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Several tumor suppressor genes (TSGs) have been cloned and found to be mutated in a variety of cancers, including breast cancer. However, few breast cancer-specific TSGs are known. The purposes of this proposal are to (1) clone novel TSGs specific to human breast cancer; (2) examine the alteration of these TSGs in primary breast tumors; and (3) identify their characteristics, regulation and function. We have constructed a cDNA library from normal human breast epithelia and are cloning this cDNA library into a vector that is negatively regulated by tet repressor (tetR). These vectors will be co-transfected into MCF-7 cells that express tetR. Upon withdrawal of tet, the repressed expression of the cDNA of interest is released, and the cDNA is expressed. Using a novel dye that is retained by nonproliferating cells, we will identify growth inhibited clones by Flow Cytometry. This will provide a functional screen for the discovery of TSGs, since these will be expressed in the growth inhibited cells. By PCR, we can obtain the insert sequence and then screen primary breast carcinomas to identify mutations in cloned TSGs.

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Le-Ping Pu 08/26/97

8/26/97

PI - Signature Date

Proposal Title: ***Tumor Suppressor Genes in Breast Cancer***

(4) Table of Contents

(5) Introduction

Breast cancer is among the most prevalent malignancies of women in Europe and North America. Many molecular biology studies have revealed genomic abnormalities in breast cancer, including amplification of proto-oncogenes, such as c-myc, c-erbB2, int-2, bcl-1, PRAD-1, EMS, EGF receptor (c-erbB 1), IGF-1 receptor, flg and bek (1,2), and intragenic mutations in tumor suppressor genes (TSGs) including p53 and Rb (3,4). TSGs constitute a relatively new class of genes that is rapidly expanding. The study of TSGs has opened an important avenue of cancer research. Many TSGs have been cloned from humans and found to be mutated in variety of human cancers (3-12). The study of TSGs should not only speed up basic cancer research, but it also may aid in the early diagnosis, prognostication, and treatment of human malignancies. Loss of heterozygosity (LOH), which is usually considered the hallmark of TSGs, has been observed in at least 15 out of the 23 pairs of chromosomes in human tumors. This result suggests that there may be numerous TSGs. However, only two genes specifically related to breast cancer have so far been cloned (BRCA-1) (13) or mapped to a specific chromosomal segment (BRCA-2) (14); moreover the prevalence of intragenic somatic mutations in BRCA-1 is not very high in sporadic breast tumors (less than 10% of cases). Therefore, it seems likely that the cloning of new tumor suppressor genes of specific importance in breast cancer will be important and promising task for future research into this common disease.

The current proposal will focus on the isolation and characterization of novel TSG(s) in human breast cancer. A human epithelial eukaryotic cDNA expression library has been constructed, and will be transfected into the human breast cancer cell line MCF-7. Gene(s) inhibiting the growth of MCF-7 cells will be considered candidate TSGs for breast cancer. These will be cloned and the full length cDNA sequence obtained. Expression of cloned genes will first be investigated in RNA populations derived from two immortalized "normal" human breast epithelial cell lines (A1N4 and MCF-10A), and in MCF-7 cells growth arrested either by antiestrogen-treatment or estrogen withdrawal. This approach provides a rapid and sensitive functional screen for growth inhibition-related activities using renewable resources, and is particularly important should a significant number of unique cDNAs be isolated. Subsequently, the expression of clones exhibiting an appropriate pattern of expression will be investigated in a series of RNA populations isolated from primary breast tumors. Once we have identified the most promising candidates, we will further screen genomic DNA from cell lines and primary breast tumors for somatic alterations, including deletion, mutation, and change in expression level. In the longer term, the most promising cDNAs will be studied to establish their characteristics and regulation. Putative TSGs that are growth-suppressive and specifically altered in breast cancers may be useful tools for the early diagnosis, prognostication, and eventual treatment of human breast cancers.

(6) Body of Report

A. Brief statement of ideas and reasoning

Tumor suppressor genes (TSGs) function in normal tissues by regulating the growth of normal cells. Mutations, deletions, or other modes of inactivation of TSGs contribute to uncontrolled growth and malignant transformation of normal cells. Many TSGs have been cloned from humans and found to be mutated in variety of human cancers, including breast cancer (3-12). Many human chromosomes show high rates of loss of heterozygosity (LOH) in breast cancer; however, very few breast cancer-specific TSGs, such as BRCA-1, have actually been cloned (13). Moreover, the mutation rate of BRCA-1 in primary human breast tumors is less than 10% (14). Therefore, additional specific tumor suppressor genes for breast cancer are likely to exist. A cDNA expression library made from mRNA of normal human mammary glands should contain potential TSGs for human breast cancer, and thus can be reasonably used to isolate TSG(s) specific to breast cancer that inhibit the growth of breast cancer cells. **We propose a functional screen for the discovery of TSGs, which dramatically**

decreases the time to isolation and a priori demonstrates the function of novel TSGs. In addition, by using a cDNA expression library from normal human breast epithelia, transfected into breast cancer cells, we hope to clone TSG(s) that are specific to breast cancer.

