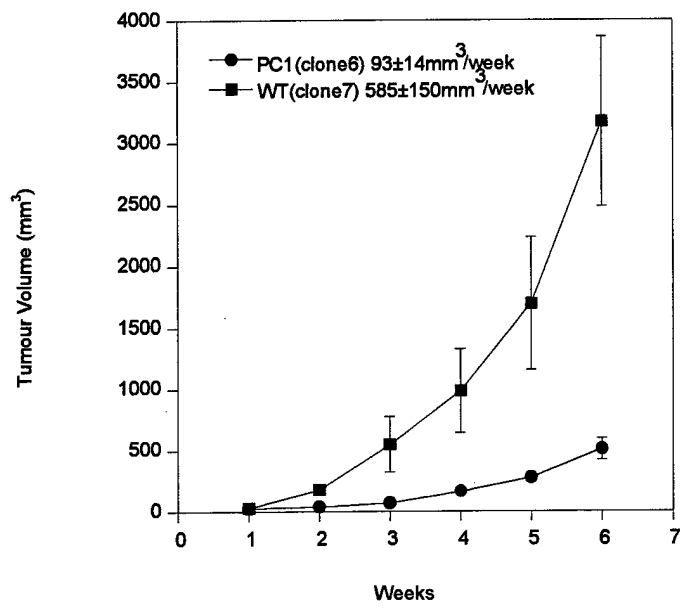


Fig.7

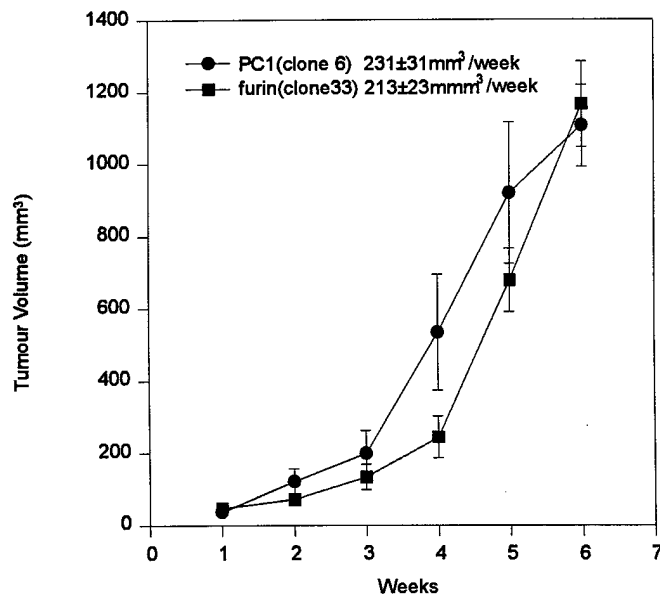


Fig.8

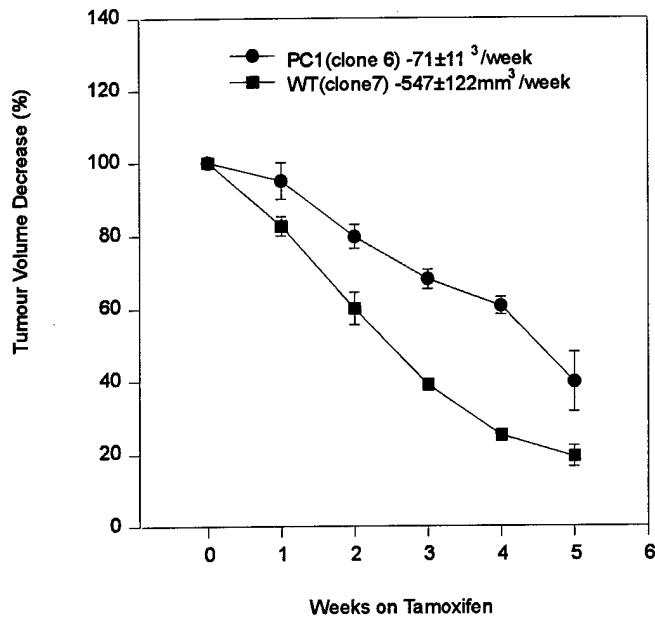


Fig.9

