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**6. ATHOR(S)**
Yijun Zhu, M.D.
Janardan K. Reddy, M.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
Northwestern University
Evanston, IL 60208-1110

E-Mail: y-zhu2@northwestern.edu

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Estrogen plays an important role in the normal breast and breast cancer development. Estrogens exert their cellular effects through ER that is a member of nuclear receptor superfamily. PRIP (Peroxisome proliferator receptor interacting protein) is a nuclear receptor coactivator that is amplified and overexpressed in breast cancer. The proposal was to investigate how the PRIP dysregulation contributes to abnormal growth and neoplastic development of breast. During the 3-year period, we disrupted PRIP gene in mice by homologous recombination. Mice nullizygous for PRIP died between embryonic day 11.5 and 12.5 (post coitum), indicating that PRIP like PBP, CBP, and p300 is an essential and nonredundant coactivator. We generated a conditional null mutation of PRIP in mammary gland. Null mutation of PRIP in mammary gland causes defective mammopoiesis, demonstrating that PRIP is important for the normal mammary gland development. We isolated PIMT and PRMT2 as two components in the estrogen receptor transcriptional activation, advancing our understanding of estrogen signaling pathway. We found that PRIP overexpression occurs in about 60% and gene amplification occurs 10% of the breast cancers, suggesting that this coactivator plays an important role in the breast cancer development.



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TITLE: The Role of PRIP in Breast Cancer

PRINCIPAL INVESTIGATOR: Yijun Zhu, M.D.
Janardan K. Reddy, M.D.

CONTRACTING ORGANIZATION: Northwestern University
Evanston, IL 60208-1110

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## Table of Contents

## INTRODUCTION

Estrogens play an important role in the normal breast and breast cancer development. Estrogens exert their cellular effects through ER which is a member of nuclear receptor superfamily. Nuclear receptors achieve transcriptional activation through the participation of additional factors known as nuclear receptor coactivators. PRIP (Peroxisome proliferator receptor interacting protein) is a nuclear receptor coactivator amplified and overexpressed in some breast cancers. The proposal was to investigate how PRIP dysregulation contributes to abnormal growth and neoplastic development of breast. Specifically, transgenic animal models overexpressing PRIP would be developed to determine if overexpression of PRIP is sufficient to cause abnormal growth of mammary glands and if such growth ultimately leads to the development of breast cancers. PRIP transgenic mouse and PRIP null mutated mouse would be used to examine the role of coactivator PRIP in determining the susceptibility of breast cancer development induced by estrogen and chemical carcinogen. The expression of estrogen responsive genes would be examined to test the role of PRIP as an estrogen receptor coactivator in vivo and identify the estrogen responsive genes that are specifically affected by PRIP overexpression and null mutation. The prevalence of PRIP upregulation and amplification in breast cancers would be examined in breast cancers. The potential polymorphisms in PRIP genes would be identified and the linkage between the identified polymorphisms and breast cancer risk would be elucidated by examining normal female and breast cancer patients. The proposed studies would generate novel information regarding the role of nuclear receptor coactivator in breast cancer development and the importance of the variation of nuclear receptor coactivator in the etiology of breast cancer, and most likely provide new insights regarding prevention and treatment strategies.

## BODY

Task 1a. Generation of transgenic animal models overexpressing PRIP to determine if overexpression of PRIP is sufficient to cause abnormal growth of mammary glands and if such growth ultimately leads to the development of breast tumors.

We made a construct MMTV-PRIP to overexpress PRIP in mammary gland. We then used this construct to develop transgenic mice. Transgenic founders were identified by PCR with primers recognizing the promoter region of the transcription unit. 7 transgenic mouse lineages were identified. The founder mice were bred with C57BL/6 mice to generate F1 progeny. The expression of PRIP transgene in mammary glands from F1 progeny was examined by RNAse protection assay. The expression level of PRIP transgene was about half of that from the endogenous PRIP. We did not detect any tumors in mammary glands from the transgenic mice after two-year observation.

Task 1b. Investigation of the role of coactivator PRIP in the susceptibility for breast cancer development to estrogen and chemical carcinogen using PRIP transgenic mouse and PRIP null mutation mouse.

We disrupted PRIP gene in mice by homologous recombination. Mice nullizygous for PRIP died between embryonic day 11.5 and 12.5 (post coitum) due in most part to defects in the development of placenta, heart, liver, nervous system and retardation of embryonic growth(1). Transient transfection assays using fibroblasts isolated from $PRIP^{-/-}$ embryos revealed a significant decrease in the capacity for ligand-dependent transcriptional activation of RXRα and to a lesser effect on PPARγ transcriptional activity. These observations indicate that PRIP like PBP, CBP, and p300 is an essential and nonredundant coactivator.

As PRIP null mutation is embryonic lethal, we generated a conditional null mutation of PRIP in mammary gland (2). In PRIP deficient mammary gland, the elongation of ducts during puberty was not affected but the number of ductal branches was decreased, which persisted long after puberty, indicating the potential of ductal branching was impaired. During pregnancy, PRIP deficient mammary gland exhibited decreased alveolar density. The lactating PRIP deficient gland contained scant lobuloalveoli with many adipocytes while wild type gland was composed virtually of no adipocytes but mostly lobuloalveoli. As a result, PRIP mammary deficient gland could not produce enough milk to nurse all pups during lactation. The ductal branching of mammary gland in response to estrogen treatment was attenuated in PRIP mutant gland. While proliferation index was similar between wild type and PRIP deficient gland, increased apoptosis was observed in PRIP deficient gland. PRIP deficient gland expressed increased amphiregulin, TGFα, and betacellulin mRNA as compared to wild type gland. The differentiated function of PRIP deficient mammary epithelial cells was largely intact, as evidenced by the expression of abundant β-casein, WAP and WNDN1 mRNA. We conclude that PRIP is important for the normal mammary gland development.

We identified PIMT (PRIP interacting protein with a methyltransferase domain) which interacts with and enhances nuclear receptor coactivator PRIP function(3). We also found that PRMT2(protein arginine methyltransferase 2) to act as a coactivator for estrogen receptor α(4).

Task 1c. Examination of the expression of estrogen responsive genes and identification of the estrogen responsive genes that are specifically affected by PRIP overexpresssion and null mutation.

Mammary glands were isolated from *ovariectomized* animals treated with 17β-estradiol or ICI 182,780 for three days. Poly(A)$^+$ RNA was isolated from the mammary glands. cDNA probe was made from poly A$^+$ RNA for hybridization to Mouse cDNA microarray filters. The filters were then exposed to a phosphor imaging system and analyzed using software to obtain the relative expression level of each gene. We found that estrogen treatment increased the mRNA levels of about fifty genes. GAS6 was picked up for further studies. The induction of GAS6 mRNA expression by estrogen was confirmed by RNAse protection assay. The mammary gland from mice deficient for the GAS6 receptors was examined and found to have increased budding and decreased branching.

Task 2. Determination of the prevalence of PRIP upregulation and amplification in breast cancers.

We have collected about 100 breast cancer specimen from the Department of Pathology, Robert Lurie cancer center. RNAse protection assay revealed that 62 % of the breast cancers have PRIP overexpression. Southern blot studies showed that 10% of the breast cancers have PRIP gene amplification.

Task 3. Investigation of the polymorphism of PRIP gene in Breast cancer.

PCR were performed to cover all exons and 500 bp promoter region of PRIP gene. The PCR products were analyzed by SSCP (single strand conformational polymorphism) and the potential polymorphisms were determined by sequencing. We found polymorphism A to G at nucleotide 289, G to A at nucleotide 2408. The significance of these polymorphisms in breast cancer development remains to be investigated.

The list of personnel receiving pay:

Dr. Rigen Mo, Research associate, 100% effort.

## KEY RESEARCH ACCOMPLISHMENTS

* PRIP gene was disrupted in mice by homologous recombination. Mice nullizygous for PRIP was found to die between embryonic day 11.5 and 12.5.
* Null mutation of PRIP in mammary gland causes defective mammopoiesis, demonstrating that PRIP is important for the normal mammary gland development.
* PIMT (PRIP interacting protein with a methyltransferase domain) which interacts with and enhances nuclear receptor coactivator PRIP function was identified.
* PRMT2(protein arginine methyltransferase 2) was found to act as a coactivator for estrogen receptor $\alpha$.
* About fifty estrogen-inducible genes including GAS6 were identified. The mammary gland from mice deficient for the GAS6 receptors was found to have increased budding and decreased branching.
* PRIP overexpression and amplification were found in about 60% and 10% of breast tumors, respectively.

## REPORTABLE OUTCOMES

Publications:

(1). **Zhu YJ**, Crawford SE, Stellmach V, Dwivedi RS, Rao MS, Gonzalez FJ, Qi C, and Reddy JK. Coactivator PRIP, the Peroxisome Proliferator-activated Receptor-interacting Protein, is a Modulator of Placental, Cardiac, Hepatic and Embryonic development. *J. Biol. Chem.* 278: 1986-1990. 2003

(2). Qi C, Kashireddy P, Zhu YT, Rao SM, and **Zhu YJ.** Null mutation of PRIP in mammary gland causes defective mammopoiesis. *J. Biol. Chem. (in press)* 2004

(3). Zhu YJ, Qi C, Cao WQ, Yeldandi AV, Rao MS, and Reddy JK. Cloning and characterization of PIMT, a protein with a methyltransferase domain, which interacts with and enhances nuclear receptor coactivator PRIP function. *Proc. Natl. Acad. Sci. USA* 98: 10380-10385, 2001.

(4). Qi C, Chang J, Zhu YW, Yeldandi AV, Rao MS and Zhu YJ. Identification of PRMT2 as a coactivator for estrogen receptor alpha. *J. Biol. Chem.* 277: 28624-30. 2002.

## CONCLUSIONS

We disrupted PRIP gene in mice by homologous recombination. Mice nullizygous for PRIP died between embryonic day 11.5 and 12.5 (post coitum), indicating that PRIP like PBP, CBP, and p300 is an essential and nonredundant coactivator. We generated a conditional null mutation of PRIP in mammary gland. Null mutation of PRIP in mammary gland causes defective mammopoiesis, demonstrating that PRIP is important for the normal mammary gland development. We isolated PIMT and PRMT2 as two components in the estrogen receptor transcriptional activation, advancing our understanding of estrogen signaling pathway. We found that PRIP overexpression occurs in about 60% and gene amplification occurs 10% of the breast cancers, suggesting that this coactivator plays an important role in the breast cancer development.

**REFERENCES**

(1). **Zhu YJ**, Crawford SE, Stellmach V, Dwivedi RS, Rao MS, Gonzalez FJ, Qi C, and Reddy JK. Coactivator PRIP, the Peroxisome Proliferator-activated Receptor-interacting Protein, is a Modulator of Placental, Cardiac, Hepatic and Embryonic development. *J. Biol. Chem.* 278: 1986-1990. 2003

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(4). Qi C, Chang J, Zhu YW, Yeldandi AV, Rao MS and Zhu YJ. Identification of PRMT2 as a coactivator for estrogen receptor alpha. *J. Biol. Chem.* 277: 28624-30. 2002.

## APPENDICES

Four articles are attached.

# Cloning and characterization of PIMT, a protein with a methyltransferase domain, which interacts with and enhances nuclear receptor coactivator PRIP function

Yijun Zhu, Chao Qi, Wen-Qing Cao, Anjana V. Yeldandi, M. Sambasiva Rao, and Janardan K. Reddy*

Department of Pathology, Northwestern University Medical School, Chicago, IL 60611-3008

Communicated by Irving M. Klotz, Northwestern University, Evanston, IL, July 9, 2001 (received for review June 11, 2001)

**The nuclear receptor coactivators participate in the transcriptional activation of specific genes by nuclear receptors. In this study, we report the isolation of a nuclear receptor coactivator-interacting protein from a human liver cDNA library by using the coactivator peroxisome proliferator-activated receptor-interacting protein (PRIP) (ASC2/AIB3/RAP250/NRC/TRBP) as bait in a yeast two-hybrid screen. Human PRIP-interacting protein cDNA has an ORF of 2,556 nucleotides, encodes a protein with 852 amino acids, and contains a 9-aa VVDAFCGVG methyltransferase motif I and an invariant GXXGXXI segment found in K-homology motifs of many RNA-binding proteins. The gene encoding this protein, designated PRIP-interacting protein with methyltransferase domain (PIMT), is localized on chromosome 8q11 and spans more than 40 kb. PIMT mRNA is ubiquitously expressed, with a high level of expression in heart, skeletal muscle, kidney, liver, and placenta. Using the immunofluorescence localization method, we found that PIMT and PRIP proteins appear colocalized in the nucleus. PIMT strongly interacts with PRIP under *in vitro* and *in vivo* conditions, and the PIMT-binding site on PRIP is in the region encompassing amino acids 773–927. PIMT binds *S*-adenosyl-L-methionine, the methyl donor for methyltransfer reaction, and it also binds RNA, suggesting that it is a putative RNA methyltransferase. PIMT enhances the transcriptional activity of peroxisome proliferator-activated receptor $\gamma$ and retinoid-X-receptor $\alpha$, which is further stimulated by coexpression of PRIP, implying that PIMT is a component of nuclear receptor signal transduction apparatus acting through PRIP. Definitive identification of the specific substrate of PIMT and the role of this RNA-binding protein in transcriptional regulation remain to be determined.**

The nuclear receptor superfamily consists of members of ligand-regulated transcriptional factors comprising receptors for steroid and thyroid hormones, vitamin $D_3$, retinoic acid, and peroxisome proliferators, among others (1, 2). All nuclear receptors share a common structure with a highly conserved DNA-binding domain consisting of two zinc fingers, a C-terminal hormone-binding domain, and two transcriptional activation function (AF) domains, termed AF-1 in the N-terminal domain and AF-2 in the hormone-binding domain (3). Transactivation of the AF-2 domain is ligand-dependent and can be blocked by the binding of antagonists. The activities of AF-1 and -2 vary depending on responsive cell types as well as a short DNA sequence, termed response element, located in the promoter regions of target genes (2, 3).

The molecular mechanism(s) by which nuclear receptors achieve transcriptional activation in a tissue-specific fashion is not fully understood. The current models call for the participation of additional factors for modifying the chromatin structure and mediating the interaction between the activated nuclear receptor and the basal transcriptional machinery in a ligand-dependent fashion. During the past few years, many nuclear receptor-binding proteins have been identified (4, 5). Most of them contain the conserved LXXLL (where L is leucine and *X* is any amino acid) signature motif, which mediates recognition of nuclear receptors (4). The nuclear receptor coactivators identified to date include the well-characterized steroid receptor coactivator-1 (SRC-1) family with three members [SRC-1 (6), TIF-2 (SRC-2, GRIP1) (7), p/CIP (ACTR, AIB1, RAC3, and SRC-3) (8–10)], CREB-binding protein (CBP)/p300 (11), PPAR-binding protein (PBP) (12), and PPAR$\gamma$ coactivator-1 (PGC-1) (13). SRC-1 and CBP/p300 possess intrinsic histone acetyltransferase activity and also recruit other proteins with histone acetyltransferase activity, indicating their role in chromatin modification (2, 4, 8, 14). In addition to histone acetylation, there is emerging evidence that suggests a role for histone methylation in the regulation of nuclear receptor transcriptional activity. A protein, designated as coactivator-associated arginine methyltransferase 1 (CARM1), which associates with SRC-2, can methylate histones, and the methyltransferase activity of CARM1 appears necessary for this protein to potentiate nuclear receptor activity (15). Furthermore, protein arginine methyltransferase 1, which shares a region of homology with CARM1, appears to enhance transcriptional activity by acting synergistically with CARM1 (16). Recent evidence suggests that coactivator proteins form distinct coactivator complexes, such as the one anchored by CBP/p300 that integrates the SRC-1 family of proteins and p/CAF to carry out histone acetyltransferase reactions, and the other thyroid hormone receptor-associated protein (TRAP)/vitamin D receptor-interacting protein (DRIP)/activator-recruited cofactor (ARC) complex anchored by PBP (TRAP220/DRIP205) (12, 17–19), which links to the basal transcription machinery (4, 20). PBP, with no histone acetyltransferase activity, serves as an anchor protein to recruit the multiprotein complex that appears to act more directly on the transcriptional apparatus, presumably after the unwinding of chromatin facilitated by the CBP/p300 anchored multiprotein complex (4). In agreement with the central role of CBP/p300 and PBP in the coactivator complex configuration and in nuclear receptor-mediated transcriptional activity, knockout experiments revealed that both CBP, p300, and PBP null mutations

Abbreviations: PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; SRC-1, steroid receptor coactivator-1; CARM1, coactivator-associated arginine methyltransferase 1; PRIP, PPAR-interacting protein; PIMT, PRIP-interacting protein with methyltransferase domain; EST, expressed sequence tag; RXR, retinoid-X-receptor for 9-*cis*-retinoic acid; GST, glutathione *S*-transferase; AdoMet, *S*-adenosyl-L-methionine; PBP, PPAR-binding protein.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY028423 and AF389908).

*To whom reprint requests should be addressed at: Department of Pathology, Northwestern University Medical School, 303 East Chicago Avenue, Chicago, IL 60611-3008. E-mail: jkreddy@northwestern.edu.

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lead to embryonic lethality (21–26), indicating that disruption of these pivotal anchoring coactivators affects the function of many nuclear receptors and possibly other transcription factors.

In this work, we report the cloning of a protein that binds the recently identified nuclear receptor coactivator peroxisome proliferator-activated receptor-(PPAR) interacting protein (PRIP) (ASC2/AIB3/RAP250/NRC/TRBP) (27–31). We cloned PRIP by using PPARγ as bait in the yeast two-hybrid system (27); others have cloned this coactivator by using thyroid hormone receptor and retinoid-X-receptor for 9-*cis*-retinoic acid (RXR) (28–31). To elucidate the role of PRIP, we set out to isolate the PRIP-interacting protein(s) with a two-hybrid system. One of the proteins identified is designated PRIP-interacting protein with methyltransferase domain (PIMT); this protein is a ubiquitously expressed nuclear protein containing a methyltransferase domain. PIMT is able to enhance the transcriptional activity of PPARγ and RXRα, which is further potentiated by overexpression of PRIP. We also show that PIMT binds *S*-adenosyl-L-methionine (AdoMet) and RNA, implying that PIMT may function as a putative RNA methyltransferase.

## Materials and Methods

**Plasmids.** GAL4-PRIP (amino acids 773-2068), GAL4-PPARγ, PCMV-PRIP, PCMV-PPARγ, peroxisome proliferator response element (PPRE)-luciferase (LUC), PCMX-RXRα, and RXRE-LUC have been described elsewhere (12, 27, 32). PCMV-PIMT was constructed by inserting the full-length coding region of PIMT cDNA into *Hin*dIII/*Xho*I site of PCDNA 3.1(+) (Invitrogen). PCMV-PIMT-FLAG was made by inserting full-length PIMT cDNA into *Hin*dIII/*Bam*HI site of p3XFLAG-CMV-14 (Sigma).

**Isolation of Human and Mouse PIMT cDNA.** A partial cDNA encoding a protein that interacts with PRIP was first isolated by yeast two-hybrid screening of a human liver cDNA library, as described previously (12, 27). We then obtained the full-length cDNA by checking human expressed sequence tag (EST) in GenBank. The full-length cDNA we cloned has been designated PIMT to reflect its ability to bind PRIP and its putative functional domain. We then searched the mouse EST database in GenBank, by using the human PIMT cDNA sequence. Several ESTs, including ESTs covering the 5′ and 3′ of PIMT cDNA, were found. The primers 5′-GAGCCCGGATAACGAAATGT-3′ and 5′-CTTCCTCCTGTCTCTCACTT-3′ were synthesized on the basis of the EST sequences. The full-length mouse PIMT cDNA was amplified by using spleen mouse cDNA and sequenced.

**Northern Blot Analysis and *in Situ* Hybridization.** Human multiple tissue Northern blot (CLONTECH) containing 2 μg of poly(A) RNA in each lane was probed with $^{32}$P-labeled PIMT full-length cDNA, according to the conditions outlined by the manufacturer. For *in situ* hybridization, tissues were fixed in 4% paraformaldehyde at 4°C for 14–18 h, dehydrated, embedded in paraffin, and 7-μm-thick sections were cut under ribonuclease-free conditions. RNA riboprobes (antisense and sense riboprobes) for PIMT were synthesized in the presence of digoxigenin-labeled UTP (Roche Diagnostics). Prehybridization, hybridization, washing, and immunological detection were performed as described (33).

**Glutathione S-Transferase (GST) Pull-Down Assays.** The GST alone or GST-fusion protein, GST-PRIP (amino acids 772–1317), was expressed in *Escherichia coli* BL21 and bound to glutathione-Sepharose-4B beads according to the manufacturer's instructions (Amersham Pharmacia). *In vitro* translation was performed by using rabbit reticulocyte lysate (Promega) and labeled with [$^{35}$S]methionine. In a GST pull-down assay, 15 μl of GST fusion protein on glutathione-Sepharose beads was incubated with 5 μl of [$^{35}$S]methionine-labeled *in vitro*-translated proteins for 2 h in 600 μl of 20 mM Tris·HCl, pH 7.5/100 mM KCl/0.7 mM EDTA/0.05% Nonidet P-40/1 mM phenylmethylsulfonyl fluoride (NETN). Bound proteins were washed four times with 800 μl of NETN, eluted by boiling for 2 min in 24 μl of SDS loading buffer, separated by SDS/PAGE, and autoradiographed.