B. Hypotheses/Purpose

We hypothesize that:

1) Normal human mammary gland epithelia should contain all normally expressed potential TSGs for breast cancer.

2) TSGs play an important role in growth regulation of breast cancer cells in culture.

3) TSGs are responsible for the carcinogenesis of at least a significant portion of breast cancer.

The purpose of this proposal is to clone TSG(s) specific to breast cancer, examine their alteration in primary breast tumors, identify their characteristics, and ultimately study their regulation and function.

C. Technical Objectives

1) To clone novel TSGs for human breast cancer from a cDNA expression library made from normal human mammary gland epithelia.

2) To characterize the cloned TSGs by sequence homology analysis and study their functional effect on *in vitro* tumorigenesis for the most promising candidates.

3) In the long term: to study the regulation of cloned TSGs by finding their promoter regions and regulatory elements.

D. Experimental Methods, Assumptions and Procedures

Outline and rationale for approach

A major problem in the identification of growth inhibitory genes is that it is the non-proliferating cells in a functional assay that are the cells containing the genes of interest. We have constructed a novel approach that we believe is optimized for the specific purpose of identifying growth suppressor genes. Thus, we will utilize the tetracycline repressor (tetR)-based gene expression system. We will directionally clone the cDNA library (see below) into an expression vector placing each cDNAs under the control of the tetracycline resistance operon that is negatively regulated by tetR (20). These vectors will be co-transfected with a plasmid expressing the hygromycin resistance marker into MCF-7 cells already transfected with a plasmid expressing both the tetR repressor and the G418 resistance marker (MCF-7$^{tetR + neoR}$). Upon withdrawal of tetracycline (tet), the tetR/VP16 binds and the repressed expression of the cDNA of interest is released, and the cDNA is expressed

(20). Double resistant cells are selected and expression of the gene of interest studied in the presence of increasing concentrations of tet.

While we have a method to regulate genes expression, we also have an approach for enriching bulk transfected cell populations for growth inhibited cells. We will use our adaptation of the dye enrichment method of Maines *et al.* (21). The dye (PKH-2; Sigma Chemical Co. St Louis, MO) is non-toxic and specifically retained in non-proliferating cells. Since Flow Cytometry can be used to viably sort cells retaining dye (cells are maintained in the absence of tet), and there is a state-of-the-art Flow Cytometry Core Facility at the Lombardi Cancer Center, we can rapidly enrich the population for the growth inhibited cells, including cells that are completely growth arrested, sorting for the most fluorescent cells (21). Thus, following the 24 hr recovery period immediately post-transfection, the cells will be selected with the antibiotic, G418. The concentration of G418 has been optimized for MCF-7 cells at 400 mg/ml. Surviving cell populations will be stained with PKH-2 and grown, now in the absence of tet, for the equivalent of several generations as described by Maines *et al.* (21). The estimated generation time for non-inhibited MCF-7 cells is 24-36 hrs (22). Subsequently, single cells will be aseptically sorted into the wells of 96-well plates, providing individual cell clones expressing growth inhibitory genes. Cell clones containing growth suppressing cDNAs will then be rapidly expanded by releasing the growth suppression by addition of tet. Growth suppression can be further confirmed, and RNA containing the expressed putative suppressor genes obtained, by reintroducing tet to the culture medium.

The Mentor (Dr. Clarke) also has generated and characterized MCF-7 cells that do not require estrogen for growth *in vitro* or *in vivo* (MCF7/MIII; MCF7/LCC1) but exhibit an antiestrogen-induced growth suppression that is reversed by estrogen (23-25) or are antiestrogen resistant (MCF7/LCC2; MCF7/LCC9 (26,27). By using both MCF7/LCC1tetR+neoR (grow without estrogen but respond to estrogen) and MCF-7tetR+neoR cells (require estrogen to grow), we can distinguish between genes that merely suppress growth (*i.e.,* MCF7/LCC1tetR+neoR cells are growth inhibited regardless of the presence of estrogen) and those that suppress estrogen-induced proliferation (*i.e.,* both MCF7/LCC1tetR+neoR and MCF-7tetR+neoR growth inhibition is reversed by estrogen). ***The use of these additional cell lines would likely constitute an alternate/additional approach, since the work with MCF-7tetR+neoR cells is sufficient for the initial time period of this application.***

There are several significant advantages to this novel approach:

(1) Growth inhibition will be apparent only upon removal of tet, and this will reduce the background due to insertional mutagenesis, which could randomly produce slowly proliferating/growth inhibited cells independent of the inserted cDNA.

(2) We can identify genes that completely suppress proliferation, as well as genes that merely reduce the rate of proliferation.

(3) We can rapidly identify, in a functional assay, genes that specifically inhibit estrogen-regulated growth.