**Immunoprecipitation.** COS-1 cells are transfected with PCMV-PIMT-FLAG by the calcium precipitation method. Twenty-four hours after transfection, the cell was harvested. The lysate was immunoprecipitated with anti-PRIP or preimmune serum. The precipitates were resolved on SDS/PAGE and subjected to Western blot analysis by using anti-FLAG (Sigma).

**Cell Culture and Transfection.** CV-1 cells ($1 \times 10^5$) were plated in six-well plates and cultured in DMEM containing 10% FCS for 24 h before transfection. Cells were transfected for 5 h with 1.5 μg of luciferase reporter plasmid DNA, 1.5 μg of appropriate expression plasmid DNA, and 0.4 μg of β-galactosidase expression vector pCMVβ (CLONTECH) DNA by using the Lipofectamine 2000-mediated transfection method (GIBCO/BRL). Cell extracts were prepared 24 h after transfection and assayed for luciferase and β-galactosidase activities (Tropix, Bedford, MA).

**Immunofluorescence.** COS-1 cells were transfected with PCMV-PIMT-FLAG by using Lipofectamine 2000 (GIBCO/BRL), and 24 h after transfection, the cells were fixed in 1% formaldehyde and washed once with PBS, pH 7.4, after which autofluorescence was quenched with 50 mM ammonium chloride in PBS. Cells were washed with PBS, permeabilized with 0.5% saponin, and then blocked with 0.2% fish skin gelatin. The cells were incubated with the primary antibody anti-FLAG (Sigma) and anti-PRIP, followed by incubation with secondary antibodies. Fluorescence microscopy and digital image collection were performed by using an Olympus (New Hyde Park, NY) microscope and a photometrix cooled charge-coupled device camera driven by DELTAVISION software from Applied Precision (Seattle).

**AdoMet-Binding Assay.** Purified GST or GST-PIMT (10 μg) was incubated with 20 μCi of *S*-adenosyl-L-[*methyl*-$^3$H]methionine (Amersham Pharmacia) in a buffer containing 20 mM Tris·HCl, pH 7.5, 150 mM NaCl, and 2 mM $MgCl_2$, at 37°C for 10 min. The protein was trapped on a HAWP 02500 filter (Millipore). Unbound *S*-adenosyl-L-[*methyl*-$^3$H]methionine was removed by washing with the buffer (20 mM Tris·HCl, pH 7.5/150 mM NaCl/2 mM $MgCl_2$). The filters were dried, and the amount of bound *S*-adenosyl-L-[*methyl*-$^3$H]methionine was quantified by liquid scintillation counting.

**RNA-Binding Gel Retardation Assay.** The degenerate $^{32}$P-labeled RNA (GGAGACCGGCCAGGAUCCAAGCUNNNNNNNN-CAUAAGGAUCCAAGCUAA) was generated by *in vitro* transcription (Ambion, Austin, TX). Reactions (20 μl) containing 0.1 μg of protein indicated, 1 μg of anti-FLAG, 10,000 cpm of RNA, and 1× buffer (20 mM Tris·HCl, pH 7.5/150 mM NaCl/2 mM $MgCl_2$) was assembled and incubated for 15 min on ice. The reactions were loaded on a native polyacrylamide gel (Invitrogen) running at 300 V at 4°C in 0.5× TBE buffer (90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3). The gels were dried and autoradiographed.

## Results

**Isolation of PIMT.** Using PRIP (amino acids 773-2068) as bait in a yeast two-hybrid system, we isolated from human liver cDNA library a partial cDNA encoding a PRIP-interacting protein, which was designated as PIMT. The rest of the cDNA was obtained from a human EST clone that was identified by

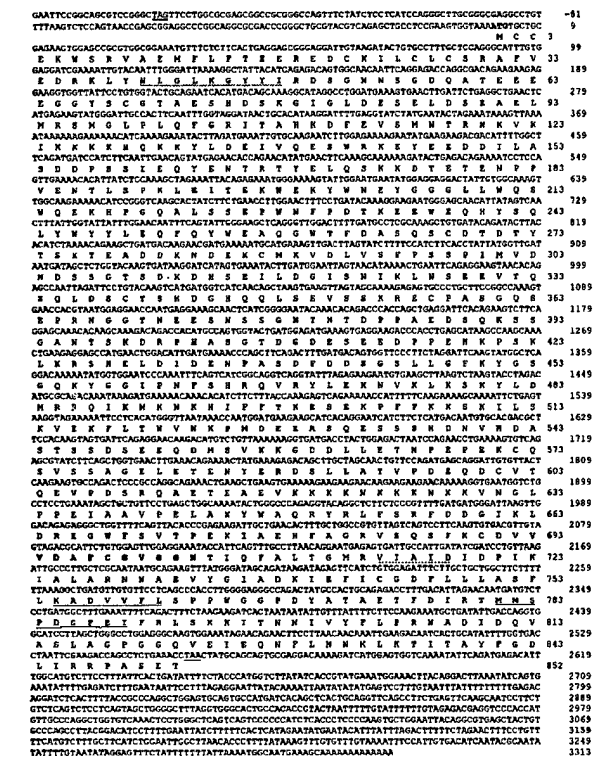

**Fig. 1.** The complete nucleotide and deducted amino acid sequence of human PIMT cDNA. The nucleotide numbering (*Upper Right*) begins as +1 from the initial ATG codon, and the amino acids for the single longest ORF are numbered starting with the first methionine (*Lower Right*). The initiation codon ATG is shown in boldface, and the termination codon TAA and the in-frame stop codon TAG at nucleotide position −150 are underlined. The consensus G*XXG*XXI found in the K-homology RNA-binding domain is underlined. Putative methyltransferase motifs I, II, and III are shown in boldface (motif I), underlined (motif II), and double underlined (motif III). Motif I and Post I (dotted) are known to interact directly with *S*-adenosyl-L-methionine.

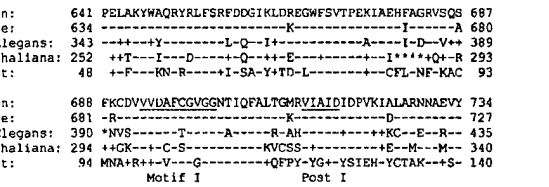

**Fig. 2.** Homology of putative methyltransferase motifs (I, II, and III), and Post I, between human PIMT (amino acids 640–853), mouse PIMT, and *C. elegans* (GenBank accession no. T24696), *Arabidopsis thaliana* (GenBank accession no. AC009917), and yeast gene YPL157WP (GenBank accession no. Z73513) proteins. Dashes represent the same amino acid as in human PIMT, and asterisks represent spaces inserted for optimum alignment.

searching for the homologous sequence in GenBank's human EST. The full-length human PIMT has an ORF of 2,556 nucleotides that encodes a protein of 852 amino acids (Fig. 1) with an estimated molecular mass of 96.5 kDa. The PIMT cDNA fragment directly recovered by yeast two-hybrid screening encodes amino acids from 1 to 384. The human PIMT cDNA contains a short 5′ (165-bp) and a long 3′ (743-bp) untranslated region (Fig. 1). The start of the coding sequence was defined by the first ATG downstream of an in-frame stop codon at position −150. The gene encoding human PIMT is localized on chromosome 8q11, spans more than 40 kb, and consists of more than 13 exons (data not presented). The full-length mouse PIMT cDNA we cloned is 2,648 bp in length, with an ORF of 2,559, which encodes a peptide of 853 amino acids that is 73% identical and 84% similar to the human PIMT (data not shown).

When the nucleotide-derived amino acid sequences of human and mouse PIMTs were used to search the databases, no protein that showed significant overall similarity was detected, but the carboxyl region of human PIMT from amino acids 640–852 shows 56% identity and 68% similarity of amino acids with a protein of unknown function in *Caenorhabditis elegans* and 35% identity and 52% similarity of amino acids with the YPL157WP of the yeast (Fig. 2). They most likely represent homologues of human PIMT. Protein with such a high homology is not found in *Drosophila*. In this region, the human PIMT contains VVDAFCGVG (amino acids 693–701), which is similar to the highly conserved motif I with a consensus hh(D/S)(L/P)F*X*G*X*G, where "h" represents a hydrophobic residue, and "*X*" represents any amino acid, found in a number of $m^5C$ and $m^5A$ methyltransferases and other enzymes that use AdoMet as substrate (34–38). The C-terminal part of human PIMT also reveals regions that are similar to methyltransferase motif II, motif III, and Post 1 (Fig. 2).

**Tissue Distribution of the PIMT Transcript.** Human PIMT is expressed as a 3.2-kb transcript in all tissues examined, with high levels of expression of PIMT mRNA found in heart, skeletal muscle, and kidney (Fig. 3). By *in situ* hybridization, using mouse PIMT as a probe, we found the presence of PIMT transcripts in several tissues of adult mouse, including liver parenchymal cells, proximal tubular epithelium of kidney, intestinal epithelium, exocrine and endocrine pancreatic tissues, thyroid follicles, and prostate epithelium, among others (not illustrated). To localize PIMT protein, a plasmid containing three FLAG-epitopes linked to the C-terminal portion of the PIMT protein was transfected into COS-1 cells derived from African green monkey kidney (American Type Culture Collection CRL1651). Immuno-

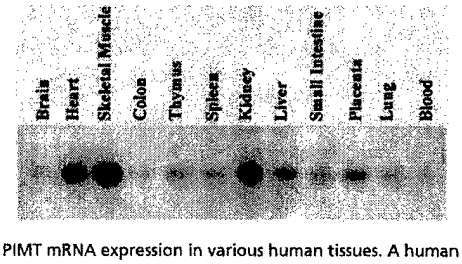


**Fig. 3.** PIMT mRNA expression in various human tissues. A human multiple tissue Northern blot (CLONTECH) containing 2 μg of poly(A) RNA for each tissue was probed with $^{32}P$-labeled full-length human PIMT cDNA and then exposed to film at −80°C with an intensifier screen for 24 h. The transcript size of PIMT is 3.2 kb in all tissues examined.

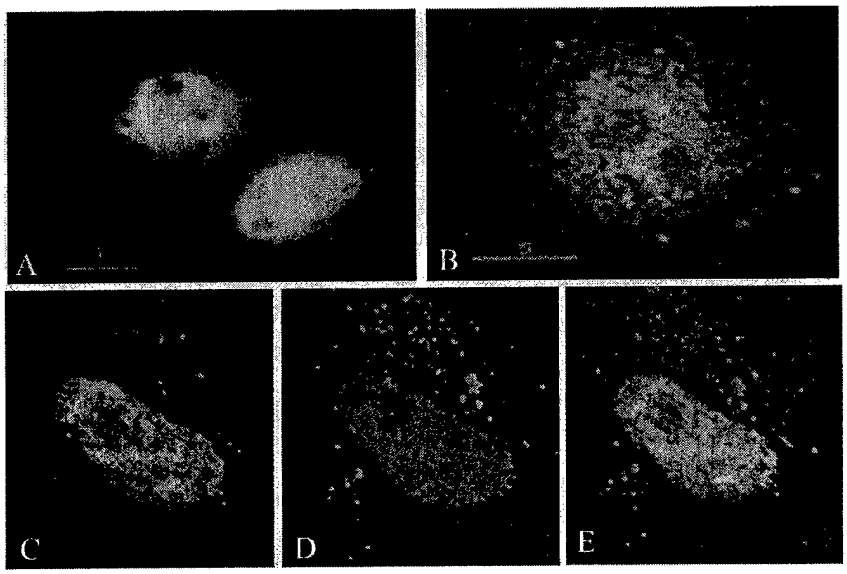


**Fig. 4.** PIMT and PRIP colocalize in the nucleus. PRIP was visualized by using conventional fluorescence microscopy (*A*) and by DELTAVISION deconvolution microscopy (*B*). By DELTAVISION microscopy, a speckled pattern of distribution of this coactivator is noted. PIMT was expressed transiently in COS-1 cells by using three FLAG epitopes linked to the C terminus of PIMT, and the FLAG epitope of PIMT was visualized by deconvolution microscopy by using anti-FLAG antibodies (*C*). Merging of the PIMT (*D*) and PRIP (*D*) localization images reveals overlapping localization (yellow) of these two proteins (*E*).

fluoresence with anti-FLAG revealed the expressed PIMT protein is localized in the nucleus (Fig. 4). Localization of PRIP by using anti-PRIP revealed that PRIP is also localized in the nucleus and, when the images of PIMT and PRIP localization were merged, an overlapping localization of PRIP and PIMT has been noted, suggesting colocalization of these molecules in the nucleus (Fig. 4).

**Interaction of PIMT with PRIP *in Vitro* and *in Vivo*.** The direct interaction between PRIP and PIMT was further tested by using *in vitro* a GST-binding assay with a bacterially generated GST-PRIP fusion protein (amino acids 773-1317) and radioactively labeled *in vitro*-translated PIMT. As shown in Fig. 5, PIMT specifically interacted with the immobilized GST-PRIP but not with GST. Using further truncated PRIP, we narrowed the PIMT-binding site on PRIP to a region encompassing amino acids 773–927 (Fig. 5*A*). To determine whether PIMT and PRIP interact within the context of intact cells, a vector encoding human PIMT with C-terminal FLAG epitopes was transfected into COS-1 cells. The potential complex between PRIP and PIMT was immunoprecipitated by using anti-PRIP, and the product was analyzed by immunoblotting by using anti-FLAG to demonstrate PIMT in the precipitate (Fig. 5*B*). The results showed that anti-PRIP precipitated PIMT but not control serum, demonstrating the existence of a PRIP and PIMT complex *in vivo* (Fig. 5*B*). Because PRIP is able to form homodimers, we examined whether PIMT can also form homodimers. A GST pull-down assay was performed with GST-PIMT fusion protein (amino acids 326–852) and *in vitro*-translated [$^{35}$S]methionine-labeled human PIMT. The results demonstrated that *in vitro*-translated PIMT interacts with GST-PIMT, suggesting that PIMT may form homooligomers (Fig. 5*C*).

**PIMT Binds AdoMet.** Human PIMT contains discernible methyltransferase motifs I, II, and III, and also a conserved motif called Post 1 (Figs. 1 and 2). Because motif I (F*X*G*X*G) is known to interact with an adenosyl moiety of the cofactor (34, 35), we assessed the ability of human PIMT to bind AdoMet by a filter-binding assay. The partial PIMT (amino acid residues 326–852), which contains the putative methyltransferase domain, binds AdoMet, whereas the N-terminal PIMT fragment (amino acids 1–384), which does not contain these motifs, failed to bind AdoMet (Fig. 6*A*).

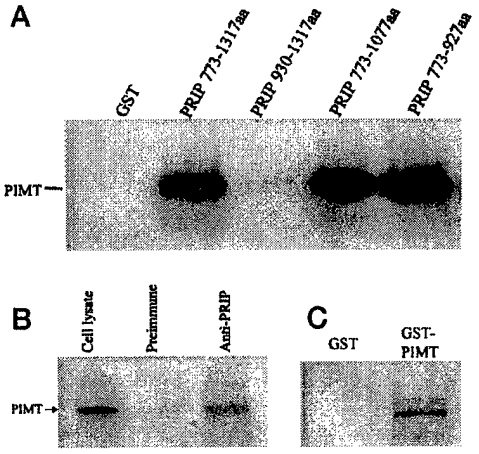


**Fig. 5.** *In vitro* interaction of PRIP with PIMT. (*A*) [$^{35}$S]Methionine-labeled full-length PIMT generated by *in vitro* translation was incubated with glutathione-Sepharose beads bound with purified *E. coli*-expressed GST-PRIP (amino acids 773-1317, 930-1317, 773-1077, and 773–927) or GST. The bound proteins were eluted and analyzed by using 10% SDS/PAGE and autoradiographed. (*B*) Coimmunoprecipitation of PIMT and PRIP in intact cells. pcDNA3.1-FLAG-PIMT were transfected into COS-1 cells and, 24 h after transfection, cells were harvested. Cell lysate was immunoprecipitated with anti-PRIP or control serum, and immunoprecipitates were subjected to immunoblotting with anti-FLAG. Epitope-tagged protein PIMT can be coprecipitated by anti-PRIP (lane 3) but not by preimmune serum (lane 2). Lane 1, 1/20 input cell lysate. (*C*) PIMT forms homodimers. GST-PIMT (326-852 aa) or GST alone was incubated with PIMT labeled with [$^{35}$S]methionine. The bound protein was electrophoresed and visualized by fluorography. GST-PIMT, but not GST alone, binds PIMT.

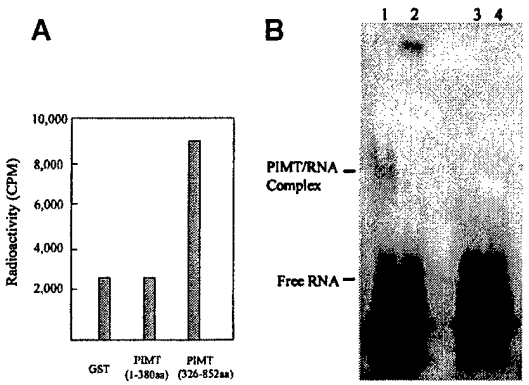


Fig. 6. PIMT binds AdoMet and RNA. (*A*) PIMT binds AdoMet. Purified protein was incubated with *S*-adenosyl-L-[*methyl*-$^3$H]methionine, and the amount of protein bound to AdoMet was trapped on a filter and quantified by liquid scintillation. GST-PIMT (amino acids 326–852), which contained the methyltransferase domain, retained a significant amount of labeled AdoMet, whereas the filter with GST-PIMT (amino acids 1–384) showed background radioactivity as that with GST alone. (*B*) PIMT is an RNA-binding protein. $^{32}$P-labeled RNA produced by *in vitro* transcription is incubated with purified FLAG-PIMT (amino acids 1–384). A PIMT–RNA complex is seen in addition to the free RNA (lane 1). The addition of anti-FLAG markedly diminished the complex; instead, antibody and PIMT–RNA formed a big complex, which hardly migrated into the gel (lane 2). Lanes 3 and 4 represent controls by using purified FLAG-PRIP (amino acids 786-1132) and purified FLAG-PRIP (amino acids 786-1132) plus anti-FLAG, respectively.

**PIMT Is an RNA-Binding Protein.** In an effort to search for the potential substrate of PIMT, we examined whether PIMT can bind to RNA by using a gel-shift assay. When purified FLAG-PIMT (amino acids 1–384) was incubated with $^{32}$P-labeled RNA produced by *in vitro* transcription, a PIMT–RNA complex was produced (Fig. 6*B*, lane 1). The addition of anti-FLAG markedly diminished the formation of the PIMT–RNA complex; instead, antibody and PIMT–RNA formed a high molecular-weight complex, which failed to migrate into the gel (Fig. 6*B*, lane 2). As a control, when purified FLAG-PRIP (amino acids 786-1132) was used in an RNA-binding assay with or without anti-FLAG, no detectable RNA binding was observed (Fig. 6*B*, lanes 3 and 4). Close examination of PIMT amino acids 1–384 revealed that they contained a consensus sequence G*XX*G*XX*I (Fig. 1), which is present in virtually all K-homology domains of RNA-binding proteins (39, 40). A gel retardation experiment revealed that FLAG-PIMT (amino acids 1–384) was unable to bind single- and double-stranded DNA (data not shown).

**PIMT Potentiates the Transcriptional Activity of PPARγ.** To determine the functional relevance of the interaction of PIMT with PRIP, we transiently overexpressed PIMT and PRIP in CV-1 cells along with PPARγ and monitored the transcriptional activity of PPARγ with expression of the PPRE-linked reporter luciferase gene. Both PIMT and PRIP, when transfected individually, consistently increased the transcription of the PPARγ-mediated luciferase gene by about 1.6-fold in the presence of PPARγ ligand BRL49653 (Fig. 7*A*). Cotransfection of PIMT and PRIP resulted in further enhancement of ligand-dependent reporter gene expression, indicating that PIMT synergizes PRIP action. The truncated PIMT (amino acids 1–384), without methyltransferase domains, can also enhance PPARγ transcriptional activity by about 1.8-fold, suggesting the methyltransferase domain is not necessary for PIMT's ability to enhance the transcriptional activity of PPARγ under the conditions of transient transfection. Similar results were observed with RXRα (Fig. 7*B*).

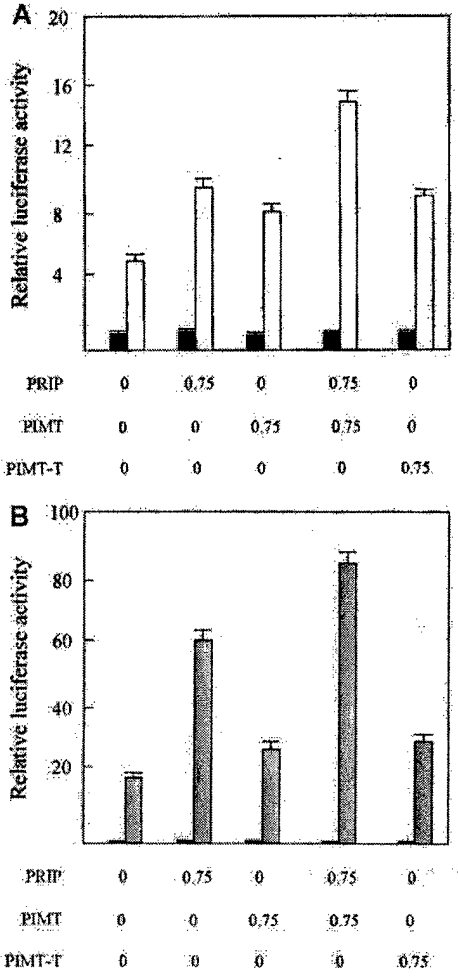


Fig. 7. PIMT increases PPARγ- and RXRα-mediated transactivation of reporter expression in CV-1 cells. (*A*) CV-1 cells were cotransfected with 1.5 μg of reporter construct PPRE-TK-LUC, 25 ng of PCMV-mPPARγ, and 0.4 μg of PCMVβ, along with the indicated plasmid in the absence (solid bar) or presence (open bar) of $10^{-5}$ M BRL49653. Transfection, without the indicated plasmid, was compensated by adding the same amount of PCDNA3.1. The activity obtained on transfection of PPRE-TK-LUC, without exogenous PIMT in the absence of ligand was taken as 1. Results are the mean of three independent transfections. (*B*) Transfection analysis for RXRα was performed in the same way as PPARγ, except for using RXRE-TK-LUC and PCMX-RXRα in the absence (solid bar) and presence (hatched bar) of ligand 9-*cis*-retinoic acid.

## Discussion

In a previous study, using the yeast two-hybrid system, we isolated and characterized mouse PRIP as a PPAR coactivator (27). The cloning of this nuclear receptor coactivator from human and rat has also been reported (28–31). PRIP has been shown to interact with a variety of nuclear receptors and also CBP and is widely expressed with the highest expression in heart, ovary, testis, prostate, and skeletal muscle (27–31). These observations suggested this coactivator may prove as indispensable as CBP, p300, and PBP in mediating the transcriptional activity of nuclear receptors and other transcription factors. In this study, we used a two-hybrid system with PRIP as bait and isolated PIMT, a PRIP-interacting protein, which has a methyltransferase domain. The mRNA of PIMT is ubiquitously expressed, and its protein appears colocalized in the nucleus

with PRIP. It appears that both PRIP and PIMT are expressed abundantly in the same tissues and cell types, suggesting these two proteins play a synergistic function *in vivo*. The PIMT-binding region in PRIP (amino acids 773–927) has also been determined by using truncated portions of PRIP in GST pull-down assays; this region avidly binds PIMT. Overexpression of PIMT enhances the transcriptional activity of PPARγ and RXR, and this enhancement is further stimulated by overexpression of PRIP, suggesting that PIMT is a component of nuclear receptor signal transduction that acts through PRIP.

PIMT reveals the presence of an AdoMet-binding site VVDAFCGVG, which is similar to the highly conserved methylltransferase motif I with a consensus hh(D/S)(L/P)F*X*G*X*G, (where "h" represents a hydrophobic residue, and "*X*" represents any amino acid) (34, 35). This motif is found in several methyltransferases and other enzymes that use AdoMet as substrate (34–36). Of considerable interest is that the motif FCGVG present in PIMT corresponds to the sequence (F*X*G*X*G) found in many RNA and DNA methyltransferases (34, 35, 41). The Phe in this motif is known to interact with the adenosyl moiety of the cofactor AdoMet (35). In other methyltransferases, particularly in protein arginine methyltransferases, Phe is often substituted by Gly (34, 35). The binding of AdoMet, the donor of methyl groups, to purified PIMT under *in vitro* conditions essentially confirms that PIMT is a putative methyltransferase, but the target of PIMT remains unknown. Although PIMT failed to methylate *in vitro* either histone, unlike CARM1 (15), or DNA (Y.Z., unpublished work), we found that it binds RNA. The segment of PIMT (amino acids 1–384) that binds RNA contains an invariant G*XX*G*XX*I motif present in the K-homology domains of RNA-binding proteins (39, 40). It is estimated that the human genome contains ≈1,500 RNA-binding proteins, but their distribution across global, group-specific, and type-specific classes is unknown (42). PIMT may function as a putative RNA methyltransferase, raising the possibility that some coactivators and/or their cofactors may influence transcription by binding and methylating RNA. PIMT shows high homology with an uncharacterized protein YPL157W (GenBank accession no. Z73513) from yeast and a functionally unknown protein (GenBank accession no. T24696) from *C. elegans*. They most probably represent the homologues of PIMT and should have similar substrates. The yeast strain with null mutation of YPL157W gene has been generated by the *Saccharomyces* Genome Deletion Project and has turned out to be viable. We have analyzed the methyl proteins in the YPL157W mutated and wild strains by *in vivo* labeling with *S*-adenosyl-L-[*methyl*-$^3$H]methionine. The methyl protein pattern in the mutated strain appeared similar to that of wild type, but we cannot rule out the possibility that the YPL157W protein methylates a less abundant protein not identified by SDS/PAGE gel (Y.Z., unpublished work).

Methylation plays an important role in the regulation of gene expression (43, 44). Methylation of the promoter participates in the inactivation of gene transcription, which contributes to the silencing of tumor suppressor genes in cancers, whereas methylation of sites downstream of transcriptional initiation is associated with increased transcription (41, 44). In addition to DNA methylation, the importance of histone methylation in the transcriptional regulation is increasingly appreciated (15, 16). Although PIMT does not methylate the histone, it could be involved in transcriptional regulation by methylating other transcriptional components. Truncated PIMT, without the methyltransferase domain, still showed its ability to enhance the transcriptional activity of PPARγ and RXRα, suggesting this enzyme activity may not be crucial to transcription under transient transfection conditions. Given the oversimplified transient transfection system, the role of PIMT's methyltransferase activity in transcriptional regulation remains to be appreciated in the context of *in vivo* physiological condition.

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# Identification of Protein Arginine Methyltransferase 2 as a Coactivator for Estrogen Receptor α*

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**Chao Qi, Jeffrey Chang, Yiwei Zhu, Anjana V. Yeldandi, Sambasiva M. Rao, and Yi-Jun Zhu‡**

*From the Department of Pathology, The Feinberg School of Medicine, Northwestern University, Chicago, Illinois 60611*

**In an attempt to isolate cofactors capable of influencing estrogen receptor α (ERα) transcriptional activity, we used yeast two-hybrid screening and identified protein arginine methyltransferase 2 (PRMT2) as a new ERα-binding protein. PRMT2 interacted directly with three ERα regions including AF-1, DNA binding domain, and hormone binding domain in a ligand-independent fashion. The ERα-interacting region on PRMT2 has been mapped to a region encompassing amino acids 133–275. PRMT2 also binds to ERβ, PR, TRβ, RARα, PPARγ, and RXRα in a ligand-independent manner. PRMT2 enhanced both ERα AF-1 and AF-2 transcriptional activity, and the potential methyltransferase activity of PRMT2 appeared pivotal for its coactivator function. In addition, PRMT2 enhanced PR, PPARγ, and RARα-mediated transactivation. Although PRMT2 was found to interact with two other coactivators, the steroid receptor coactivator-1 (SRC-1) and the peroxisome proliferator-activated receptor-interacting protein (PRIP), no synergistic enhancement of ERα transcriptional activity was observed when PRMT2 was coexpressed with either PRIP or SRC-1. In this respect PRMT2 differs from coactivators PRMT1 and CARM1 (coactivator-associated arginine methyltransferase). These results suggest that PRMT2 is a novel ERα coactivator.**

The estrogen receptor (ER)[1] is a transcription factor that belongs to the nuclear receptor superfamily (1, 2). Upon estrogen binding, ER regulates the transcription of specific target genes by binding to specific DNA response elements referred to as estrogen response elements (EREs) in their promoters or by interacting with other transcription factors such as Jun and Fos (1, 2). In addition to hormone-mediated activation, ER is also activated by growth factors including epidermal growth factor and insulin-like growth factor-1 probably through phosphorylation (3, 4). ER contains two transcriptional activation function (AF) domains: AF-1 located in the N terminus and the ligand-dependent AF-2 located in the ligand binding domain (5). The ability of AF-1 and AF-2 to activate transcription varies according to the promoter context and the cell type (6). There are two isoforms of estrogen receptors, namely ERα and ERβ (7). ERα and ERβ recognize identical DNA elements and have similar affinity for a certain estrogen, but exhibit distinct tissue distribution (7). Evidence provided by gene knock experiments indicates that ERα is the receptor responsible for the estrogen-induced growth of mammary gland and the reproductive tract (8).

The precise mechanism by which ER modulates cell- and gene-specific transcription is not fully understood. Recent evidence suggests that ER activates transcription by recruiting coactivators that appear to act by modifying chromatin structure or facilitating the formation of transcriptional initiation complexes (9, 10). Among a growing list of cofactors that regulate nuclear receptors, including ER, are the well studied coactivators of the SRC-1 family (9), CREB-binding protein (CBP/p300) (11, 12), and PBP (13). PBP is a component of the thyroid hormone receptor-associated protein (TRAP)/vitamin $D_3$ receptor-interacting protein (DRIP) complexes (14–16). Both SRC-1 and CBP/p300 have intrinsic histone acetyltransferase activity and recruit other acetyltransferases (17–20). The acetylation of histone results in the modification of chromatin and increases the access of the DNA to other components of transcription apparatus. The multiprotein TRAP/DRIP complexes exhibit no intrinsic histone acetyltransferase activity and appear to function through the direct interaction with general transcriptional machinery (15, 16). The observation that certain coactivators such as SRC-3 (AIB1; ACTR, p/CIP, RAC3) (21–24), AIB3 (PRIP, ASC2, RAP250, NRC, TRBP) (25–29), and PBP (30) are amplified and overexpressed in some breast cancers underscores the importance of nuclear receptor coactivators in transcriptional activation and also points to their possible role in neoplastic conversion.

Post-translational modification of proteins by arginine methylation has recently been implicated in a variety of cellular processes including nuclear receptor transcriptional regulation (31). Among the five members of protein arginine methyltransferases (PRMTs) identified thus far based on protein sequences, PRMT1 is the first identified and the predominant PMRT in mammalian cells (32). PRMT1 has been shown to interact with SRC-2 (GRIP1) and enhance the nuclear receptor transactivation function (33). Coactivator-associated arginine methyltransferase 1 (CARM1)/PRMT4 was identified by its interaction with nuclear receptor coactivator SRC-2 (GRIP1) (34). PRMT1 and CARM1 are able to methylate the histones H4 and H3, respectively, suggesting their role in modulating

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‡ To whom correspondence should be addressed: Dept. of Pathology, Northwestern University Medical School, 303 E. Chicago Ave., Chicago, IL 60611-3008. Tel.: 312-503-3113; Fax: 312-503-8249; E-mail: y-zhu2@northwestern.edu.

[1] The abbreviations used are: ER, estrogen receptor; ERE, estrogen responsive element; AF, activation function; CREB, cAMP response element-binding protein; CBP, CREB-binding protein; PRMT, protein arginine methyltransferase; CARM1, coactivator-associated arginine methyltransferase 1; TK, thymidine kinase; LUC, luciferase; PPAR, peroxisome proliferator-activated receptor; PRIP, PPAR-interacting protein; PPRE, peroxisome proliferator response element; GST, glutathione *S*-transferase; SRC-1, steroid receptor coactivator-1; RXR, retinoid-X receptor; TR, thyroid hormone ($T_3$) receptor; PR, progesterone receptor.

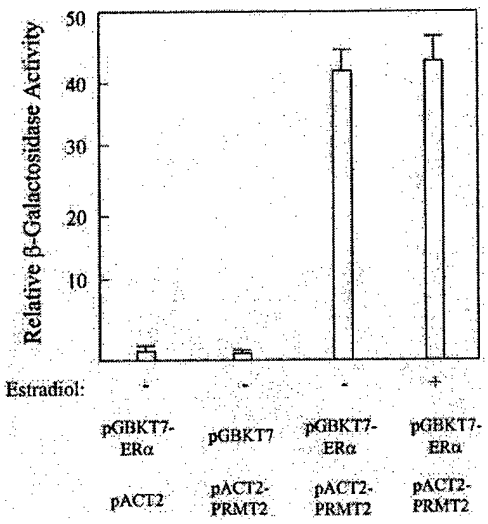


FIG. 1. **The estrogen-independent interaction of PRMT2 with ERα in yeast.** pACT2 expressing GAL4 activation domain alone or pACT2-PRMT2 expressing the fusion protein between the GAL4 activation domain and PRMT2 was cotransformed into yeast HF7C with PGBTKT7 expressing GAL4 DNA binding domain alone or PGBKT7-ERα expressing fusion protein between GAL4 DNA binding domain and ERα into yeast HF7C. The β-galactosidase activities from equal number of cells were measured as an indication of the relative strength of interaction in the presence or absence of ligand 17β-estradiol.

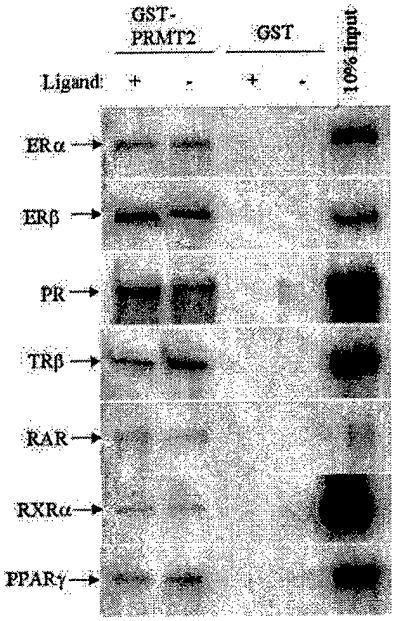


FIG. 2. ***In vitro* interaction of PRMT2 with ERα, ERβ, PR, TRβ, RARα, PPARγ, and RXRα.** GST-Sepharose beads bound with purified *E. coli* expressed GST-PRMT2 or with GST were incubated with [$^{35}$S]methionine-labeled ERα, ERβ, PR, TRβ, RARα, PPARγ, and RXRα in the presence (+) or absence (−) of ligand. The ligands used were: 17β-estradiol for ERα and ERβ, progesterone for PR, $T_3$ for TRβ, 9-*cis*-retinoic acid for RARα and RXRα, and Invitrogen 49653 for PPARγ. Following four times washing with NETN solution, the bound proteins were eluted and separated using 10% SDS-polyacrylamide gel electrophoresis and autoradiographed.

the chromatin structure (34, 35). In addition, CARM1 also methylates the CBP/p300, which disables the interaction between CREB and CBP/p300 and blocks the CREB activation (36). PRMT2 was isolated based on its sequence similarity with PRMT1 (37). So far no methyltransferase activity has been revealed for PRMT2 (37). Here we report the identification of PRMT2 as a new ERα-binding protein through yeast two-hybrid screening. We now demonstrate that PRMT2 binds to ERα directly and also enhanced both its AF-1 and AF-2 transcriptional activity. We also demonstrate that the potential methyltransferase activity was pivotal for PRMT2 coactivator function. These results suggest that PRMT2 is a new ERα coactivator.

## EXPERIMENTAL PROCEDURES

*Plasmids*—pcDNA3.1-ERα, ERE-TK-LUC, PCMX-RARα, RARE-TK-LUC, PCMV-PPARγ, and PPRE-TK-LUC and the vectors for *in vitro* translation of PR, RXRα, TRβ1 have been described elsewhere (26). ERβ is a gift from Dr. Laird D. Madison (Northwestern University). pGBKT7-ERα was constructed by inserting the full-length coding region of ERα cDNA into *NcoI*/*SalI* site of pGBKT7 (CLONTECH). PCMV-PRMT2 was an IMAGE clone purchased from Invitrogen and confirmed by sequencing. GST-PRMT2 was generated by inserting PRMT2 coding region into the *EcoRI*/*SalI* site of PGEX-5X-2. PRMT2 M, which encodes mutated PRMT2 with the mutation on the most conserved motif GCGTG (amino acids 145–149) (from GCGTG to GCRTR), was produced from PCMV-PRMT2 using a PCR site-directed mutagenesis kit (Stratagene). GST-ERα (1–184), GST-ERα (185–250), GST-ERα (251–301), and GST-ERα (302–595) were generated by inserting the corresponding ERα fragment amplified by PCR into the *EcoRI*/*SalI* site of PGEX-4T-3 and were confirmed by sequencing. Gal4-DBD-ERα (1–184) and Gal4-DBD-ERα (250–595) were created by inserting the corresponding fragments into *EcoRI*/*SalI* site of Gal4-DBD vector (CLONTECH) and were confirmed by sequencing. pCMV-FLAG-PRMT2, GST-PRMT1, and PRMT2ΔSH3 expression vectors encoding PRMT2 with the SH3 domain deletion are gifts from Dr. Julia Kzhyshkowska (Regensburg University).

*Yeast Two-hybrid Screening*—Yeast two-hybrid screening was performed using the matchmaker two-hybrid system kit (CLONTECH). Briefly, the yeast strain HF7C was cotransformed with a human matchmaker mammary gland cDNA expression library and pGBKT7-ERα. The positive clones were selected by their growth in medium lacking histidine and the expression of β-galactosidase in the presence of $1 \times 10^{-7}$ M of 17β-estradiol.

*Quantitative β-Galactosidase Assays*—Appropriate plasmids were cotransformed into yeast strain HF7C, plated on selective media plates in the presence or absence of $10^{-7}$ M 17β-estradiol, and then incubated for 4 days at 30 °C. Ten colonies from each plate were suspended in 150 μl of buffer Z (60 mM $Na_2HPO_4$, 40 mM $NaH_2PO_4$, 10 mM KCl, 1 mM $MgSO_4$, 35 mM 2-mercaptoethanol). An equal number of cells in suspension was collected by centrifugation, and β-galactosidase activity was determined (Galacto-light kit, Tropix, Bedford, MA). Three independent assays were performed.

*GST Pull-down Assays*—The GST alone and GST fusion proteins were produced in *Escherichia coli* BL21 and bound to glutathione-Sepharose beads according to the manufacturer's instructions (Amersham Biosciences). *In vitro* translation was performed using rabbit reticulocyte lysate (Promega) and labeled with [$^{35}$S]methionine. In GST pull-down assays, a 25-μl aliquot of GST fusion protein loaded on glutathione-Sepharose beads was incubated with 5 μl of [$^{35}$S]methionine-labeled *in vitro* translated proteins for 2 h in 500 μl of NETN (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.7 mM EDTA, 0.05% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride). The binding was assayed in the presence or absence of specific ligands: 17β-estradiol ($1 \times 10^{-6}$ M) for ER; BRL49653 ($1 \times 10^{-5}$ M) for PPARγ; 9-*cis*-retinoic acid ($1 \times 10^{-6}$ M) for RXRα and RARα; Wy-14, 643 ($1 \times 10^{-5}$ M) for PPARα; and $T_3$ ($1 \times 10^{-6}$ M) for TRβ1. Bound proteins were washed five times with binding buffer, eluted by boiling for 2 min in 20 μl of SDS sample buffer, analyzed by SDS-PAGE, and autoradiographed.

*Immunoprecipitation*—COS-7 cells were transfected with 5 μg of pCDNA3.1-ERα and 5 μg of pCMV-FLAG-PRMT2 using LipofectAMINE 2000 (Invitrogen). 24 h after transfection, the cells were harvested. The lysate was immunoprecipitated with anti-FLAG (Sigma) or control serum. The precipitates were resolved by SDS-PAGE and subjected to Western blot analysis using anti-ERα.