Each transfected clone will initially be examined for the integrity and copy number of transfected cDNAs by Southern analysis (there may be more than one plasmid in some transfectants), and the appropriate mRNA expression by Northern analyses. The Northern analysis will provide critical information on the size of the expressed transcript(s). Since we have used a regulable promoter approach, we will perform dose response analyses with increasing concentrations of the regulating agent (i.e., tet). This will enable us to assess the potency of the gene, i.e., what level of expression is associated with a corresponding level of growth inhibition. The effects of tet on expression of mRNA from the derepressed promoter will be monitored by Northern analysis. Controls will consist of parallel cultures of nontransfected cells and cells transformed with the tetR operator expression vectors without the cDNA inserts and that are treated ± tet.

We cannot exclude the possibility that the level of expression required for growth suppression is below the limit of detection by Northern. Where this occurs we will use RNase protection or semiquantitative PCR to detect product. We will use primers from the portion of the regulable promoter sequence that is transcribed in the final product, and a site internal to the inserted cDNA sequence. This also will enable us to distinguish those products amplified from newly transcribed RNA from those derived from the endogenous gene.

Construction of a cDNA expression library from normal human breast epithelia

To successfully isolate mRNA from normal human breast tissues, there are three important concerns: 1) effective disruption of tissue and denaturation of nucleoprotein complexes, 2) inactivation of RNase activity, and 3) purification of mRNA away from contaminating DNA and protein. Thus, we have chosen to use PolyA TractR system 1000 (Promega Corp. Cat.#Z5410), since this procedure yields an essentially pure fraction of mature mRNA without extractions or precipitations. This method combines guanidine thiocyanate (GTC) and β-Mercaptoethanol to inactivate RNase. Then GTC is associated with SDS to disrupt nucleoproteins and allows for hybridization between the poly(A) sequence of mRNAs and a synthetic biotinylated oligo(dT)probe. The biotinylated oligo(dT):mRNA hybrids were captured with Streptavidin Paramagnetic Particles (SA-PMPs). The particles were washed at high stringency and purified mRNA was eluted by the addition of nuclease-free deionized water (15).

To construct a cDNA library, we used The CapFinderTM PCR cDNA Library Construction Kit (CLONTECH Laboratories, Inc., Cat.# K1051-1). This is a novel, PCR-based method for making high-quality libraries from a small quantity of RNA. This technique also utilizes the unique CapSwitchTM oligonucleotide in the first-strand synthesis, followed by long distance PCR amplification to allow to produce high yields of full-length, double-strand (ds) cDNA (16-19). Therefore, we performed the reverse transcription (RT) to transcribe 100ng poly A+ mRNA into single-strand (ss) DNA by using reverse transcriptase, a modified oligo (dT) primer (CDS/3' PCR primer) (17,18) and a CapSwitch oligonucleotide. The CDS/3' PCR primer served to primer the first-strand reaction and the CapSwitch oligonucleotide was used as a short, extended template at the 5' end for the RT. When the RT reached the 5' end of the mRNA, enzyme switched templates and continued replicating to the end of the CapSwitch oligonucleotide. The resulting full-length ss cDNA contained the complete 5' end of the mRNA and the sequence complementary to the CapSwitch oligonucleotide, which then served as a PCR priming site (CapSwitch anchor).

The PCR was performed by directly using the CapSwitch anchor. In this reaction, only those oligo (dT)-primed ss cDNAs having a CapSwitch anchor sequence at the 5' end served as templates and was amplified using the 3' and 5' PCR primers and Advantage KlenTaq Polymerase (19). This selective amplification did not allow incomplete cDNAs and cDNA transcribed from polyA- RNA to be amplified, therefore eliminated library contamination by genomic and polyA- RNA.

The ds cDNAs were ligated using T4 ligase to specific-adaptors which contains a pre-existing EcoRI "sticky end" and phosphorylated blunt end for efficient ligation to the blunt-ended cDNA. Such ligation eliminated the need to methylate and EcoRI-digest the cDNA, and left internal EcoRI sites intact. Following adaptor ligation, the ds cDNAs were phosphorylated at the EcoRI sites and size-fractionated using columns to remove small (0.5kb) cDNA fragments and non-cDNA contaminants (0.1kb) (unincorporated primers and unligated adaptors). The resulting cDNA was then cloned into (λgt11 which is an EcoRI-digested and phosphorylated) phage vector. We titered the three test ligations, compared the titers and determined the optimal ratio of vector to cDNA insert. Phage packaging reaction was performed according to the λ-DNA *in vitro* packaging module instructions (Amersham LIFE SCIENCE, Cat.#RPN 1717). In this procedure, we used cell extracts derived from two induced lysogens whose prophages carry different, but complementing, mutations in the genes required for assembly of mature phage particles. Subsequently, we mixed these cell extracts together with λ-DNA, the DNA was packaged into infectious phage particles and then introduced into *E.coli* host cells by infection processes. From the five ligations combined, we obtained the unamplified library which contains 1×10^6 independent clones. This library was then amplified, and the titer of amplified library was determined to be 1×10^{10} pfu/ml.