*S-Adenosyl-L-methionine Binding Assay*—Purified GST, GST-PRMT2, GST-PRMT2M, or GST-PRMT1 (10 μg) was incubated with 20 μCi of *S*-adenosyl-L-[*methyl*-$^3$H]methionine (Amersham Biosciences) in the buffer (20 mM Tris-HCL, pH 7.5, 150 mM NaCl, and 2 mM $MgCl_2$) at 37 °C for 10 min. The protein was trapped on HAWP 02500 filter (Millipore). The filter was washed with the buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 2 mM $MgCl_2$) to remove unbound *S*-adenosyl-L-

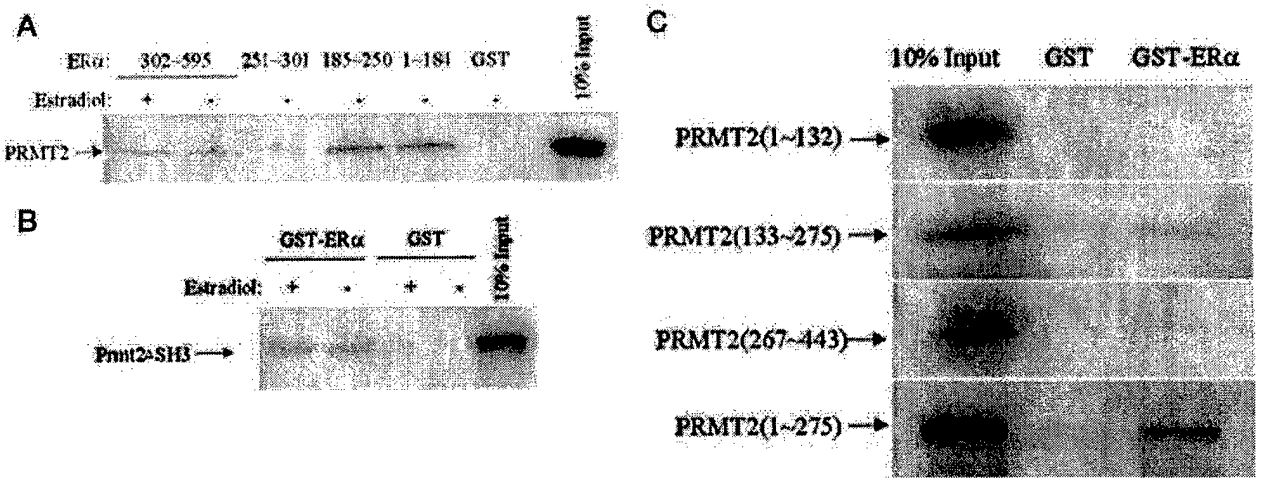


FIG. 3. **Mapping the regions for ERα and PRMT2 interaction.** *A*, GST pull-down assay was performed using [$^{35}$S]methionine-labeled PRMT2 and fusion proteins between GST and four different ERα fragment. Three ERα regions including AF-1 (amino acids 1–184), DNA binding domain (amino acids 185–250), and hormone binding domain (amino acids 302–595) interact with PRMT2. The binding of PRMT2 to the hormone binding domain is estrogen-independent. *B*, the SH3 domain on PRMT2 is dispensable for PRMT2 and ERα interaction. [$^{35}$S]methionine-labeled PRMT2 with SH3 domain deletion is able to bind to the GST-ERα fusion protein but not GST alone. *C*, the interaction region of PRMT2 to ERα was mapped to the fragment from amino acid 133 to 275 by GST pull-down assay using [$^{35}$S]methionine-labeled truncated PRMT2 and GST-ERα fusion protein.


FIG. 4. **PRMT2 interacts with ERα *in vivo*.** Plasmids expressing FLAG-tagged PRMT2 and ERα were cotransfected into COS-7 cells in the presence or absence of 17β-estradiol. The cell extracts were immunoprecipitated with either anti-FLAG or control serum. The precipitates were then analyzed by Western blot using anti-ERα antibody.

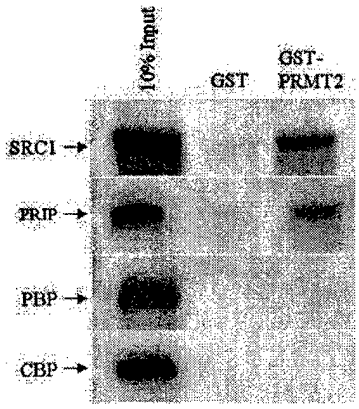


FIG. 5. **PRMT2 interacts with SRC-1 and PRIP.** GST or GST-PRMT2 fusion protein was incubated with [$^{35}$S]methionine-labeled SRC-1, PRIP, PBP, or CBP. Bound proteins were resolved by SDS-PAGE and detected by autoradiography.

[*methyl*-$^{3}$H]methionine. The filters were dried, and the amount of bound *S*-adenosyl-L-[*methyl*-$^{3}$H]methionine was quantified by liquid scintillation counting.

*Cell Culture and Transfection*—CV-1 cells ($1 \times 10^5$) were plated in 6-well plates and cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum for 24 h before transfection. Cells were transfected for 5 h with 1.25 μg of luciferase reporter DNA, 20 ng of plasmid expressing the receptor, and 1.25 μg of appropriate expression plasmid DNA or as indicated in the figure legends using LipofectAMINE 2000 (Invitrogen); 0.1 μg of β-galactosidase expression vector pCMVβ (CLONTECH) DNA was always included as an internal control. Cell extracts were prepared 24 h after transfection and assayed

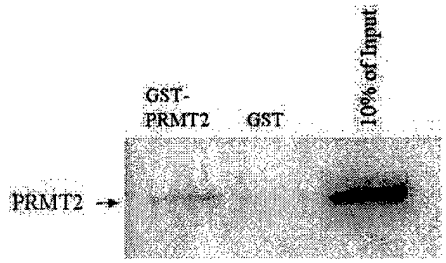


FIG. 6. **PRMT2 forms homodimer or oligodimer.** GST pull-down assay demonstrated that GST-PRMT2 fusion protein but not GST alone retained [$^{35}$S]methionine-labeled PRMT2.

for luciferase and β-galactosidase activities (Tropix). Three independent transfections were performed for each assay.

## RESULTS

*Isolation of PRMT2 as an ERα-binding Protein by Two-hybrid Screening*—Using full-length ERα as a bait in yeast two-hybrid system, we isolated from human mammary gland cDNA library a partial cDNA encoding PRMT2 (amino acids 10–433). To examine the influence of estrogen on the interaction, pACT2-PRMT2, which was isolated by yeast two-hybrid screening and expressed as fusion protein between GAL4 activation domain and PRMT2 (amino acods 10–433), or pACT2 was cotransformed with PGBKT7-ERα expressing fusion protein between GAL4 DNA binding domain and ERα or PGBTKT7 into yeast HF7C. The β-galactosidase activity was measured as an indication of the relative strength of interaction in the presence or absence of ligand. In the absence of ligand, we observed an interaction between ERα and PRMT2 that resulted in a ~40-fold increase in the β-galactosidase activity (Fig. 1). The presence of the ligand estrogen did not significantly affect the interaction between PRMT2 and ERα (Fig. 1).

*Interaction of PRMT2 with ERα and Other Nuclear Receptors in Vitro*—The direct interaction between PRMT2 and ERα was further tested by *in vitro* GST binding assay. The immobilized GST-PRMT2, but not GST alone, retained [$^{35}$S]methionine-labeled ERα both in the presence and absence of estrogen (Fig. 2). Moreover, PRMT2 also showed the ligand-independent in-

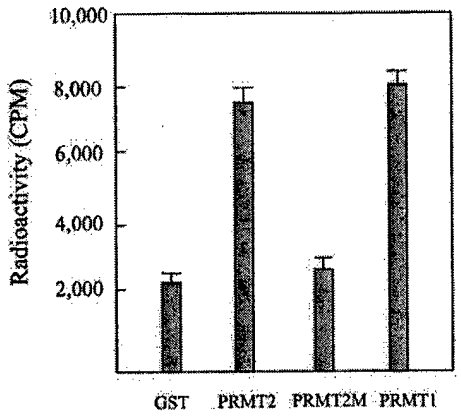


FIG. 7. **PRMT2 binds *S*-adenosylmethionine.** Purified protein was incubated with *S*-adenosyl-L-[*methyl*-$^3$H]methionine. The protein was trapped on a filter and the bound *S*-adenosyl-L-[*methyl*-$^3$H]methionine was quantified by liquid scintillation. GST-PRMT2, just as GST-PRMT1, retained a significant amount of *S*-adenosyl-L-[*methyl*-$^3$H]methionine, while the filter with GST-PRMT2M showed the same background radioactivity as that with GST alone.

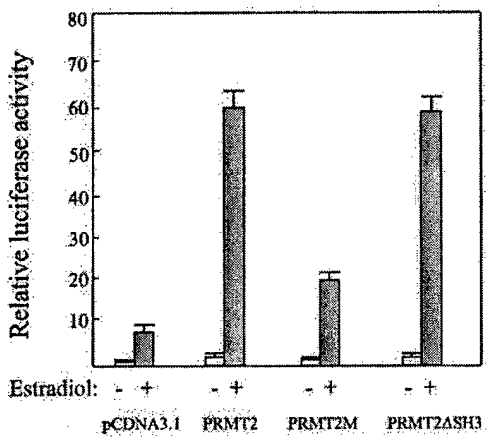


FIG. 8. **PRMT2 enhances ERα-mediated transactivation and its potential methyltransferase activity is pivotal while its SH3 domain is dispensable for this function.** 1.5 μg of PRMT2, PRMT2M, PRMT2ΔSH3 expression vector or control vector PCDNA3.1 was transfected with 1.5 μg of the reporter construct ERE-TK-LUC and 25 ng of PCDNA3.1-ERα into CV-1 cells in the absence or presence of $10^{-7}$ M 17β-estradiol. The activity obtained on transfection of the ERE-TK-LUC without exogenous PPMT2 in the absence of ligand was taken as 1.

teraction with ERβ, PR, TRβ, RARα, PPARγ, and RXRα (Fig. 2).

To determine which region of ERα binds to PRMT2, a GST pull-down assay was performed using fusion proteins between GST and different regions of ERα. As shown in Fig. 3*A*, PRMT2 bound to the AF1 region, DNA binding domain, and hormone binding domain but not to the hinge region. The binding to ERα hormone binding domain was ligand-independent. The interaction between PRMT2 and ERα AF-1 region or DNA binding domain is stronger than that between PRMT2 and ERα hormone binding domain.

PRMT2 contains a Src homology 3 (SH3) domain that binds to proteins with a proline-rich motif and plays a pivotal role in a wide variety of biological processes (38). A GST pull-down assay revealed that PRMT2 with a SH3 domain deletion was still able to bind to the GST-ERα fusion protein but not GST alone. Therefore, this domain is not considered necessary for PRMT2 and ERα interaction (Fig. 3*B*). The region of PRMT2 that interacts with ERα was further defined by GST pull-down assay using different truncated PRMT2 fragments. A fragment from amino acid 133 to 275 was found to interact with ERα (Fig. 3*C*).

*PRMT2 Interacts with ERα in Vivo*—The potential interaction between PRMT2 and ERα in the intact cell was examined by coexpressing ERα and FLAG-tagged PRMT2 in COS-7 cells followed by immunoprecipitation and Western blot analysis. As shown in Fig. 4, PRMT2 interacts with ERα both in the presence and absence of estrogen.

*Interaction of PRMT2 with PRIP, SRC-1, and with PRMT2 Itself*—The potential interaction between PRMT2 and other known nuclear receptor coactivators was investigated using the GST pull-down assay. We detected the interaction of PRMT2 with PRIP and SRC-1 (Fig. 5). No interaction was observed between PRMT2 and PBP or PRMT2 and CBP (Fig. 5).

The methyltransferase PRMT1 is able to form homodimer or homooligomers (39, 40). A GST pull-down assay was performed to see if PRMT2 exhibits this property. GST and PRMT2 fusion protein but not GST alone retained [S$^{35}$]methionine-labeled PRMT2 suggesting that PRMT2 is capable of forming homodimer or homooligomers (Fig. 6)

*PRMT2 Binds S-Adenosylmethionine*—PRMT2 was initially isolated by its protein sequence similarity to other PRMTs and so far no methyltransferase activity has been revealed. Using bacterially expressed GST-PRMT2 fusion protein, we did not demonstrate that PRMT2 was capable of methylating histone and ERα (data not shown). We then tested the ability of PRMT2 to bind the methyl donor *S*-adenosylmethionine by a filter binding assay. Just like PRMT1, PRMT2 was found to be able to bind *S*-adenosylmethionine, whereas PRMT2 with point mutation in the *S*-adenosylmethionine binding motif (41) failed to bind *S*-adenosylmethionine (Fig. 7).

*PRMT2 Potentiates ERα Transcriptional Activity and Its Potential Methyltransferase Activity Is Pivotal While Its SH3 Domain Is Dispensable for This Function*—Having established that PRMT2 is an ERα-binding protein, we investigated the effect of increased levels of PRMT2 upon ERα transcriptional activity in CV-1 cells. The luciferase activity expressed from ERE-TK-LUC that contains one copy of ERE serves as the indicator of the ERα transcriptional activity. Expression of PRMT2 increased the estrogen-dependent transcription of luciferase gene by about 8-fold with minimal effort on basal transcription, which provided evidence that PRMT2 acts as a coactivator for ERα (Fig. 8). However, the mutated PRMT2 that was incapable of binding *S*-adenosylmethionine enhanced the ERα transcriptional activity by about 2.5-fold, which is much less than the 8-fold obtained with wild-type PRMT2 indicating the importance of the potential methyltransferase activity for the role of PRMT2 as a coactivator (Fig. 8). On the other hand, PRMT2 with deletion of the SH3 domain increased ERα activity to the same extent as that for wild-type PRMT2, indicating that the SH3 domain is dispensable for its coactivator function (Fig. 8).

*PRMT2 Increases Both AF-1 and AF-2 Transcriptional Activity of ERα*—As ERα contains the autonomous activation domain AF-1 and ligand-dependent activation domain AF-2, we further examined the effect of increased expression of PRMT2 on their individual activities. The AF-1 (1–184) and AF-2 (251–595) were fused to GAL4 DNA binding domain, respectively, and then cotransfected with GAL4 responsive element-directed luciferase as the reporter gene. In comparison with the GAL4 DNA binding domain alone, AF-1 increased the luciferase activity by about 3-fold. The expression of PRMT2 further increased AF-1-mediated luciferase expression by about 4.5-fold (Fig. 9*A*). Therefore, PRMT2 is able to enhance the ERα AF-1 activity. Just as other nuclear receptors show transcription repression in the absence of their corresponding

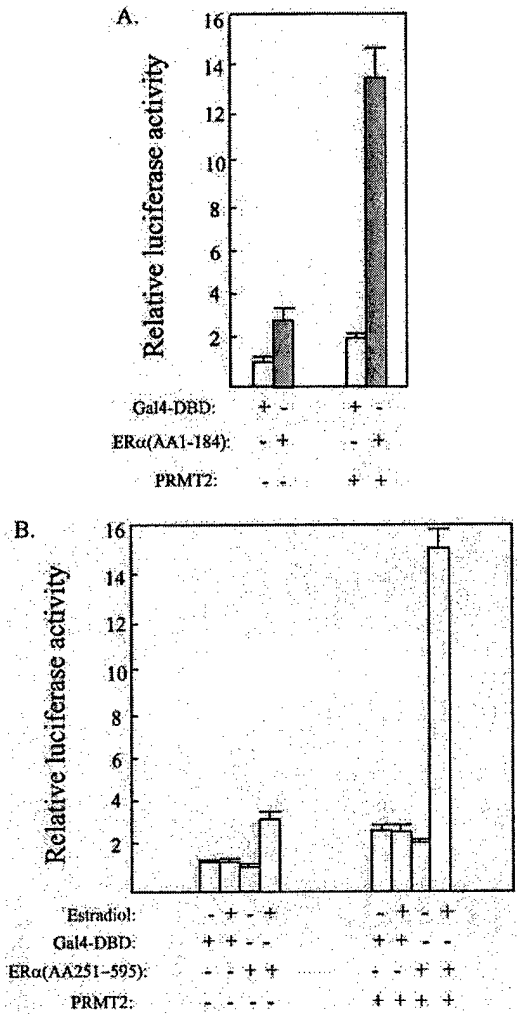


FIG. 9. **The effects of PRMT2 on AF-1 and AF-2 transcriptional activities of ERα.** Gal4-DBD-ERα (1–184) and Gal4-DBD-ERα (251–595) were generated by inserting the corresponding ERα cDNA fragment downstream of the Gal4 DNA binding domain (Gal4-DBD). The Gal4 responsive element-directed luciferase vector (Gal4-TK-LUC) was used as the reporter of activities. *A*, PRMT2 increases AF-1 activity. CV-1 cells were cotransfected with 1.5 μg of GAL-TK-LUC, 25 ng of Gal4-DBD or Gal4-DBD-ERα (1−184), and 1.5 μg of PRMT2 expression vector or control PCDNA3.1. The activity obtained on transfection of GAL-TK-LUC and Gal4-DBD without exogenous PRMT2 was taken as 1. *B*, PRMT2 also increases AF-2 activity. CV-1 cells were cotransfected with 1.5 μg of GAL-TK-LUC, 25 ng of Gal4-DBD or Gal4-DBD-ERα (251−595), and 1.5 μg of PRMT2 expression vector or control PCDNA3.1. The activity from transfection of GAL-TK-LUC and Gal4-DBD without exogenous PPMT2 in the absence of ligand was taken as 1.

ligands, the hormone-dependent AF-2 slightly decreased the luciferase activity without estrogen over the control. The addition of estrogen increased the AF-2-mediated luciferase expression by about 3-fold, which is further enhanced by coexpression of PRMT2 by about 5-fold, demonstrating that PRMT2 also potentiates the AF-2 activity (Fig. 9*B*).

*No Synergistic Enhancement of ERα Activity by Coexpression of PRMT2 and SRC-1 or PRIP*—Given that PRMT2 binds to SRC-1 and PRIP, we sought to determine whether there was synergistic enhancement of ERα activity by PRMT2 and SRC-1 or PRIP. In transient transfection assay with ERα and its reporter gene, PRMT2, SRC-1, and PRIP all enhanced the expression of reporter gene to different levels (Fig. 10). When PRMT2 was cotransfected with either SRC-1 or PRIP, the expression of the reporter gene luciferase was modestly decreased in comparison with PRMT2 alone (Fig. 10). Therefore, there appears no synergistic activation when PRMT2 are coexpressed with either PRIP or SRC-1.

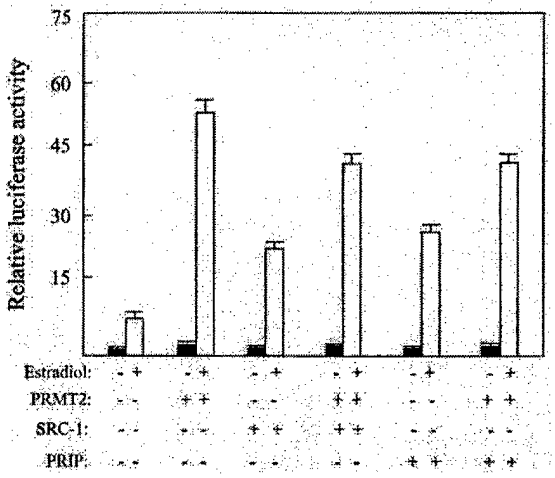


FIG. 10. **No synergistic activation was observed for coexpression of PRMT2 and SRC-1 or PRIP.** CV-1 cells were transfected with 1.5 μg of reporter construct ERE-TK-LUC, 35 ng of PCDNA3.1-ERα, and 0.8 μg of expression vector as indicated in the absence (−) or presence (+) of $10^{-7}$ M 17β-estradiol. Transfection without indicated expression vector was compensated by adding the same amount of PCDNA3.1. The activity obtained on transfection of ERE-TK-LUC without exogenous PPMT2 in the absence of ligand was taken as 1.

*PRMT2 Also Enhances PR, PPARγ, and RARα-mediated Transactivation*—To investigate whether PRMT2 acts as a coactivator for other nuclear receptors, transient transfection assays were performed with PR, PPARγ, and RARα responsive element-directed luciferase gene. PRMT2 enhanced the PR ligand-dependent transcriptional activity by about 8-fold. In comparison with ERα and PR, PRMT2, which also increased the ligand-dependent PPARγ and RARα transactivation by about 5- and 4.5-fold, respectively, showed less effect on PPARγ and RARα transactivation (Fig. 11).

*PRMT2 Contains No Intrinsic Transcriptional Activity*—In an effort to define the mechanism by which PRMT2 acts as a coactivator, we tested if PRMT2 contains intrinsic transcriptional activity similar to that reported with other coactivators such as SRC-1 (42). PRMT2 was linked to the GAL4 DNA binding domain and transfected into CV-1 cells along with GAL4 responsive element-directed reporter gene luciferase. In comparison with GAL4 DNA binding domain alone, GAL4-PRMT2 fusion protein produced no additional activity whereas GAL4-SRC-1 fusion protein increased the luciferase activity by about 7-fold and served as a positive control (Fig. 12). Therefore, PRMT2 does not have intrinsic transcriptional activity.