E. Results and Discussion

For cDNA library construction we utilized a strategy similar to that already used to generate a normal human esophageal epithelia cDNA library. Normal human breast tissues were collected from reduction mammoplasties performed at Georgetown University Hospital. These specimens were immediately snap frozen in liquid nitrogen. Based on histological analysis and our prior experience, we obtained several specimens each of which contain sufficient glandular epithelium to yield several micrograms of mRNA. Thus, we can generate more than one library if necessary. The quality of mRNA was verified by formaldehyde-agarose gel electrophoresis and RT-PCR using a probe for human GADPH.

A λ phage library was constructed, and is being subsequently converted into the appropriate plasmid library by *in vivo* excision with the assistance of a helper phage. The quality of the cDNA library was checked by PCR for the size of inserts (Fig. 1,2), as well as by endonuclease restriction enzyme (Fig. 3). Size-fractionation of synthesized cDNA showed the peak size to lie in the range of 600 to 2,000 bp. Using PCR and endonuclease restriction enzyme digestion, we determined the average length of our cDNAs to be approximately 1.3 kb. This expression library is being used to obtain the cDNAs that are being cloned into the tet responsive plasmids for subsequent transfection into MCF-$7^{tetR+neoR}$ cells.

Transfection of MCF-7 cells: The MCF-7$^{tetR+neoR}$ cells have already been generated in the Mentor's laboratory for other studies not related to this application. We utilized a previously cloned MCF-7 cell population (MCF-7 clone #2) that exhibits a total dependence upon estrogen for growth both *in vivo* and *in vitro* (Table 1) (Dr. Clarke, unpublished data). These cells will be used for the initial transfection.

Cell Line	Treatment	G0/G1	δG0/G	G2/M	δG2/M	S	δS
MCF-7	Control: 1 nM E2	31%	-	27%	-	42%	-
	Vehicle	89%	+58%	4%	-23%	7%	-35%

Table 1: Representative analyses of E2-withdrawal on cell cycle distribution in MCF-7 cells as determined by Flow Cytometry. The % change in each phase (δ) is indicated, where % distribution in control treatment = 100%.

F. Progress on the Statement of Work

In the past few months, we have successfully followed our previous plans as described below:

Technical Objective 1: Identify putative TSG(s)

Task 1: Months 1-6: Construct and characterize cDNA library.

Task 2: Months 6-8: Transfect MCF-7tetR+neoR cells.

Tasks 3/4: Months 8-24: Identify cells containing growth inhibitory genes and clone TSG(s).

Technical Objective 2: Characterize putative TSG(s)

Task 5: Months 24-36: cDNA sequencing and sequence analysis.

Task 6: Months 30-48*: Screen tumors for mutations in putative TSG(s).

*We anticipate that completion of these studies will take longer than the three year period. However, it is likely that we will have sufficient data to enable the Fellow to apply for additional funding, e.g., NCI R29 application.

We should mention that up-to-now, we have not had any special problems in accomplishing any of our tasks. In the near future (next few months), we will focus our attention on identifying cells containing growth inhibitory genes and cloning TSG(s), as indicated in the work statement.

We have successfully constructed and performed our initial characterization of the first cDNA library from a reduction mammoplasty (normal breast tissue). We also have obtained two clones of MCF-7 cells transfected with the tetR vector. Consequently, we are now on track to begin isolating TSGs as described in the original application.

It should be noted that the original application was awarded to Dr. Junyi Lei. However, Dr. Lei decided to take a clinical position elsewhere, and we obtained permission to transfer the award to Dr. Le-ping Pu. We lost about six months of time in transferring the award and bringing Dr. Pu onboard. Nevertheless, we are essentially ontrack with regard to the original time frame, being only 12 weeks or so behind the anticipated schedule. We believe this to be highly encouraging, and supporting our ability to perform the proposed studies within the anticipated time frame. Dr. Pu's biographical sketch is included in section 9 (page no.15).

Other Training

In addition to the training obtained in the laboratory, Dr. Pu has been participating in several other research activities within the Lombardi Cancer Center. Dr. Pu has been attending the regular center-wide research Journal Club, and is required to present 1-2 times per year. She also has been attending and participating in the center-wide Research Data Meetings, at which she also is required to present 1-2 times per year, and will begin attending the Gene Regulation Data Meeting when it restarts next semester. Dr. Pu continues to interact with the other scientists within Dr. Clarke's laboratory and to work and consult with other investigators within the Cancer Center. She attended the AACR meeting in April of this year, shortly after joining the mentor's laboratory. Dr. Pu will be expected to attend the AACR and USAMRMC meetings next year and to submit abstracts of her preliminary data. She also may attend other meetings as necessary or appropriate.

7. Conclusions

This is a postdoctoral fellowship application by an individual who only recently joined Dr. Clarke's laboratory. She has previously worked in another field, and was not the original recipient of the award. Consequently, we have lost several months of research time. Nevertheless, Dr. Pu has already demonstrated her ability to quickly learn new techniques and we are confident that she will be able to obtain the training and expertise in the technical skills to complete each aspect of the proposed study.

We consider the major problems associated with this application to be either (i) an inability to identify putative TSGs in our first library, (ii) identifying too many putative TSG genes.

(i) There are several alternative strategies we have devised should our first library not contain suppressor genes. While we would consider this unlikely, it would become apparent within the first six months, providing sufficient time to generate several additional libraries or apply another alternative approach.

We would initially utilize the PCR-based differential-display technique (28) already used by the PI to identify potential retinoid regulated genes (used as preliminary data in the successful breast cancer SPORE application). We have chosen to use this as an alternative approach due to the very high incidence of false positives and the generally poor reproducibility of the technique. However, it can be used as a rapid screen to determine differences between RNA populations. We would first generate a second cDNA library from the "normal" MCF-10A mammary epithelial cell line (29), and then confirm that there are differences in gene expression by applying the differential display technique to the RNA from the reduction mammoplasty (used to generate the first cDNA library), from MCF-10A cells (used to generate the second cDNA library) and from MCF-7 cells. We then would have the opportunity to pursue any differentially regulated PCR fragments, and/or to repeat the analysis as described in the application with the cDNA library exhibiting the greatest number of differences with the MCF-7 cells. While we do not anticipate having to adopt this approach, based on our prior experience we are confident that between our primary and alternative approaches we can rapidly and effectively identify putative breast cancer TSGs.

(ii) It is equally unlikely that we will obtain too many clones for analysis. We believe that our prioritization scheme (above) will adequately address the issue of too many sequences for selection. It also seems likely that some of the putative TSGs will be represented by more than one plasmid, thus the DNA sequencing studies also will help reduce the number of clones for analysis.

In conclusion, we have successfully constructed and performed our initial characterization of the first cDNA library from a reduction mammoplasty (normal breast tissue). We also have obtained two clones of MCF-7 cells transfected with the tetR vector. Furthermore, the Fellow continues to receive training in the application of molecular and cellular techniques to breast cancer research. Her participation in local and national meetings provides a further level of training and exposure to breast cancer research. In these regards, we believe that we are making satisfactory progress towards the successful completion of the aims and goals of the original application.

(8) References:

1. van de Vijver, M.J. Molecular genetic changes in human breast cancer. Adv. Cancer Res. 61:25-56, 1994
2. Slamon, D.J., Clark, G.M., Wong, S.G., Levin, W.J., Ullrich, A., and McGuire, W.L.Science. 235:177-182, 1987
3. Devilee, P., and Cornelisse, C.J. Somatic genetic changes in human breast cancer. Biochim Biophys Acta. 1198:113-30, 1994.
4. Elledge R.M., and Allred, D.C. The p53 tumor suppressor gene in breast cancer. Breast Cancer Res. Treat. 32:39-47, 1994
5. Greenblatt M.S., Bennett W.P., Hollstein M., Harris C.C. Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. (review). Cancer Res. 54:4855-78, 1994.
6. Knudson, A.G., Jr. A two-mutation model for human cancer. In: Advances in viral oncology, Vol. 7. G. Klein Ed. Raven Press, New York, 1987, pp.1-17.
7. Xiao S., Li D., Corson J.M., Vijg J., and Fletcher J.A. Codeletion of p15 and p16 genes in primary non-small cell lung carcinoma. Cancer Res. 55:2968-71, 1995.
8. Toyooka M., Konishi M., Kikuchi-Yanoshita R., Iwama T., and Miyaki M. Somatic mutations of the adenomatous polyposis coli gene in gastroduodenal tumors from patients with familial adenomatous polyposis. Cancer Res. 55:3165-70, 1995.
9. Zhou X., Tarmin L., Yin J., Jiang H-Y., Suzuki H., Rhyu M-G., Abraham J.M., Meltzer tS.J. The MTS1 gene is frequently mutated in primary human esophageal tumors. Oncogene. 9:3737-41, 1994.
10. Suzuki H., Zhou X., Yin J., Lei J-Y., Jiang H-Y., Suzuki Y., Chan T., Hannon G.J., Mergner W.J., Abraham J.M., Meltzer S.J. Intragenic mutations of CDKN2B and CDKN2A in primary human esophageal cancers. Human Mol. Genet. (in press)
11. Viskochil, D., Buchberg, A.M., Xu, G., Cawthon, R.M., Stevens, J., Wolff, R.K., Culver, M., Carey, J.C., Copeland, N.G., Jenkins, N.A., White, R., O'Connell, P. Deletions and a translocation interrupt a cloned gene at the neurofibromatosis type 1 locus, Cell 62:187-92, 1990
12. Culotta E., and Koshland D.E., Jr. p53 sweeps through cancer research. Science. 262:1958-61, 1993.
13. Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P.A., Skolnick, M.H., *et al.* A strong candidate for the breast and ovarian cancer susceptibility gene BRCA 1. Science. 266:66-71, 1994
14. Futreal, P.A., Liu, Q., Shattuck-Eidens, D., Cochran, C., Harshman, K., Tavtigian, S., et al. BRCA 1 mutations in primary breast and ovarian carcinomas. Science. 266:120-2, 1994.
15. Sambrook, J. *et al.* Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 1989.
16. Fromomt-Racine,M., Bertrand, E., Pictet, R. and Grange, T. A highly sensitive method for mapping the 5' termini of mRNAs. Nucleic Acids Res. 21:1683-1684, 1993
17. Borson, N.D., Sato, W.L. and Drewes, L.R. A lock-docking oligo (dT) primer for 5' and 3' RACE PCR. PCR Methods & Appl. 2:144-148, 1992.
18. Chenchik, A., Diatchenko, L., Chang, C. and Kuchibhatla, S. Great Lengths cDNA synthesis kit for high yields of full length cDNA. Clontechniques IX (1):9-12, 1994.