## DISCUSSION

Using a yeast two-hybrid system with ERα as bait to screen a human mammary gland cDNA library, we isolated PRMT2 as a new ERα-interacting protein. The interaction between PRMT2 and ERα was confirmed by *in vitro* binding and *in vivo* immunoprecipitation assay. A transient transfection assay demonstrated that PRMT2 increased the ERα transcriptional activity. In agreement with the finding that PRMT2 bound to both ERα AF-1 domain and the hormone binding domain, PRMT2 enhanced both ERα AF-1 and AF-2-mediated transactivation. These results established that PRMT2 is a coactivator of ERα. However, unlike other coactivators such as SRC-1 family and CBP that show ligand-dependent binding to the nuclear receptors, PRMT2 binds to ERα both in the presence and absence of estrogen but enhances the ERα activity only

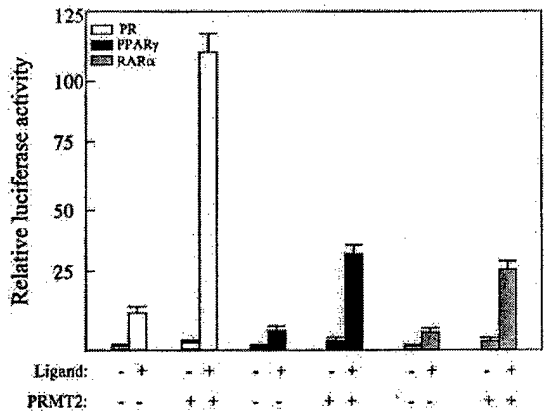


FIG. 11. **PRMT2 also enhances PR, PPARγ, and RARα-mediated transactivation.** CV-1 cells were transfected with 1.5 μg of reporter vector (PRE-TK-LUC for PR, PPRE-TK-LUC for PPARγ, RARE-TK-LUC for RARα), 25 ng of receptor expression vector, and 1.5 μg of control PCDNA3.1 or PRMT2 expression vector in the presence or absence of ligand ($10^{-7}$ M progesterone for PR, $10^{-5}$ M BRL49653 for PPARγ, and $10^{-6}$ M 9-*cis*-retinoic acid for RARα). The activity obtained on transfection of the reporter vector without exogenous PPMT2 in the absence of ligand was taken as 1.

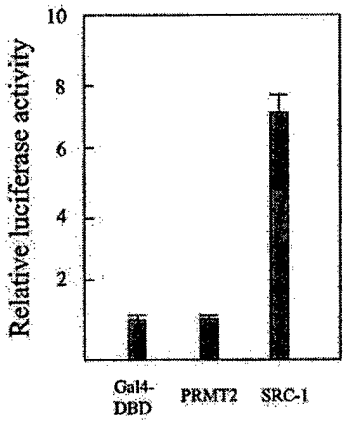


FIG. 12. **PRMT2 does not have intrinsic transcriptional activity.** 1 μg of Gal4-DBD, Gal4-DBD-PRMT2, or Gal4-DBD-SRC-1 was cotransfected with 2 μg of GAL-TK-LUC into CV-1 cells. The activity of luciferase from transfection of GAL-TK-LUC with Gal4-DBD was taken as 1.

with the estrogen. It appears that the interaction between ERα and PRMT2 is not enough for ERα transcriptional activation, which occurs only after the binding of estrogen resulting in most probably the recruitment of other ligand-dependent coactivators.

Two types of PRMT activities have been identified in mammalian cells (31). Type 1 PRMT enzymes including PRMT1, PRMT3, and CARM1 catalyze the formation of monomethylarginine and asymmetric dimethylarginine. Type 2 PRMT enzymes catalyze the formation of monomethylarginine and symmetric dimethylarginine. PRMT5/JBP1 is the only type II enzyme identified so far (43). Based on the protein sequence, PRMT2 was identified as a methyltransferase most probably belonging to type I enzyme, but so far its methyltranferase activity has not been identified (37). Although we demonstrated PRMT2 is capable of binding *S*-adenosylmethionine, we failed to detect any methyltransferase activity using bacterially expressed GST-PRMT2 fusion protein with substrates including histone and ERα (data not shown). A systematic approach to identify the substrates for PRMT2 will be required, and it is also possible that some modification such as phosphorylation or some cofactor may be required for its activity. Nevertheless, the mutation in the conserved PRMT2 binding site for *S*-adenosylmethionine, which would abolish the potential methyltransferase activity, substantially diminished the PRMT2 coactivator function. The finding that PRMT2 does not have any intrinsic transcriptional activity favors the hypothesis that PRMT2 acts by modifying chromatin structure or the transcriptional apparatus through methylation. The elucidation of the substrates will be crucial for the understanding of PRMT2 coactivator function.

PRMT1 and CARM1 are two arginine methyltransferases that have been found to participate in the nuclear receptor transcriptional activation. Both PRMT1 and CARM1 interact with the carboxyl-terminal activation domain of coactivator GRIP1 and are able to methylate histones H4 and H3, respectively. PRMT1 or CARM1 enhance the nuclear receptor activity mildly by itself but substantially when coexpressed with GRIP1, suggesting that PRMT1 and CARM1 act as the secondary coactivators that are recruited by the first coactivator to modify the chromatin structure. Instead, PRMT2 strongly enhances the ERα transcriptional activity by direct interaction with ERα. Although PRMT2 was found to interact with other coactivators SRC-1 and PRIP, no synergistic activation was found with coexpression of PRMT2 and PRIP or SRC-1. The coexpression of SRC-1 or PRIP even modestly decreased PRMT2 coactivation. Therefore, although PRMT2 is a protein arginine methyltransferase highly homologous to PRMT1 and CARM1, PRMT2 may have a very different mechanism by which it acts as a coactivator, possibly because it has different substrates involved in transcriptional activation.

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# Coactivator PRIP, the Peroxisome Proliferator-activated Receptor-interacting Protein, Is a Modulator of Placental, Cardiac, Hepatic, and Embryonic Development*

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**Yi-Jun Zhu‡, Susan E. Crawford‡, Veronica Stellmach‡, Rama S. Dwivedi§, M. Sambasiva Rao‡, Frank J. Gonzalez¶, Chao Qi‡, and Janardan K. Reddy‡‖**

*From the ‡Departments of Pathology and §Pediatrics, Northwestern University, the Feinberg School of Medicine, Chicago, Illinois 60611-3008 and ¶The Laboratory of Metabolism, NCI, National Institutes of Health, Bethesda, Maryland 20892*

**Nuclear receptor coactivator PRIP (peroxisome proliferator-activated receptor (PPARγ)-interacting protein) and PRIP-interacting protein with methyltransferase activity, designated PIMT, appear to serve as linkers between cAMP response element-binding protein-binding protein (CBP)/p300-anchored and PBP (PPARγ-binding protein)-anchored coactivator complexes involved in the transcriptional activity of nuclear receptors. To assess the biological significance of PRIP, we disrupted the PRIP gene in mice by homologous recombination. Mice nullizygous for PRIP died between embryonic day 11.5 and 12.5 (postcoitum) due in most part to defects in the development of placenta, heart, liver, nervous system, and retardation of embryonic growth. Transient transfection assays using fibroblasts isolated from PRIP$^{-/-}$ embryos revealed a significant decrease in the capacity for ligand-dependent transcriptional activation of retinoid X receptor α and to a lesser effect on PPARγ transcriptional activity. These observations indicate that PRIP like PBP, CBP, and p300 is an essential and nonredundant coactivator.**

Our understanding of the mechanisms underlying transcriptional activation by nuclear receptors has been advanced by the identification of nuclear receptor coactivators or coregulators that appear to influence embryonic development, cell proliferation, and differentiation (1). These include p160/SRC-1[1] (steroid receptor coactivator-1) family with three members (SRC-1, TIF/GRIP1/SRC-2, and pCIP/AIB1/ACTR/RAC3/TRAM1/SRC3) (2–6), CREB-binding protein (CBP) (7), adenovirus E1A-binding protein p300 (8), peroxisome proliferator-activated receptor-γ (PPARγ)-binding protein (PBP) (9), PPAR-interacting protein (PRIP/ASC-2/RAP250/TRBP/NRC) (10–14) and PPARγ coactivator-1 (PGC-1) (15), among others. Nuclear receptor coactivators contain one or more conserved LXXLL (where L is leucine and *X* any amino acid) signature motif, which has been found to be necessary and sufficient for ligand-dependent interactions with the activation function-2 domain present in the C-terminal hormone-binding region of the nuclear receptors (1, 6). It is generally held that coactivators play a central role in mediating nuclear receptor transcriptional activity by functioning as at least two large multiprotein complexes formed either sequentially or combinatorially (1). The first complex anchored by CBP/p300 and containing p/160 cofactors/SRC-1 cofactors exhibits histone acetyltransferase activity necessary for remodeling chromatin (1, 4, 7, 16), while the second multiprotein complex, variously referred to as TRAP/DRIP/ARC mediator complex, which is anchored by PBP (17–19), facilitates interaction with RNA polymerase II complexes of the basal transcription machinery (1). Deletion of CBP/p300 and PBP genes in the mouse results in embryonic lethality around E11.5 days, indicating that disruption of these pivotal anchoring coactivators affects the integrity of the cofactor complexes, thus altering the function of many nuclear receptors and most likely of other transcription factors (20–24).

Of interest is that the recently identified coactivator designated PRIP/ASC2/RAP250/NRC/TRBP has also been shown to interact with several nuclear receptors and with CBP/p300 and TRAP130 of the TRAP/DRIP/ARC complex (10–14). Thus, PRIP appears to serve as a bridge between the first complex anchored by CBP/p300 and the downstream TRAP/DRIP/ARC mediator complex anchored by PBP. Furthermore, the recently isolated PRIP-interacting protein with RNA methyltransferase activity, designated PIMT (25), forms a complex with CBP, p300, and PBP (26), further attesting to the possibility that two major multiprotein cofactor complexes anchored by CBP/p300 and PBP, respectively, merge into one megacomplex on DNA template (28). Since PRIP and PRIP-binding protein PIMT appear to link the two cofactor complexes under *in vitro* conditions, we have found it necessary to explore the biological function of PRIP by generating mice with PRIP null phenotype. We now demonstrate that PRIP is critical for the embryonic development, since disruption of the PRIP gene in the mouse leads to embryonic lethality around E11.5 to E12.5 days, implying that PRIP (like CBP/p300 and PBP) is also critical for embryonic development and survival.

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‖ To whom correspondence should be addressed: Dept. of Pathology, Northwestern University, the Feinberg School of Medicine, 303 East Chicago Ave., Chicago, IL 60611-3008. Tel.: 312-503-8144; Fax: 312-503-8249; E-mail: jkreddy@northwestern.edu.

[1] The abbreviations used are: SRC-1, steroid receptor coactivator-1; PPAR, peroxisome proliferator-activated receptor; PRIP, PPAR-interacting protein; PBP, PPAR-binding protein; PIMT, PRIP-interacting protein with methyltransferase activity; RXR, retinoid X receptor; PPRE, peroxisome proliferator response element(s); RAR, retinoic acid receptor; RARE, retinoic acid response element; CREB, cAMP response element-binding protein; CBP, CREB-binding protein; TRAP, thyroid hormone receptor-associated protein(s); DRIP, vitamin $D_3$ receptor-interacting proteins(s); ARC, activator-recruited cofactor; PRIC, PPARα-interacting cofactor complex; ES cells, embryonic stem cells; RT, reverse transcriptase; PCNA, proliferating cell nuclear antigen; MEF, mouse embryonic fibroblast.

## MATERIALS AND METHODS

*Construction of Targeting Vector*—The genomic DNA fragment containing the full-length PRIP gene was isolated from a mouse 129/Sv P1 bacteriophage library (Genome Systems, St. Louis, MO), using polymerase chain reaction with primers 5′-CAATGCAGCCTGTTCCTGT-3′ and 5′-GCTGCTGCATCACCATGAAA-3′ designed from mouse PRIP cDNA sequence (10). A Cre-LoxP system was employed to delete exon 7 from the PRIP gene. For this purpose, we constructed a triple-LoxP PRIP targeting vector to generate a "floxed" mouse PRIP-targeted locus.

*Generation of PRIP Null Mice*—The targeting vector was linearized and electroporated into HM1 embryonic stem (ES) cells (28). Transfected ES cells were selected in the medium with G418 (200 μg/ml), and the surviving colonies were screened for homologous recombination by PCR with primers P1 5′-CCTACAGCTGCAAGCAAATC-3′ and P2 5′-TATACGAAGTTATGCGGCCG-3′. ES cells with the appropriate PRIP floxed targeted locus were further confirmed by Southern blot analysis (Fig. 1*B*), and the euploid selected ES cells were used for injection into 3.5-day-old blastocysts derived from C57/B6 mouse by the Northwestern University Targeted Mutagenesis Facility to generate chimeric mice. Chimeric male mice were bred with wild type C57/B6 female to produce heterozygous mice, which were then crossed with EIIa-Cre transgenic mice (29) to delete the DNA fragment between LoxP1 and LoxP3. EIIa-cre-mediated recombination occurs early in development (2–8 cells), and mice carrying the allele with deletion were crossed with wild type C57/B6 to achieve germ line transmission. The heterozygous mice with expected deletion were interbred to generate homozygous mutants.

*Genotyping of Mice and Embryos*—DNA extracted from the tail tips of mice and from the yolk sac of embryos was used for genotyping by PCR. The mice carrying the recombination between LoxP1 and LoxP3 on one allele were identified by PCR with primers P1/P2 and P3 (5′-CGGCCGCATAACTTCGTATA-3′)/P4 (5′-TTCTTCTTCCGAGGCGGTTT-3′). Presence of P1/P2 product, while lacking P3/P4 amplification, indicated the deletion of PRIP gene on one allele. The homozygosity for the deletion was detected by the absence of exon 7 as ascertained by PCR with primers P5 5′-ACGGGCCACCAAATATGATG-3′ and primer P4 (see above) (Fig. 1*C*).

*RT-PCR and Western Blots*—For RT-PCR, total RNA was extracted from embryos with TRIzol reagent (Invitrogen). Primers 5′-CCTACAGCTGCAAGCAAATC-3′ and 5′-CGAACATGCTGCATGAGCTGA-3′ were used to amplify the region between exon 6 and exon 8 from the PRIP homozygous mutant. To detect PRIP protein in embryos, whole cell lysates were prepared from the embryos by homogenization and probed with anti-mouse PRIP antiserum. The signal was detected by ECL detection system.

*Histological Analysis and Immunohistochemistry*—Age-matched embryos were fixed in paraformaldehyde or 10% of buffered formalin, embedded in paraffin, serially sectioned at 5-μM thickness in sagittal or transverse planes, and stained with hematoxylin and eosin. Immunohistochemical staining for the localization of proliferating cell nuclear antigen (PCNA) was performed using a standard avidin-biotin-peroxidase complex protocol as described previously (24). Giemsa stain was done using the standard protocol.

*Isolation of Fibroblasts from Embryos and Transfection of Primary Fibroblasts*—Mouse embryonic fibroblasts (MEF) were isolated from E11.5 embryos and cultured in Dulbecco's modified Eagle's medium with 10% of fetal bovine serum as described (22). For transfection, $2 \times 10^5$ of MEFs were plated in six-well plates for overnight culture. The transfections were carried out with LipofectAMINE-2000 Plus reagent according to manufacturer's instruction (Invitrogen). Plasmids pCMV-PPARγ, PPRE-TK-LUC, pCMV-RXR, RXRE-TK-LUC, pCMV-RAR, and RARE-TK-LUC were as described previously (10, 25, 26). β-Galactosidase expression vector pCMVβ was used as a cotransfectant, which served as control for transfection efficiency. Cell extracts were prepared 36 h after transfection and were assayed for luciferase and β-galactosidase activities.

## RESULTS

*Disruption of PRIP Gene in Mice*—We constructed a conditional knock-out allele of PRIP by using the Cre/*loxP* recombination system according to the strategy in Fig. 1*A*. To generate conventional PRIP knock-out mice (in which the gene is permanently inactivated at the germ cell stage), it was necessary to induce recombination between loxP sites. To achieve heterozygosity, PRIP-targeted mice were bred with homozygous Cre transgenic mouse line, EII-cre (29). The EII-cre mice carry

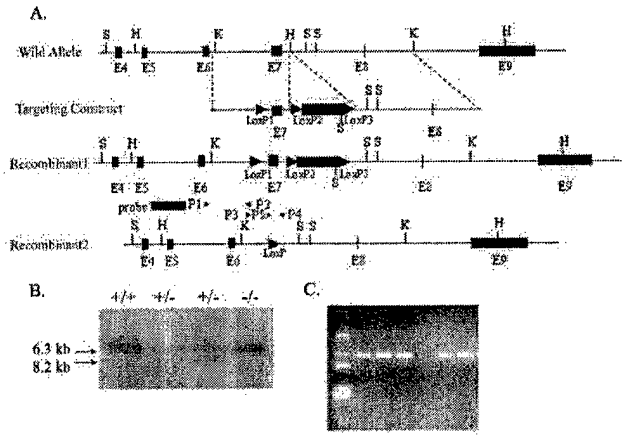


FIG. 1. **Disruption of mouse PRIP gene.** *A*, schematic drawing of the targeting vector and the recombinant alleles. Restriction sites *SpeI* (*S*), *Hin*dIII (*H*), *Kpn*I (*K*), and the location of primers and probes used for ES cell screening and mouse genotyping are indicated. *Dashed lines* show the regions of homologous recombination between the vector and the endogenous gene. *B*, Southern blot analysis of genomic DNAs from ES cells is digested with *SpeI* and hybridized to show PRIP wild type allele (6.3 kb) and recombinant allele (8.2 kb). *C*, DNA from mice derived from heterozygous mating are screened by PCR with primers P4/P5 to detect homozygous deletion of exon 7 (*lane 4*).

the Cre transgene under the control of the adenovirus EIIa promoter and express Cre recombinase only in early mouse embryos (2–8 cell stage), and it induces the recombination between the two loxP sites with the same orientation (29). We detected the expected, all types of recombinants among the offspring (29). The recombination between loxP1 and loxP3 resulted in the deletion of PRIP exon 7 and a reading frameshift to generate a stop codon right after the fusion between exon 6 and exon 8. The chimeras were crossed to wild type mice to produce heterozygous mice carrying one recombinant PRIP allele, and the homozygous mice were obtained from heterozygous mating. By sequencing the RT-PCR products, exon 7 was not found in mRNA transcribed from the recombinant PRIP allele, and the reading frameshift was introduced by the deletion leading to a premature stop codon (data not shown). As the result, only 488 amino acids at the N terminus containing no L*XX*LL motif can possibly be translated from the mRNA, but no PRIP protein was detected by Western blot analysis (data not shown).

*Embryonic Lethality and Growth Retardation of PRIP Null Mice*—Among 26 new-born pups, and 54 mice that were 3 weeks old generated from intercrosses between heterozygous PRIP mutant mice, no homozygous mutants were detected. Genotyping the embryos at different stages of gestation showed that no PRIP null embryos survived beyond E13.5 (13.5 days postcoitum). However, heart beating was observed among the majority of viable PRIP null embryos recovered between E11.5 and E12.5 and few were moribund or dead. These observations indicate lethality occurred in a relatively narrow window of time as no viable PRIP null embryos were seen at E13.5. The viable $PRIP^{-/-}$ embryos recovered at E11.5 and E12.5 exhibited clear evidence of growth retardation compared with the wild type and heterozygous littermates. $PRIP^{-/-}$ embryos appeared strikingly different at the gross level at E12.5, they were pale and smaller in size than their $PRIP^{+/+}$ and $PRIP^{+/-}$ littermates (Fig. 2, *A* and *B*). Normally, extraembryonic mesoderm of the yolk sac gives rise to blood and endothelial cells, which form blood islands. The extraembryonic membrane covering PRIP null embryos contained fewer vessels in contrast to wild type yolk sac with its well developed blood vessels (Fig. 2*B*). In addition, superficial vasculature was less obvious in

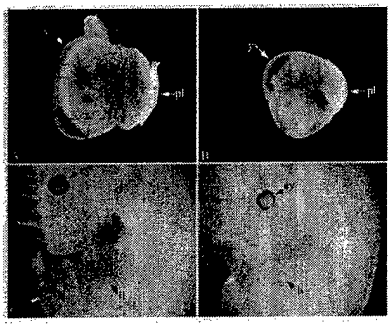

FIG. 2. **PRIP$^{-/-}$ mutants are developmentally retarded.** Wild type (*A*) embryo at E12.5 reveals yolk sac with well developed blood islands, while the PRIP$^{-/-}$ littermate (*B*) is smaller in size with fewer blood islands in its yolk sac (*ys*). *C*, external appearance of eye, blood-enriched liver, and distal part of hind limb with separated fingers (*white arrow*) in wild type embryo. *D*, PRIP$^{-/-}$ embryo shows dilated leaking small vessels (white arrowhead) and few large blood vessels. Pigmented tissue is thinner in the dorsal portion of the eye (*ey*). The liver (*li*) mass is small and pale in the PRIP$^{-/-}$ embryo. Finger separation does not occur in the distal part of hind limb (*white arrow*). Each pair of photographs was taken at the same magnification.