19. Barnes, W,M, PCR amplification of up to 35-kb DNA with high fidelity and high yield from (bacteriophage templates. Proc. Natl. Acad. Sci. USA 91:2216-2220, 1994.

Gossen M, Bujard H: Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci USA 89:5547-5551, 1992.

21. Maines JZ, Sunnarborg A, Rogers LM, Mandavilli A, Spielmann R, Boyd FT: Positive selection for growth inhibitory genes. Cell Growth Diff 6:663-671, 1995.

22. Clarke R, Brünner N, Katz D, Glanz P, Dickson RB, Lippman ME, Kern F: The effects of a constitutive production of TGF- on the growth of MCF-7 human breast cancer cells *in vitro* and *in vivo*. Mol Endocrinol 3:372-380, 1989.

23. Clarke R, Brünner N, Katzenellenbogen BS, Thompson EW, Norman MJ, Koppi C, Paik S, Lippman ME, Dickson RB: Progression from hormone dependent to hormone independent growth in MCF-7 human breast cancer cells. Proc Natl Acad Sci USA 86:3649-3653, 1989.

24. Clarke R, Brünner N, Thompson EW, Glanz P, Katz D, Dickson RB, Lippman ME: The inter-relationships between ovarian-independent growth, antiestrogen resistance and invasiveness in the malignant progression of human breast cancer. J Endocrinol 122:331-340, 1989.

25. Brünner N, Boulay V, Fojo A, Freter C, Lippman ME, Clarke R: Acquisition of hormone-independent growth in MCF-7 cells is accompanied by increased expression of estrogen-regulated genes but without detectable DNA amplifications. Cancer Res 53:283-290, 1993.

26. Brünner N, Frandsen TL, Holst-Hansen C, Bei M, Thompson EW, Wakeling AE, Lippman ME, Clarke R: MCF7/LCC2: A 4-hydroxytamoxifen resistant human breast cancer variant which retains sensitivity to the steroidal antiestrogen ICI 182,780. Cancer Res 53:3229-3232, 1993.

27. Brünner N, Boysen B, Kiilgaard TL, Frandsen TL, Jirus S, Clarke R: Resistance to 4OH-Tamoxifen does not confer resistance to the steroidal antiestrogen ICI 182,780, while acquired resistance to ICI 182,780 results in cross resistance to 4OH-TAM. Breast Cancer Res Treat 27:1351993;

28. Liang P, Pardee AB: Differential display of eucaryotic messenger RNA by means of the polymerase chain reaction. Science 257:967-971, 1992.

29. Soule HD, Maloney TM, Wolman SR, Peterson WD, Brenz R, McGrath CM, Russo J, Pauley RJ, Jones RF, Brooks SC: Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. Cancer Res 50:6075-6086, 1990.

(9) Appendices

1. Biographical Sketch: Dr. Le-ping Pui

2. Illustrations: Figures 1, 2 and 3.

Figures 1-3:

BIOGRAPHICAL SKETCH

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME	POSITION TITLE
Le-ping Pu	Postdoctoral Fellow

EDUCATION *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Lanzhou Medical School, Gansu, China	MD	1983	Medicine
Universite Lyon-Sud, Lyon, France	PhD	1992	Neuroscience
Laboratory of Developmental Neurobiology, NICHHD, NIH, Bethesda MD	Postdoctoral	1996	Molecular Biology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

Awards and Professional Experience:

1990-91	Research Award, Fundation pour La Recherche Medicale
1991	Data featured on the cover of an issue of the peer review journal Neuroendocrinology
1992	Data featured on the cover of an issue of the peer review journal Neuroendocrinology
1993-date	Member, Society for Neuroscience
1993-date	Member, American Association for the Advancement of Science
1997-date	Associate Member AACR

Publications:

1. Cawley, N.X*., **Pu, L.P*.,** and Loh, Y.P. Immunological identification and localization of yeast aspartic protease 3-like prohormone processing enzymes in mammalian brain and pituitary. ***Endocrinology***, in press. * The first and second authors had equal contributions to this paper.
2. **Pu, L.P.,** Ma, W., Barker, J.L., and Loh Y.P. Differential co-expression of genes encoding prohormone convertases (PC1 and PC2) and PRO-TRH in adult rat brain neurons: Implications for differential processing of PRO-TRH. ***Endocrinology*** 137:1233-1241, 1996.
3. **Pu, L.P.,** Van Leeuwen, F.W., Sonnemans, M.A.F., Tracer, H., and Loh, Y.P. Localization of vasopressin mRNA and immunoreactivity in the pituicytes of pituitary stalk-transected rats after osmotic stimulation. ***Proc. Natl. Acad. Sci. USA*** 92:10653-10657, 1995
4. Cawley, N.X., Wong, M., **Pu, L.P.,** Tam, W., and Loh, Y.P. Secretion of yeast aspartic protease 3 (YAP3p) is regulated by its carboxy-terminus tail:Characterization of secreted YAP3p. ***Biochemistry*** 34:7430-7437, 1995.
5. **Pu, L.P.,** Hayes, W.P., Mill, J., Ghose, S., Friedman, T.C., and Loh, Y. P. Frog Prohormone Convertase PC2 mRNA has a Mammalian-Like Expression Pattern in the CNS and is Co-localized With a Subset of Neurons That Express Thyrotropin-Releasing Hormone. ***J. Comp. Neurol.*** 354:71-86, 1995.
6. Bamberger, A.M., Bamberger, C.M., Pu, **L.P.,** Puy, L.A., Loh, Y.P., and Asa, S.L. Expression of Pit-1 in Human Placenta and Choriocarcinoma Cells. ***J. Clin. Endocrinol. Metab.*** 80: 2021-2026, 1995

Publications: continued

7. Bamberger, A.M.*, **Pu, L.P.***, Cool, D., and Loh, YP. The Neuro-2a neuroblastoma cell line expresses enkephalin and vasopressin mRNA and protein. ***Mol. Cellular Endocrinol*** 113:155-163, 1995. * The first and second authors had equal contributions to this paper.

8. Hill, R.M. Ledgerwood, E.C., Brennan, S.O., Pu, L.P., Loh, Y.P., Christie, D.L., and Birch, N.P. Characterization of the molecular forms and membrane association of Kex-2/subtilisin-like serine proteases in the neuroendocrine secretory vesicles. ***J. Neurochem.*** 65:2318-2326, 1995

9. Loh, Y.P., Cawley, N.X., Friedman, T.C., and Pu, L.P. Yeast and mammalian basic residue-specific aspartic proteases in prohormone conversion. In: Aspartic Proteinases. Ed. K. Takahashi. Plenum, New York. pp 519-527, 1995

10. Loh, Y.P., Kon, O.L., and **Pu, L.P.** Increase in desacetyl-a-MSH cells in rat anterior pituitary following adrenal enucleation and adrenalectomy. ***Endocrine J.*** 2:115-121, 1994.

11. **Pu, L.P.,** and Dubois MP. The distribution and development of delta sleep-inducing peptide (DSIP)-like immunoreactivity in postnatal prepubertal guinea pig brain. ***J. Neuroendocrinology*** 4:45-50, 1992.

12. **Pu, L.P.,** and Dubois M.P. The fetal development of delta sleep-inducing peptide (DSIP)-likeimmunoreactivity in hypothalamus of guinea pig with special regard to the prenatal colocalization with gonadotropin-releasing hormone (GnRH)-like immunoreactivity. ***Neuroendocrinology*** 55:332-338, 1992. (Cover for that issue)

13. **Pu, L.P.,** Charnay, Y., Leduque, P., Morel, G., and Dubois, M.P. Light and electron microscopic immunocytochemical evidence that delta sleep-inducing peptide (DSIP) and gonadotropin- releasing hormone (GnRH) are coexpressed in the same structures in the guinea pig median eminence. ***Neuroendocrinology*** 53:332-338, 1991. (Cover for that issue)

14. **Pu, Le-Ping.** Ultrastructural changes of synapses inparietal cortex of aged rat. Acta Academic Medicine of Lanzhou, 1988, (China)

15. **Pu, Le-Ping.** Ultrastructural changes of neurons inparietal cortex of aged rat. J. Anat. Neurol., 1989 (China)

Manuscripts in Preparation:

16. **Pu, L.P.,** Hayes, W.P., Bamberger, A.M., and Loh, Y.P. Prohormone convertase 2 is expressed in proenkephalin containing neurons in developing and adult rat cerebellum. Manuscript in preparation for J. Comp. Neurol.

17. Hayes, W.P., Chin, H., Gallo, V., Mill, J.F., **Pu, L.P.,** Kim, D.S., Taira, M., and Dawid I.B. Neuronal progenitors and their progeny express the LIM-class homeobox gene rlim-1 in developing rat cerebellum. Manuscript in preparation.