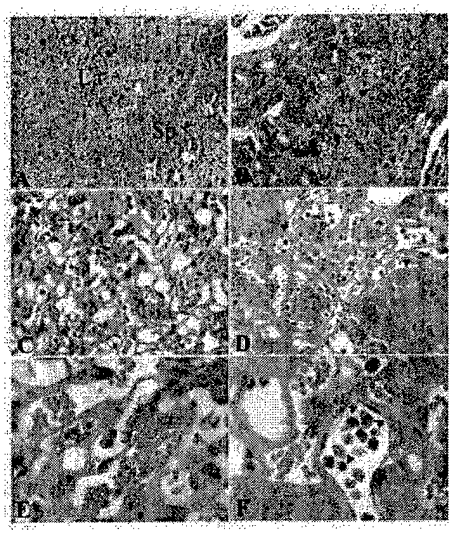

FIG. 3. **Frontal sections of placenta from E12.5 wild type (*A*, *C*, and *E*) and PRIP$^{-/-}$ mutant (*B*, *D*, and *F*) embryos.** *A*, H&E staining of wild type placenta shows labyrinth region (*La*) with extensive well developed fetal vessels surrounded by blood sinuses containing enucleated maternal erythrocytes. The spongiotrophoblast layer (*Sp*) is distinct in wild type placenta. *B*, in PRIP$^{-/-}$ placenta, maternal blood sinuses are generally absent in most areas of labyrinth zone. No organized spongiotrophoblast layer is present. *C* and *D*, close-up view of labyrinth region shows cells with multinucleus clusters in PRIP$^{-/-}$ placenta (*black arrowhead*). Cellularization is less extensive compared with the control. Nucleated fetal erythrocytes in the PRIP$^{-/-}$ placenta (*black arrow*) exhibit irregularly shaped nuclei and have scant cytoplasm compared with control.

PRIP null mutants (Fig. 2*D*). While the liver of wild type embryos was easily visualized by its rich vasculature through the skin, only a pale primitive liver bud was discerned in PRIP$^{-/-}$ embryos (Fig. 2*D*). Failure of palatal shelf to fuse, abnormal finder separations, and developmental abnormalities in brain were detected in PRIP$^{-/-}$ mutants (not illustrated).

*Lack of Organized Spongiotrophoblast Layer in PRIP Mutant Placenta*—In wild type placenta at E12.5 contains three distinct trophoblast cell structures: the innermost labyrinthine layer, the intermediate spongiotrophoblast layer, and the outermost trophoblast giant cell layer. The labyrinthine zone formed by the fusion of chorion with allantois is composed of extensively branched fetal blood vessels and maternal blood sinuses among strands of diploid trophoblast cells that separate the maternal blood sinuses from fetal blood vessels. In PRIP null placenta, no compact layer of spongiotrophoblast cells was observed between labyrinth zone and trophoblast giant cell layer. Instead, islands of spongiotrophoblast-like cells dissociated from trophoblast giant cell layer and migrating into the labyrinth zone were common occurrence in PRIP$^{-/-}$ placenta (Fig. 3*B*). In wild type placenta blood sinuses are filled with maternal blood cells throughout the labyrinthine layer (Fig. 3, *A*, *C*, and *F*), whereas most of the tortuous vessels in the PRIP$^{-/-}$ placenta were enlarged, ruptured, and generally empty (Fig. *3*, *B*, *D*, and *F*). While the chorioallantoic fusion appeared to occur in PRIP mutant placenta, chorionic trophoblast cells clustered in labyrinth had multiple nuclei, and these clusters showed insufficient blood vessel branching. These changes are reminiscent of some of the placental defects observed in PBP null mutants (22, 24). However, changes in PRIP$^{-/-}$ placenta appeared less profound when compared with PBP$^{-/-}$ placenta (24). Nucleated fetal erythroblasts in PRIP$^{-/-}$ placenta had irregular shaped nuclei with very little cytoplasm (Fig. 3*F*). Trophoblast cell proliferation in PRIP null placenta as assessed by PCNA immunostaining was significantly lower than that observed in PRIP$^{+/+}$ placenta (data not shown).

*PRIP$^{-/-}$ Embryos Manifest Cardiac Defects*—Inefficient pumping by the heart leading to circulation failure is one of the major causes of embryonic lethality during middle gestation. The development of heart requires coordinated differentiation of several embryonic lineages, including the myocytes of myocardium, the endothelial cells of the endocardium, and the cells of the neural crest that form the outflow tract. At E12.5, the heart of PRIP$^{-/-}$ embryos exhibited defects involving all three

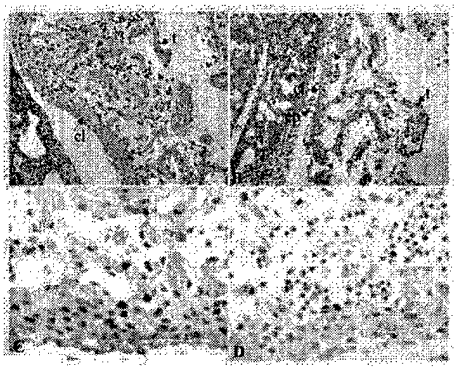

FIG. 4. *A* and *B*, histological analysis of sagittal sections of heart ventricle from E12.5 of wild type (*A*) and PRIP$^{-/-}$ (*B*) embryos. Trabeculation (*t*) is less extensive in PRIP$^{-/-}$ embryo than in the wild type littermate. The compact layer (*cl*) of the mutant is significantly thinner. Epicardium (*ep*) in mutant is separated from underlying myocardium. In some regions of compact layer of PRIP$^{-/-}$, the adhesion of myocytes was disrupted and red blood cells penetrated through myocardium (*arrowheads*). *C* and *D*, PCNA staining for the proliferating cells in ventricles of E12.5 wild type (*C*) and PRIP$^{-/-}$ (*D*) embryos. Fewer *dark brown* PCNA-positive cells are present in the compact layer of PRIP$^{-/-}$ ventricle.

lineages (Fig. 4, *A* and *B*). In PRIP$^{-/-}$ heart, the epicardium, consisting of a single layer of mesothelial cells lining against the compact layer of myocardium, appeared to separate from underlying myocardium and this space is filled with blood cells (Fig. 4*B*). Pericardial space surrounding the heart in mutant was much smaller than their wild type littermate. Unlike the ventricles in wild type heart, which consisted of multicell thick compact layer with well developed trabeculae, the ventricles in PRIP$^{-/-}$ heart only contained one or two cell layers of myocardium (Fig. 4). Cell-cell adhesion among cardiocytes appeared to

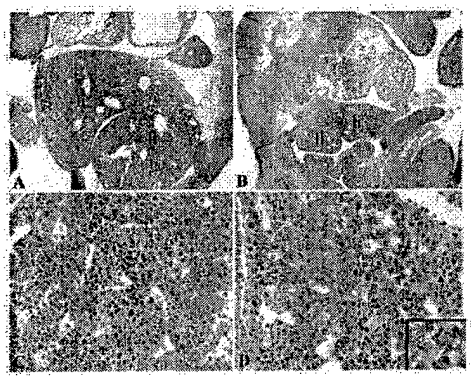


FIG. 5. **H&E staining of liver sections from wild type and PRIP$^{-/-}$ embryos.** At low modification, liver (*li*) in PRIP$^{-/-}$ embryo (*B*) is greatly reduced in size than that of wild type liver (*A*). Liver of mutant contains large numbers of megakaryocytes (*white arrowhead*), fewer of erythrod progenitors (*black arrowhead*), and many cells with apoptotic bodies (*white arrowhead*), in contrast to the normal liver (*C*). The *inset* in *D* shows a closer view of the apoptotic cells in liver PRIP$^{-/-}$ liver.

be defective, which possibly contributes to leakage of blood cells into epicardial spaces. Cell proliferation in PRIP$^{-/-}$ myocardium was markedly reduced as compared with PRIP$^{+/+}$ myocardium (Fig. 4, *C* and *D*). The failure of ventricular myocardium of PRIP$^{-/-}$ embryos to stratify into the multilayer compact structure required to sustain adequate cardiac function appears similar to that noted in PBP null and PPARγ null mutants (22, 24, 30).

*Defective Hepatopoiesis and Hepatic Hematopoiesis in PRIP$^{-/-}$ Mutants*—Liver of PRIP null mutants appeared considerably smaller in size when compared with their littermates (Fig. 5, *A* and *B*). The function of the liver at E12.5 days is to become the major site of hematopoiesis so as to gradually replace yolk sac based hematopoiesis. Histological examination of PRIP$^{-/-}$ liver revealed reduction in hepatocyte population and an increase in hepatocyte apoptosis (Fig. 5*D*). A marked decrease in the number of erythroid progenitors was also evident, and these cells had large nuclei with scant minimally hemoglobinized cytoplasm. Liver exhibited large numbers of megakaryocytes.

*Differential Reduction of Transactivation by Nuclear Receptors in PRIP$^{-/-}$ Primary Fibroblasts*—To assess the impact of loss of PRIP on transcriptional activities of nuclear receptor, we isolated MEFs from PRIP$^{-/-}$ and PRIP$^{+/+}$ embryos. They were used for assaying the transcriptional activities of PPARγ, RXR, and RAR. In wild type MEFs transfected with a RXR expressing vector and RXR-responsive element-linked reporter, the addition of RXR-ligand 9-*cis*-retinoic acid induced marked increase (~76-fold) in the transcription (Fig. 6*A*). In PRIP$^{-/-}$ MEFs, the induction of ligand-mediated RXR transcription was markedly reduced (~3-fold). Transcription assays with PPARγ (Fig. 6*B*) and RARα (data not shown) showed that the influence of PRIP was only modest. These results demonstrated that nuclear receptors require PRIP to achieve their full transcriptional potential, although the contribution of PRIP to the activities of the three nuclear receptors examined here differed somewhat in that PRIP seemed to influence RXR maximally.

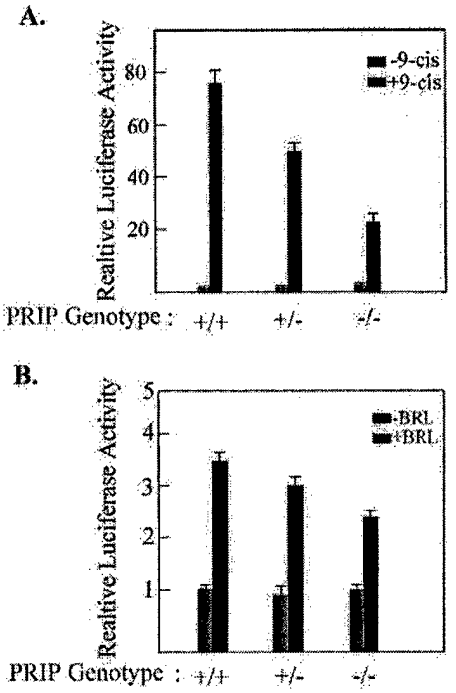


FIG. 6. **Attenuated transcriptional activity of nuclear receptors in PRIP$^{-/-}$ MEFs.** MEFs isolated from E11.5 wild type, heterozygous, and homozygous embryos were cotransfected with 1.5 μg of reporter constructs, 20 ng of vectors expressing nuclear receptors, and 0.1 μg of pCMVβ in the presence or absence of the ligands. The activities of the reporter obtained from transcfection with MEFs from wild type embryos in the absence of ligand was taken as 1. The transfections performed with RXR/RXRE (*A*), PPARγ/PPRE (*B*), and RAR/RARE (not illustrated). The results are the mean ± S.D. of three independent transfections and are normalized to the internal controls of β-galactosidase expression.

## DISCUSSION

The nuclear hormone receptors comprise a superfamily of transcription factors that regulates coordinated expression of gene networks involved in developmental, physiological, and metabolic processes (1). Notable among this nuclear receptor superfamily is PPAR subfamily comprising of three isoforms, PPARα, PPARγ, and PPARβ/δ, since these receptors have emerged in recent years as a critical player in regulating energy metabolism. In an effort to understand the factors controlling cell and gene specific transcriptional events initiated by nuclear receptors, the ligand-binding domain of nuclear receptors was used in the yeast two-hybrid screen to identify receptor interacting proteins (2–7, 31). During the past 7 years, more than 25 nuclear receptor coactivators have been cloned raising the issue of redundancy, since these coactivators generally appear promiscuous in their coactivation potential.

To fully appreciate the *in vivo* biological functions of these coactivators, molecular genetic approaches are being increasingly employed. Previous studies have demonstrated that mice lacking SRC-1 or p/CIP/SRC-3 are viable and manifest either partial or full redundancy for certain nuclear receptor actions (32–36). In contrast, deletion of more general coactivators such as CBP/p300 and PBP in mice leads to embryonic lethality implying that these are essential coactivators (20–24). Thus, there appear to be at least two broad classes of coactivators: essential and redundant. Our observations reported here now add coactivator PRIP to the class of essential coactivators because of the embryonic growth retardation, defects in placental, cardiac and hepatic development, and embryonic lethality. Embryonic lethality was noted between E11.5 and E12.5 days with no viable embryos at E13.5. Defects were noted in heart (reduced amount of myocardium and noncompaction), liver (small liver with reduction in hepatocyte population, and hepatocyte apoptosis), and defects in erythropoiesis (reduced hemoglobinization) and placenta (maturation block of trophoblast with vascularization defect). The placental defects, although not as pronounced as those encountered in PBP$^{-/-}$ placenta (22, 24),

nevertheless compromise fetal-maternal exchange of nutrients, growth factors, and oxygen, which may contribute to embryonic growth retardation. The death of embryos at about mid-gestation has been reported in a number of null mutants induced by gene targeting, including PPARγ (30), RXRα (37), PBP (22–24), and N-myc (38).

It should be noted that we isolated both PRIP and PBP using PPARγ as bait in the yeast two-hybrid screen and identified them as nuclear receptor coactivators (9, 10). PBP has since emerged as a central piece in large TRAP-DRIP-ARC-PRIC multiprotein cofactor complex (17–19, 27). Recent studies have established that PBP is indispensable for embryonic development because PBP null mutation leads to embryonic death around E11.5 of mouse development (22–24). PBP null mutation also causes defects in the development of placental vasculature similar to those encountered in PPARγ mutants, supporting the requirement of PBP for PPARγ function *in vivo* (22, 24, 30). PBP null mutants also exhibited cardiac failure because of noncompaction of the ventricular myocardium and resultant ventricular dilatation (22–24). There was also paucity of retinal pigment, excessive systemic angiogenesis, a deficiency in the number of megakaryocytes, and an arrest in erythrocyte differentiation (24). We showed that PBP interacts with GATA family of transcription factors and thus influences the development of vital organ systems (24). Consistent with this view is that the gene encoding PBP is amplified and overexpressed in breast cancer (39). Like PBP, PRIP is also highly amplified/overexpressed in human breast and colon tumors (11), suggesting that both PBP and PRIP by virtue of their coactivating function may augment cell proliferation and neoplastic progression. Finally, the PRIP null MEFs exhibited marked repression of RXR-mediated transcriptional activity as compared with PPAR and RAR. These observations strongly suggest that PRIP has better preference for RXR than other nuclear receptors, and some of the abnormalities noted in PRIP null mutants may be due to inhibition of RXR function. Further studies are needed to examine the role of PRIP in various tissues by generating PRIP conditional null mice.

*Addendum*—Deletion of the AIB3 (ASC-2) gene has been described recently (40).

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# Null mutation of PRIP in mammary gland causes defective mammopoiesis

Chao Qi, Papreddy Kashireddy, Yiwei Tony Zhu, Sambasiva M. Rao, and
Yi-Jun Zhu[¶]

*From the Department of Pathology,*
*Northwestern University,*
*Feinberg School of Medicine,*
*Chicago, Illinois 60611*

**Running title**: Mammary gland, PPAR interacting protein, estrogen receptor, progesterone receptor.

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[¶]To whom correspondence should be addressed:
Dept. of Pathology,
Feinberg School of Medicine,
Northwestern University
303 East Chicago Avenue
Chicago, IL 60611-3008
Tel.: 312- 503- 3113
Fax: 312- 503- 8249
E-mail: y-zhu2@northwestern.edu

[1]The abbreviations used are: ER, estrogen receptor; PR, progesterone receptor; WAP, whey acidic protein; PPAR, peroxisome proliferator-activated receptor; TUNEL, terminal deoxynucleotidyl transferase mediated-dUTP nick end labeling; SRC-1, steroid receptor coactivator-1; PRIP, PPAR Interacting Protein.

## ABSTRACT

To investigate the role of nuclear receptor coactivator PPAR-interacting protein (PRIP) in mammary gland development, we generated a conditional null mutation of PRIP in mammary gland. In PRIP deficient mammary gland, the elongation of ducts during puberty was not affected but the number of ductal branches was decreased, which persisted long after puberty, indicating the potential of ductal branching was impaired. During pregnancy, PRIP deficient mammary gland exhibited decreased alveolar density. The lactating PRIP deficient gland contained scant lobuloalveoli with many adipocytes while wild type gland was composed virtually of no adipocytes but mostly lobuloalveoli. As a result, PRIP mammary deficient gland could not produce enough milk to nurse all pups during lactation. The ductal branching of mammary gland in response to estrogen treatment was attenuated in PRIP mutant gland. While proliferation index was similar between wild type and PRIP deficient gland, increased apoptosis was observed in PRIP deficient gland. PRIP deficient gland expressed increased amphiregulin, TGFα, and betacellulin mRNA as compared to wild type gland. The differentiated function of PRIP deficient mammary epithelial cells was largely intact, as evidenced by the expression of abundant β-casein, WAP and WNDN1 mRNA. We conclude that PRIP is important for the normal mammary gland development.

## INTRODUCTION

The multiple-step mammary gland development is controlled by a combination of peptide and steroid hormones (1, 2). During puberty, the accelerated ductal growth which finally fills the entire fat pad is stimulated by estrogen. While the exact down-stream signals responsible for estrogen-stimulated ductal growth remain elusive, analysis of gene knock out mice suggests that estrogen receptors (ER) in both the stromal and epithelial cells, are required for optimal ductal growth under physiological conditions (3, 4). During pregnancy, mammary epithelial cells undergo an extensive alveolar proliferation. The early lobuloalveolar proliferation requires both estrogen and progesterone, while prolactin is responsible for the mammary gland development during late pregnancy and lactation (5, 6). Genetic ablation and reciprocal transplantation experiments suggest that the presence of progesterone receptors (PR) in mammary epithelial cells but not in stromal cells, is essential for alveolar branching and that progesterone receptors act in a paracrine fashion (7-9). Besides their essential role in normal mammary gland development, steroid hormones also play an important role in breast cancer development (10). The over-exposure to estrogen is associated with increased risk of breast cancer (11). The pregnancy provides certain protection against breast cancers probably through formation of secretory alveoli (12).

ER and PR are members of nuclear receptor super-family. Recent studies established that nuclear receptors require a combination of nuclear receptor coactivators for their transcriptional functions (13, 14). Coactivators, such as SRC-1 and CBP, harboring histone acetyltransferase activities, act by modifying the chromatin structure (15, 16), while coactivator PBP serves as the anchor for a large multisubunit protein complex, which facilitates the transcriptional initiation (17, 18). PPAR interacting protein (PRIP/ ASC-2/RAP250/TRBP/NRC) is one of the nuclear receptor coactivators isolated by our lab and others (19-23). PRIP interacts with PIMT (PRIP-interacting protein with RNA methyltransferase activity) and its function is stimulated by PIMT (24, 25). PRIP is a component of a large protein complex including trithorax group proteins ALR-1, ALR-2, HALR and ASH-2, and retinoblastoma-binding protein RBQ-3 (26). In addition, PRIP was found to be amplified and overexpressed in some breast cancers and could play a role in tumorigenesis (19). It would be important to elucidate the physiological function of PRIP in mammary gland development and its contribution to estrogen and progesterone signaling pathways during mammary gland development. However, PRIP null mutation is embryonic lethal (27-29), which prevents the analysis of the role of PRIP in mammary gland development.

Here we report the generation of PRIP null mutation in mammary gland. PRIP deficient mammary gland exhibits decreased number of ductal branches, impaired lobuloalveolar development, severely defective lactation, and attenuated ductal branching of mammary gland in response to estrogen treatment

with relatively intact expression of milk gene proteins. These results suggest that PRIP is an important coactivator for the normal mammary gland development.

## MATERIALS AND METHODS

*Generation of PRIP conditional null mutation in mammary gland-* MMTV-Cre transgenic mice (30) was obtained from the MMHCC Repository at NCI-Frederick. The heterozygous loxP-PRIP mice (27) were bred with MMTV-Cre transgenic mice to delete the DNA fragment between LoxP1 and LoxP3. The heterozygous mice with expected deletion were interbred to generate homozygous mutants.

*Whole-mount, histology and Immunostaining*-Whole-mount examination of the inguinal mammary gland was performed as reported previously (31). Briefly, mammary glands were fixed in Carnoy's fixative and stained overnight in hematoxylin. Samples were then cleared in xylene and mounted. For routine histological assessment, formalin-fixed mammary glands were embedded in paraffin, sectioned and stained with hematoxylin and eosin. Immunohistochemical analysis was performed on paraffin-embedded tissues. Sections were deparaffinized and rehydrated. After the inactivation of endogenous peroxidase activity and antigen retrieval, sections were blocked with 10% normal bovine serum in PBS, followed by sequential incubation at room-temperature with anti-PRIP antibodies for 3 hours, biotinylated goat anti-rabbit IgG for 1 h, streptavidin-linked horseradish peroxidase for 30 min, and finally 3,3'-diaminobenzidine tetrahydrochloride solution for 4 min. Sections were counterstained with hematoxylin.

*Bromodeoxyuridine (BrdU) staining and terminal deoxynucleotidyl transferase mediated-dUTP nick end labeling (TUNEL) assay-* Mice were given an intraperitoneal injection of BrdU (30 μg of BrdU/g of body weight) two hours before sacrifice. Mammary glands were isolated, fixed, embedded and sectioned. BrdU immunostaining was performed using the Cell Proliferation kit (Amersham Life Science Inc.) as described by the manufacturer. Apoptotic cells were detected by TUNEL assay using in situ cell death detection kit from Roche as instructed. A total of 6000 cells were counted from six different sections of a sample and final counts were expressed as the percentage of epithelial cells positive for BrdU or TUNEL. Only intensely stained nuclei were scored as positive.

*RNA isolation and Semi-quantitative RT-PCR-* The inguinal glands were collected from wild type and PRIP-mutant mice. Mammary epithelial cells were separated from stromal cells by collagenase digestion and percoll gradient centrifugation (32) and used for isolation of total RNA by TRizol (Invitrogen) method. RT-PCR was performed with SuperScript one-step RT-PCR kit from Invitrogen as instructed. 1 μg of total RNA was reversely transcribed with SuperScript II reverse transcriptase followed by PCR amplification consisting of 35 cycles of denaturing at 94 °C for 15 sec, annealing at 55 °C for 30 sec, and extension at 68 °C for 1 min. For each 100 μl-reaction, 10 μCi of [$S^{35}$]-dATP was included and 20 μl of

amplified product was taken out at cycle 25 and 30. 5 μl of PCR product was separated on PAGE, which was then dried and exposed to X-ray film. The primers used are as follows:

EGF: 5'-ACCAGCAATTGGTGGTGGAT-3' and 5'-ATGTAAGCGTGGCTTCCTTC-3'.

Betacellulin: 5'-GTAGCAGATGGGAACACAAC-3' and 5'-CTTGCCACCAGCTTGTGATA-3'

TGFα: 5'-GTATCCTGTTAGCTGTGTGC-3' and 5'-CAAATTCCTCCTCTGGGATC-3'.

Amphiregulin: 5'-AGCTGCTTTGGAGCTCAATG-3' and 5'-ATTGCATGTCACCACCTCCA-3'

β-actin: 5'-CCATCTACGAGGGCTATGCT-3' and 5'-GCAAGTTAGGTTTTGTCAAAGA-3'

*Northern blot analysis*-10 μg of total RNA was separated through the formaldehyde denaturing agarose gel and was transferred onto a Nylon membrane (BioRad Laboratories). Filters were hybridized with [$\alpha$-$^{32}$P]dCTP-labeled cDNA probes including β-casein, WDNM1 and whey acidic protein (WAP).

## RESULTS

*Conditional null mutation of PRIP in mammary gland*-As PRIP null mutation results in an embryonic lethality which precludes studying the role of PRIP in mammary gland development, a conditional null mutation in mammary gland was generated by crossing mice with LoxP integrated recombinant PRIP gene and MMTV-Cre transgenic mice. MMTV-Cre specifically expressed Cre recombinase in mammary epithelial cells, which deleted exon 7 of PRIP gene flanked by two LoxP sites (Fig 1 A). The deletion of PRIP exon 7 led to a reading frame shift and generated a stop codon right after the fusion between exon 6 and exon 8. The successful deletion of PRIP gene in mammary epithelial cells was revealed by PCR using primers specifically for the deleted and undeleted gene (Fig. 1B). RT-PCR showed that PRIP mRNA from mammary epithelial cells with deleted PRIP gene was absent (Fig. 1C). Immunostaining with anti-PRIP confirmed that mammary epithelial cells with deleted PRIP gene did not express PRIP protein (Fig. 1D).

*Defective mammopoiesis during puberty, pregnancy and lactation*-Mammary glands at different developmental stages were examined in age- and weight-matched wild type mice and PRIP-mutant mice. The mammary ducts extend and branch into fat pad under the stimulation of gonadal hormone during puberty. In comparison with wild type mammary gland, PRIP deficient mammary glands from 5-week-old mice also exhibited ductal growth with similar extent of ductal elongation. However, the number of ductal branches and the density of ducts were greatly reduced in PRIP deficient mammary gland (Fig. 2). Histological analysis revealed no appreciable difference between wild type and PRIP deficient gland except that PRIP deficient gland contained less ducts (data not shown). By 10 weeks, ductal growth almost reached the edge of the fat pad in both wild type and PRIP mutant mice with greatly decreased branching observed in PRIP mutant mammary gland (Fig. 2). The mammary duct completely fills the mammary fat pad in mature virgin mice and ceases active growth afterwards. An examination of mammary gland whole-mount revealed that the extent of ductal branching was still substantially reduced

in 15-week–old PRIP mutant female (Fig. 2), indicating that the potential of branching was compromised in PRIP deficient mammary gland. During pregnancy, mammary glands undergo further ductal branching, lobuloalveolar proliferation and differentiation. Whole mount preparation of glands from 15-day pregnant mice revealed that PRIP deficiency caused impairment of lobuloalveolar development (Fig. 3). The density of alveoli in PRIP–null glands was markedly reduced as compared to that in wild type glands (Fig. 3). However, the morphology of the alveolus, as revealed by H & E section, was indistinguishable in wild type and PRIP–null gland (data not shown). Mammary gland completes lobuloalveolar development and functional differentiation during lactation. The lobuloalveoli from PRIP deficient gland were still much less abundant and smaller than that from wild type gland as revealed by whole mount (Fig. 4). PRIP deficient gland at day 8, revealed numerous adipocytes, in contrast virtually no adipocytes were present in wild type gland (Fig. 4). PRIP mutant mice delivered regular-sized litters but only two or three pups could survive. Most pups died within 48 h after birth with no milk detected in their stomachs. However, pups could be fostered by wild-type females, indicating that PRIP deficient glands are unable to produce sufficient quantities of milk to nurse pups due to defective mammary gland development.

*Attenuation of ductal branching of mammary gland in response to estrogen treatment*-To examine the mammary gland development in response to estrogen treatment, mice were ovariectomized and estrogen pellets were implanted. Three weeks later, uterine weight was increased ~11-fold in both wild type and mutant mice, confirming the successful implantation of estrogen pellets. The whole mount of the fourth pair of mammary glands from 4 wild type mice and 4 PRIP mutant mice consistently demonstrated that PRIP deficient gland exhibited much less extensive ductal branching as compared to wild type gland (Fig.5 as the representative). Therefore, PRIP is required for efficient ductal branching of mammary gland in response to estrogen.

*Proliferation and apoptosis of PRIP deficient mammary epithelial cells*- To determine if the retarded mammapoiesis was caused by altered proliferation and survival of mammary epithelial cells, DNA synthesis and apoptosis were examined in terminal end buds of inguinal mammary gland from both wild type and PRIP mutant mice. Six-week-old wild type and PRIP deficient mice were injected with bromodeoxyuridine to label the proliferating cells in mammary gland. Immunostaining with anti-BrdU revealed no quantitative differences in mitotic index between wild type and PRIP deficient mammary gland (Fig.6). The apoptotic index was determined by TUNEL assay. A slight increase in apoptosis was found in the terminal end buds from PRIP deficient gland as compared to wild type mammary gland (Fig. 6), raising the possibility that abnormal apoptosis contributes to the impaired ductal branching.

*Altered expression of growth factors in PRIP deficient gland*- ERBB signaling pathways are essential for mammary gland development (33, 34). EGF-like growth factors including amphiregulin, EGF, TGFα,

and betacellulin are the ligands for ERBB receptors. To understand the underlying mechanism for defective mammapoiesis, the levels of amphiregulin, EGF, TGFα, and betacellulin mRNA from PRIP deficient gland were examined by semi-quantitative PCR. The level of EGF mRNA from wild type gland was slightly higher than that from PRIP deficient gland (Fig. 7). However, PRIP deficient gland expressed markedly increased amphiregulin (Areg), TGFα, and betacellulin (Btc) mRNA (Fig. 7), indicating that PRIP deficient mammary epithelial cells may have impaired response to growth factors.

*Expression of milk protein genes in PRIP deficient gland-* While lobuloalveolar development was retarded, the morphology of individual alveolus from PRIP deficient lactating gland was indistinguishable from that of wild type alveolus. This prompted us to investigate the differentiated functions of mammary epithelial cells. We examined the mRNA expression of milk protein genes β-casein, WAP and WDNM1 from 8 day-lactating mammary gland. Northern blot showed PRIP deficient gland still expressed high level of β-casein, whey acidic protein (WAP) and WNDN1 (Fig. 8). While the expression level of WDNM1 from PRIP deficient gland was slightly higher than that from wild type gland, the expression of β-casein and WAP was reduced in PRIP deficient gland.

## DISCUSSION

To define the role of PPAR interacting protein (PRIP) in mammary gland development, we created a conditional PRIP null mutation in mammary gland. PRIP deficient gland presented with the normal elongation of ducts but the decreased number of ductal branches during puberty, which remained long after puberty, indicating the potential of ductal branching was impaired. PRIP deficient gland exhibited decreased density of alveoli during pregnancy. The lactating PRIP deficient gland contained decreased number of lobuloalveoli with many adipocytes and failed to nurture all pups. This study demonstrated that PRIP is pivotal for the normal mammary gland development.

PRIP deficient mammary epithelial cells expressed abundant mRNAs of milk protein genes, but these mice appeared unable to produce enough milk to nurse all pups. Therefore, the major defect leading to reduced milk production is most probably the impaired side branching, which generated too few and smaller alveoli to produce milk rather than the mildly affected differentiated function of mammary epithelial cells.

ERBB signaling pathways activated by EGF-like factors are essential for mammary gland development. We found that expression of several of EGF-like factors was increased in PRIP deficient gland. The defect in PRIP deficient gland should not be attributed to the lack of these EGF-like factors. Other growth factors may be the targets for PRIP protein and remain to be identified. Another possibility is that the PRIP deficient mammary epithelial cells have intrinsic defect which causes inefficient response to growth factors for the ductal branching and alveologenesis. Consistent with this hypothesis, microarray

studies found that STAT5a and HIF-1 mRNAs were significantly decreased in PRIP deficient gland (Qi, unpublished). Gene-knock out experiment revealed that STAT5 is essential for the lobuloalveolar development (35). Loss of HIF-1 was demonstrated to impair the mammary epithelial differentiation and lipid secretion, resulting in failure of lactation (36). Therefore these two genes are good candidates whose decreased expressions are responsible for the mammary gland defect associated with PRIP deficiency.

The ductal growth during puberty and the side branching and alveologenesisis during pregnancy is stimulated by estrogen and progesterone, respectively. In PRIP mutant mice, ductal branching and alveolar development were still present but severely impaired. Moreover, the ductal branching stimulated by direct estrogen treatment was attenuated in mutant mice. These phenotypes of PRIP deficient gland were consistent with that from mammary gland with compromised function of estrogen and progesterone receptor, although it is possible that these defects were caused by the loss of PRIP function unrelated to its role as the coactivator of estrogen and progesterone receptor in mammary gland. Analysis of estrogen and progesterone target genes would provide direct in vivo evidence that PRIP is required for the function of estrogen and progesterone receptor. Although a few estrogen and progesterone target genes have been identified in breast cancer cell lines (37-40), the direct estrogen and progesterone target genes in mammary gland remain elusive. With the identification of estrogen and progesterone target genes in mammary gland, the function of PRIP as the nuclear receptor coactivator can be further analyzed to see how it is involved in the transcriptional regulation of different target genes.

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## FIGURE LEGENDS

**Fig. 1.** Conditional PRIP null mutation in mammary gland. (A). Schematic drawing of Loxp-PRIP gene and PRIP null mutation with exon 7 deletion. The exon 7 flanked by two Loxp sites was deleted by Cre recombinase when Loxp-PRIP mouse was crossed with MMTV-Cre mouse to produce Loxp-PRIP plus MMTV-Cre mice. (B). Genomic DNA was isolated from mammary epithelial cells from Loxp-PRIP mice or Loxp-PRIP plus MMTV-Cre mice. PCR was performed with primers specific for Loxp-PRIP transgene and deleted PRIP gene. A 7 KB band was amplified from the Loxp-PRIP mice (Lane 2) while only a 4 KB band was present in Loxp-PRIP plus MMTV-Cre mice(lane 1), which resulted from Cre-mediated deletion. (C). Total RNA was isolated from mammary epithelial cells from Loxp-PRIP mice or Loxp-PRIP plus MMTV-Cre mice. RT-PCR was done with PRIP specific primers. A band was observed in Loxp-PRIP mice (Lane 2) but not in Loxp-PRIP plus MMTV-Cre mice (Lane 1). (D). Immunohistochemical staining with anti-PRIP showed that mammary epithelial cells from Loxp-PRIP mice were positive (D1) while mammary epithelial cells from Loxp-PRIP plus MMTV-Cre mice were negative (D2).

**Fig. 2.** Retarded ductal branching of PRIP deficient gland during puberty. The fourth pair of mammary gland was isolated, fixed and whole mounted from 5-week-old(A,B), 10-week-old(C,D,E,F), 15-week-old(G,H) wild type mice or PRIP mutant mice. Note that the PRIP deficiency (B, D, F, H) caused defective branching from early puberty (B) to the end of puberty (D, F), which persisted after the puberty (H). (E) and (F) represent higher magnification of the gland structure in (C) and (D), respectively.

**Fig. 3.** Morphology of PRIP deficient gland at 15-day pregnancy. The fourth inguinal mammary glands from wild type mice (A, C) and PRIP mutant mice (B, D) were isolated and whole amounted. Note that PRIP deficient gland had underdeveloped lobuloalveolar structure with smaller and fewer alveoli. (C) and (D) represent higher magnification of the glands in (A) and (B), respectively.

**Fig. 4.** The defective development of lactating PRIP deficient gland. The fourth pairs of mammary gland from day 8 lactating wild type (A, C, E) or PRIP mutant (B, D, F) mice were isolated and then whole mounted or fixed for H&E section. Whole mount revealed that PRIP deficient gland (B) contained less and smaller lobuloalveoli than wild type gland (A). (C) and (D) represent higher magnification of the lobuloalveolar structure in (A) and (B), respectively. On H & E section(E,F), PRIP deficient gland showed abundant adipocytes surrounding alveoli while wild type gland contained well developed alveoli with no recognizable adipocytes.

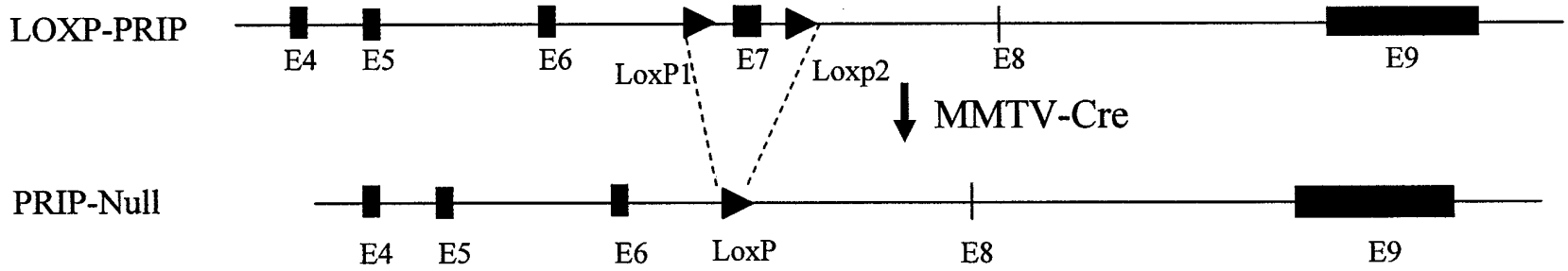
**Fig. 5.** Attenuated response of PRIP deficient gland to estrogen treatment. Five-week-old wild type or PRIP mutant females were ovariectomized and implanted with an estrogen pellet under the skin behind neck. Three weeks later, the fourth pair of mammary gland was isolated, fixed and whole mounted. Note the difference in the number of ductal branches between wild type mammary gland (A) and PRIP deficient mammary gland (B). (C) and (D) represent higher magnification of the ductal structure in (A) and (B), respectively.

**Fig. 6.** Cell proliferation and apoptosis in terminal end buds of wild type and PRIP mutant mice. (A and B), 6-week-old mice were injected with BrdU and the inguinal mammary glands were prepared, fixed, sectioned and stained with anti-BrdU. No difference in cell proliferation was observed between wild type (A) and PRIP deficient (B) mammary gland. (C and D), Immunohistochemistry was performed on mammary gland sections to detect TUNEL positive cells undergoing apoptosis. Note the increased apoptosis in terminal end buds from PRIP deficient gland (D) as compared to that from wild type mammary gland(C). (E), Quantitation of BrdU and TUNEL staining in wild type (WT) and PRIP deficient (KO) gland. 6000 cells were counted from six different sections of a sample and the percentage of labeled cells was determined. The mean $\pm$ standard deviations were calculated. No quantitative differences in mitotic index were observed ($P>0.05$) while significantly increased apoptosis was found in PRIP deficient gland as compared to wild type mammary gland ($P<0.001$).

**Fig. 7.** Altered expression of growth factors in PRIP deficient gland. Total RNA was made from mammary epithelial cells isolated from wild type mice (WT) or PRIP mutant mice (KO) 8 days after parturition. Semi-quantitative RT-PCR was performed with addition of $S^{35}$-dATP. For each PCR reaction, 5 ul of PCR products at 25 cycles, 30 cycles, and 35 cycles were resolved on PAGE which was then exposed to X-ray film. β-actin served as the control of equal amount of RNA for PCR.

**Fig. 8.** Expression of β-casein, WAP, and WDNM1 mRNA. Mammary glands were isolated from 8-day lactating wild type mice (WT) or PRIP deficient mice (KO). Total RNA was prepared and 10 ug of total RNA was subject to Northern blot analysis. The equal amount of RNA loading was demonstrated by the intensities of 28S rRNA band.

A.


LOXP-PRIP
E4
E5
E6
LoxP1
E7
Loxp2
E8
E9
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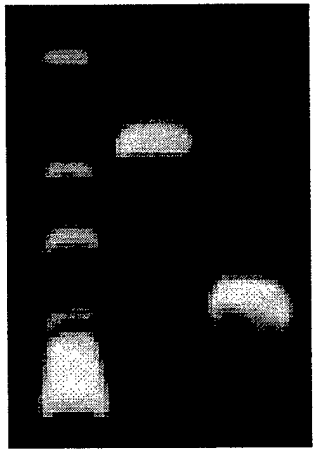
PRIP-Null
E4
E5
E6
LoxP
E8
E9

Fig. 1A

B. 1 2



C. 1 2

D.