18. **Pu, L. P.,** and Rosen, J.B. Kindling induced changes in mRNA expression of pro-TRH and prohormone convertases in dentate gyrus. Manuscript in preparation.

Selected Abstracts:

Pu, L.P., Ma, W., Barker, J.L., and Loh, Y.P. Expression of Genes Encoding Prohormone Convertases (PC1 and PC2) and PRO-TRH in Adult Rat Brain Neurons: Implications for Differential Processing of PRO-TRH. Abstract for GORDON RESEARCH CONFERENCE, 1994.

Bamberger, A.M., Bamberger, C.M., Pu, L.P., Puy, L.A., Loh, Y.P., and Asa, S.L. Pit-1 is expressed in Normal Human Placenta and Choriocarcinoma Cells. Abstract for The Endocrine Society, 1995.

Loh, Y.P., Pu, L.P., Tracer, H.L., Sonnemans, M.A.F., and Van Leeuwen, F.W. Localization of vasopression mRNA and immunoreactivity in pituicytes of pituitary stalk-transected rats after osmotic stimulation. Abstract for Society for Neuroscience, 1995.

Pu, L. P., and Rosen, J.B. Kindling induced changes in mRNA expression of pro-TRH and prohormone convertases in dentate gyrus. Abstract for Society for Neuroscience, 1995

Chodobski, A., Loh, Y.P., Pu, L. P., Johnson, C.E., Corsetti, S., and Sznydynger-Chodobski, J. The presence of arginine vasopressin (AVP) and its mRNA in rat choroid plexus. Abstract for Society for Neuroscience, 1996.

OTHER COMMITTMENTS OF TIME: No sabbatical or extended leave anticipated.

PROPORTION OF TIME DEVOTED TO RESEARCH: **This application:** 100% effort. **Current Applications:** None. **Pending Applications:** None.

CURRENT OR PRIOR FEDERAL GOVERNMENT SERVICE: None.

GRADUATE STUDENTS: None.

ASSISTANTS: None.

E. Existing/Pending Support: None

Figures and Figure Legends

Figure 1: PCR screening inserts in λgt11 cDNA library. Lambda DNA was prepared by picking up a plaque with a micropipette and transferring into deionized H_2O. PCR was performed basically according to CLONTECH's LD-Insert Screening Amplimer Sets (CLONTECH Laboratories, Inc., Cat # PT1579-1). This PCR showed different size of inserts. Lanes 1-4 used 5% DMSO, and lanes 5-7 used 10% DMSO. Note the arrow that the size of insert (lane 5) is approximately 1 kb.

Figure 2: PCR screening the size of inserts in λgt11 cDNA library. Lambda DNA was prepared from Midi-Prep DNA extraction. This PCR showed the peak size to lie in the range of 600 to 2,000 bp.

Figure 3: Endonuclease restriction enzyme checking the average length of inserts in λgt11 cDNA library. This reaction showed the average length of inserts of cDNAs to be approximately 1.3 kb.

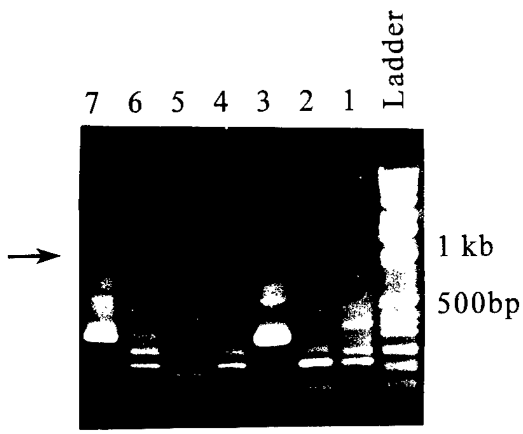

Figure 1.

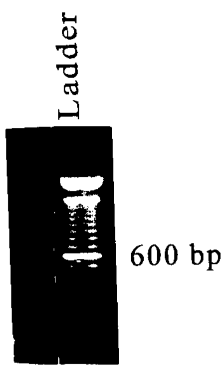

Figure 2.

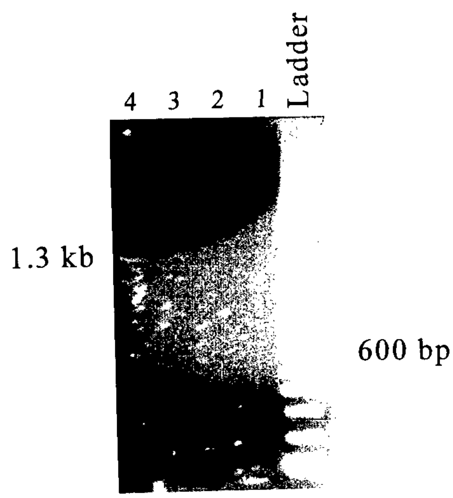

Figure 3.