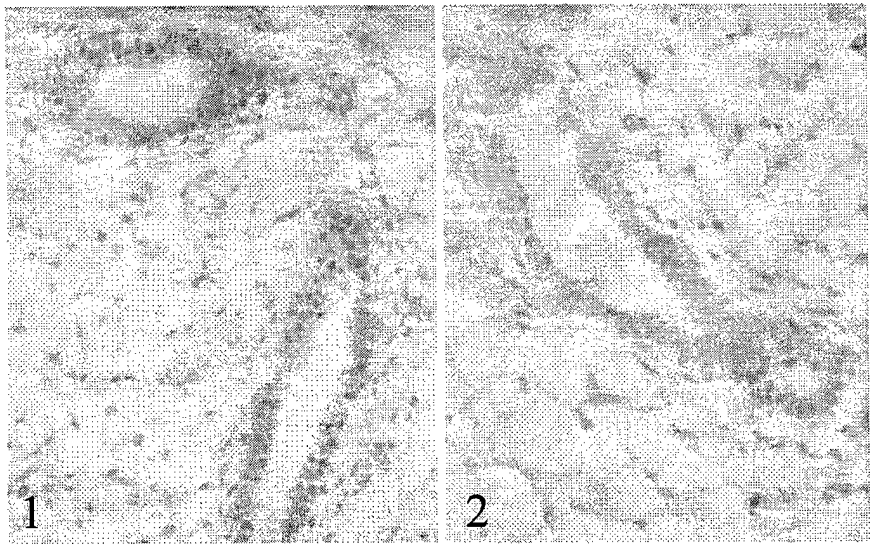



Fig.1B, C, D

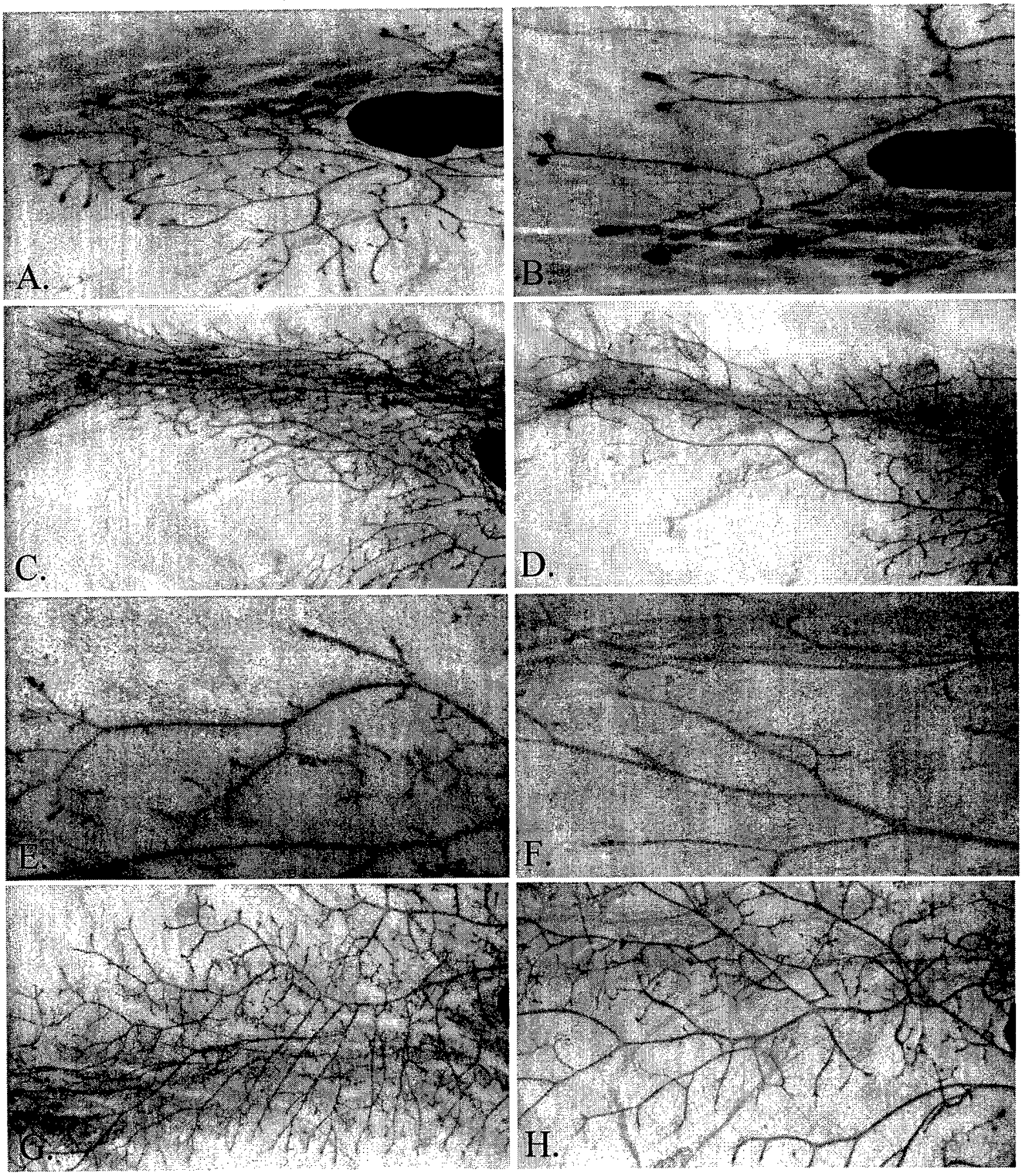



Fig. 2

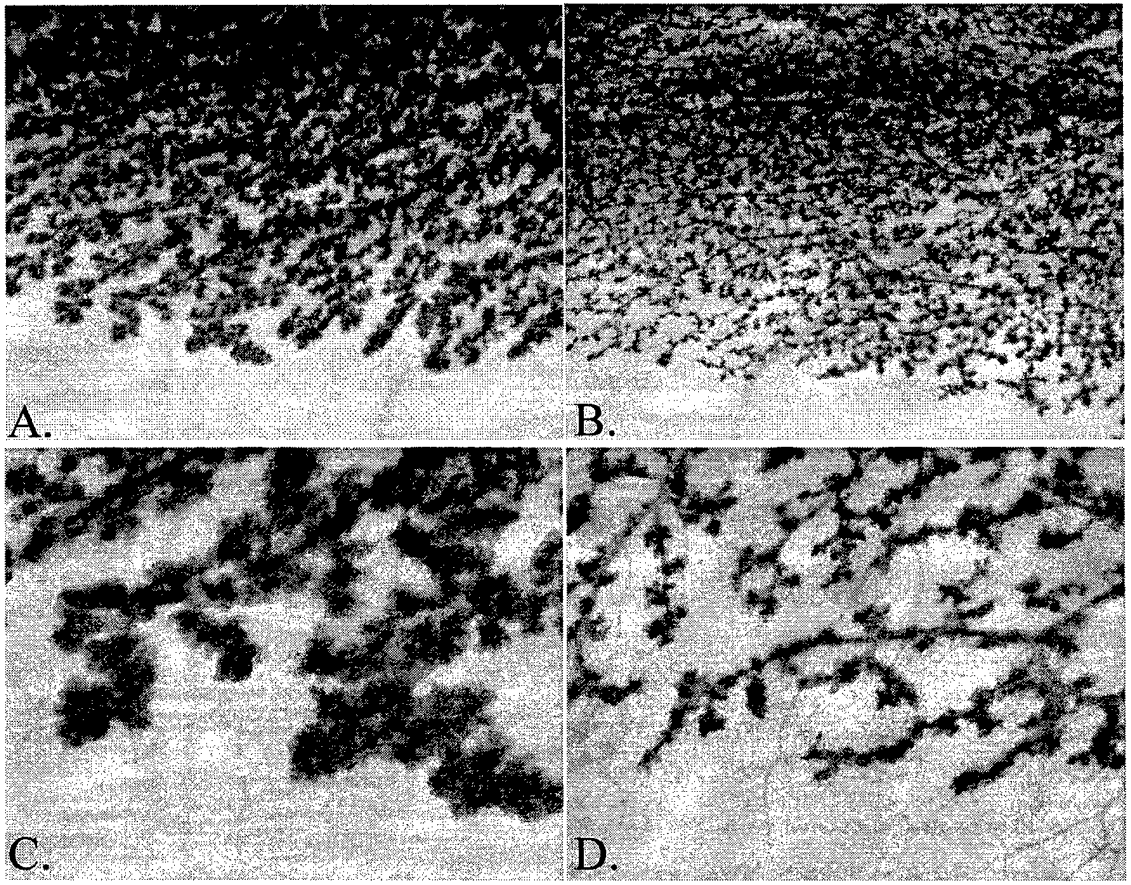



Fig. 3

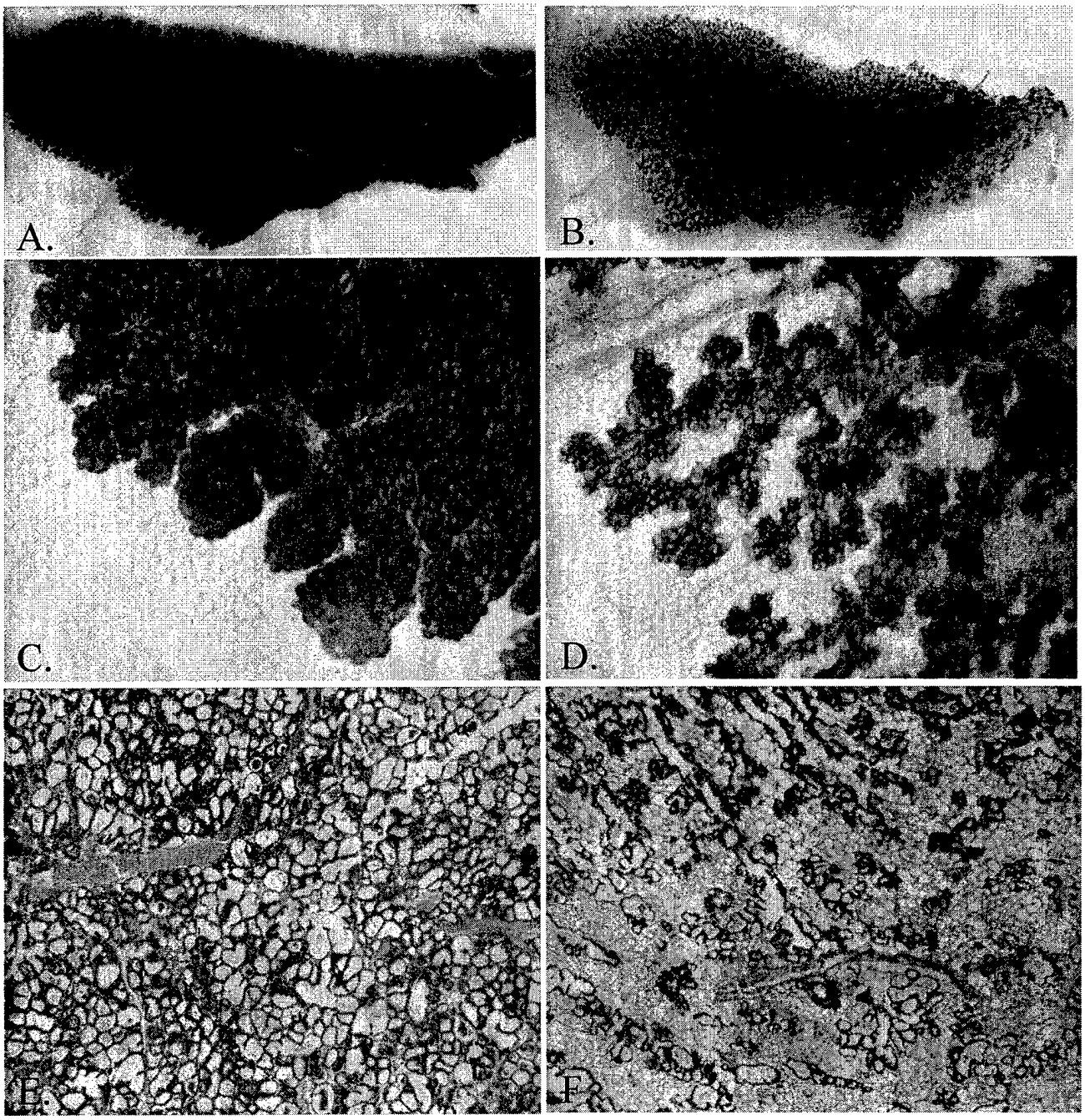



Fig. 4

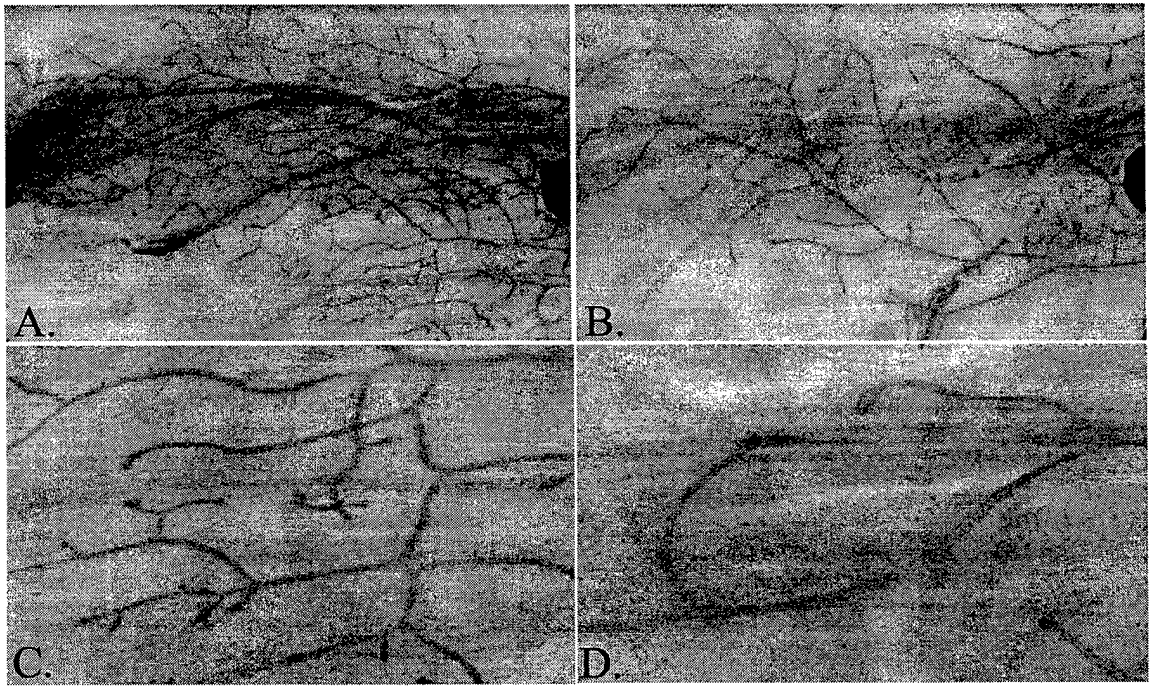



Fig. 5

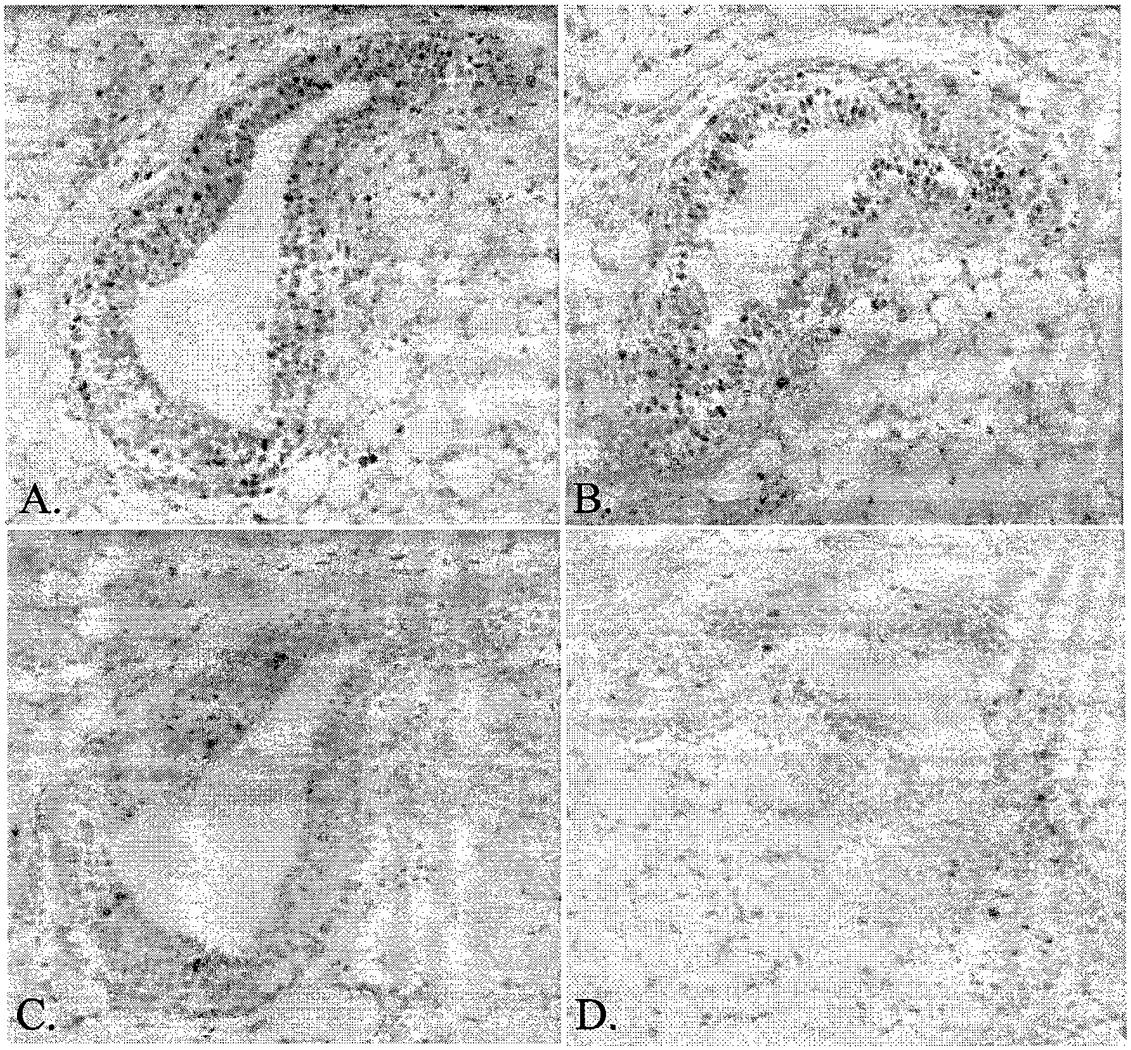



Fig. 6A, B, C, D

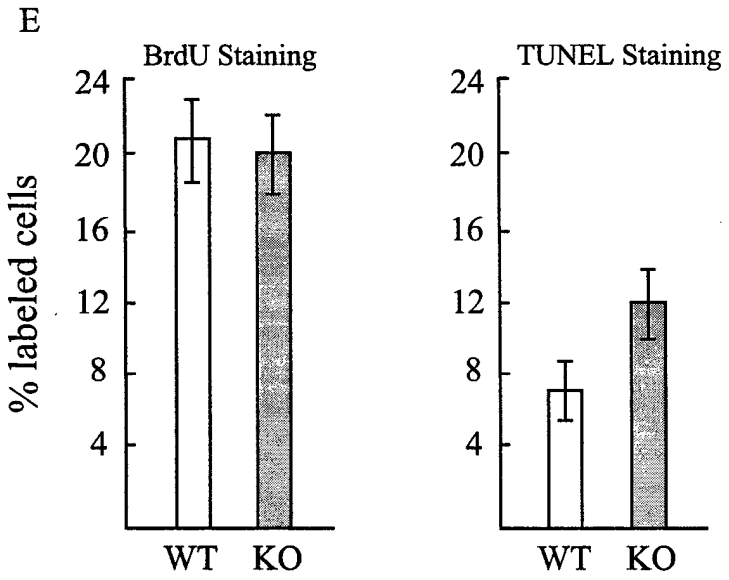

E
BrdU Staining
TUNEL Staining
% labeled cells
24
20
16
12
8
4
WT
KO
WT
KO

Fig. 6E

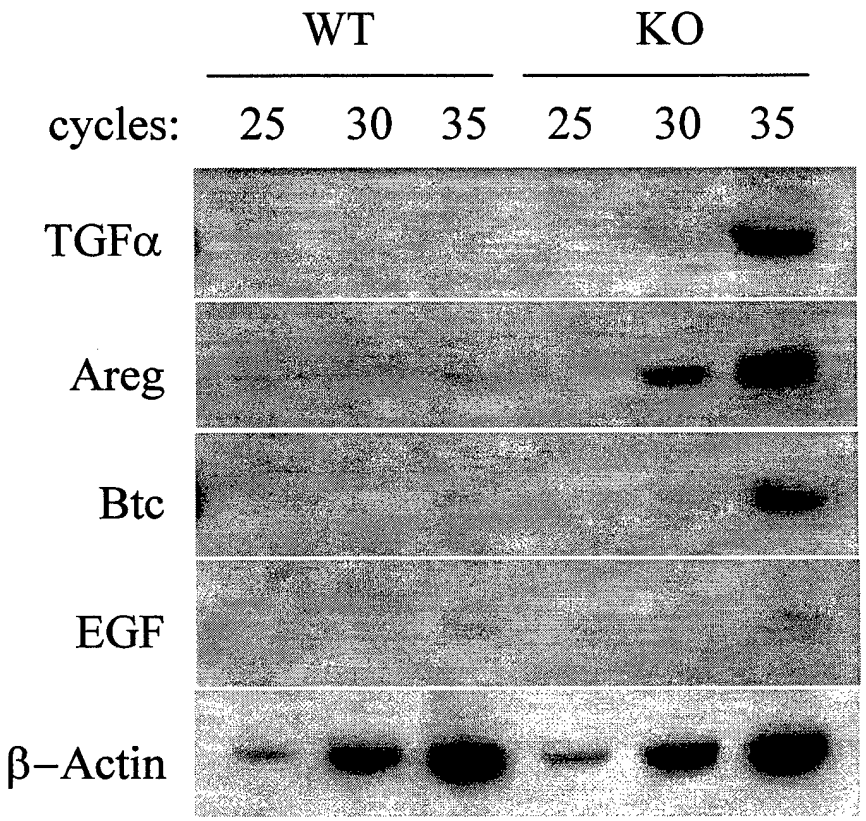



Fig. 7

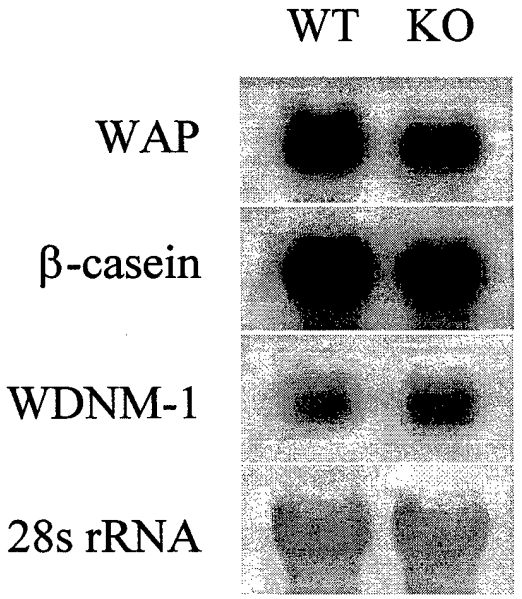



Fig. 8