Identification of apolipoprotein D as a novel inhibitor of osteopontin-induced neoplastic transformation

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Abstract. Osteopontin that associates with metabolism of calcium is one of the important factors in the development and prognosis of human breast cancer. The aim of this study was to detect potential binding partners of osteopontin to illustrate its functional mechanism. By screening a human breast cDNA library with a bacterial two-hybrid system, apolipoprotein D was isolated as a novel interacting protein of osteopontin. This interaction was confirmed by mammalian two-hybrid assay and co-immunoprecipitation. To elucidate the influence of ApoD on cellular neoplastic specifications, adhesion, soft agar, invasion and MTT growth assays were performed with Rama37 cells. The results revealed that expression of apolipoprotein D in Rama37 cells could significantly inhibit the malignant phenotype in osteopontin-transformed Rama37 cells. These findings provide better knowledge of the osteopontin signaling pathway and suggest that apolipoprotein D could be a prospective therapeutic agent for human breast and/or other carcinomas.

Introduction

Osteopontin (OPN) is a secreted negatively-charged, glycosylated adhesive phosphoprotein of 298 amino acids with a molecular weight of its polypeptide backbone of 32,000. Because glycosylation and phosphorylation occur to a variable extent, its overall molecular weight varys from approximately 32.5 kDa to 70 kDa (1,2). OPN was initially described as a phosphorylated protein secreted by transformed cells but not by their non-transformed counterparts (3). It is expressed in various tissues, including bone, cartilage, kidney, placenta, smooth muscle cells of arteries, and cells of the immune system (4). In addition, many tumor cell types, both *in vitro* (5) and *in vivo* (6,7), produce OPN. Several observations have suggested that OPN expression is significantly altered in cancer and that OPN is important for tumorigenesis and metastasis (6-12). For example, OPN expression is substantially increased in experimental cell lines following neoplastic transformation by several agents (5,13-17) and is induced in mouse epidermis with a tumor promoter (18,19). In a study described by Tuck *et al* (7), OPN protein was detected in breast cancer cells and was significantly inversely correlated with the disease-free state and overall survival in those examined lymph node-negative breast cancer patients. Immunopositivity of OPN in another group of 333 breast cancer patients also showed a negative correlation with survival (20).

In another study, OPN levels were significantly up-regulated in sera of multiple cancers, of which breast cancer was the highest, followed by lung and then prostate cancer (21). However, in the first demonstration that OPN could play a functional role in malignancy, transfection with an OPN anti-sense RNA expression vector inhibited the tumorigenic or metastatic properties of various malignant cell types, such as ras-transformed NIH 3T3 cells, neoplastic transformed Rat1 fibroblasts and transformed mouse JB6 epidermal cells (22-25). On the contrary, transfection of OPN cDNA in rat mammary epithelial cells was found to increase their metastatic ability. Recently, increasing evidence has indicated that OPN may play a pivotal role in tumor progression and metastasis (26-30). The function of OPN in human cancer is highly complex and far from clear. Although it is known that OPN has been implicated in cancer development and metastasis, the mechanism needs further investigation. As known, protein-protein interactions play an important role in virtually all cellular processes. A powerful strategy to explore the mechanism of a protein's action is to search for its interacting proteins. In this study, a bacterial two-hybrid library screen was carried out to detect unknown interacting proteins of OPN. Then a series of cellular functional experiments were performed using Rama37, a benign rat mammary epithelial cell line, to detect how the cellular behavior was influenced by the interactions (31).

Materials and methods

Cell lines and cell culture. The stable diploid rat mammary epithelial cell line, Rama37, was obtained from Liverpool University and cultured as previously described (31). The C9-

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Abbreviations: OPN, osteopontin; VDR, vitamin D receptor; ApoD, apolipoprotein D; PTH, parathyroid hormone; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IRF-1, interferon regulatory factor-1; MMP-9, matrix metalloproteinase 9

Key words: osteopontin, apolipoprotein D, neoplasms, co-repressor, bacterial two-hybrid, cDNA library

Met-DNA cell line is a Rama37 cell line permanently transfected by C9-Met-DNA (32), which can induce metastasis in rat model cell lines and other malignant phenotypes (32). All cells used in these experiments were routinely maintained in DMEM supplemented with 1% L-glutamine and 10% heat-inactivated fetal bovine serum (all from Gibco-BRL, Gaithersburg, MD). Working cultures were maintained at 37˚C in a humidified atmosphere of 5% CO_2, 95% O_2.

Transfection. For the transient transfection assays, the cell lines were cultured in DMEM, 10% (v/v) FCS, 100 μg/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc., Glasgow, UK), harvested, and seeded in multiwell plates at $2.5x10^5$ cells/3.5-cm well in 1 ml of serum-free medium. After 24 h, cells were co-transfected using Lipofectamine and Plus reagent (Invitrogen Ltd, Paisley, UK) following manufacturer's instructions with specific DNA recombinants as described below. CAT activity was normalized relative to ß-galactosidase activity. Maximum activity was reached by 48 h. Establishment of stable pBKCMV-OPN transformed Rama37 cell lines and co-transfectant with pcDNA6/HisC-ApoD was performed as previously described by adding G418 or Blasticidin as selective antibiotics following the manufacturer's instructions (33).

Human breast plasmid cDNA library. The plasmid-based cDNA library was bought from Stratagene Ltd. (La Jolla, USA) with cloning sites at *Xho*I and *Eco*RI. Primary colonies are $2.46x10^6$ colony formation unit (cfu). Estimated titer is $1.43x10^9$ cfu/ml. Host strain genotype is XL-1Blue MRF' Kan which is deficient in all known restriction systems, endonuclease deficient and recombination deficient. The mcrA, mcrCB, and mrr mutations prevent cleavage of cloned DNA that carries cytosine and/or adenine methylation. All cDNA of genes were cloned unidirectionally into the BacterioMatch™ pTRG XR vector (Stratagene).

cDNA plasmid construction. All cDNAs were made according to standard methods and verified by sequencing. The multicopy bacterial plasmids used in the bacterial two-hybrid assays were pBT and pTRG. In these plasmids the target fusion genes are expressed under control of the lac-UV5 and lpp/lac-UV5 promoters, respectively. cDNA of rat OPN coding sequence was amplified by PCR using 5'-CTC GAG TAT GAG ACT GGC AGT GGT T-3' and 5'-CTC GAG TAA TTG ACC TCA GAA GAT-3' as forward and reverse primers, respectively. The cDNA of OPN was subcloned into the pBT plasmid as a bait plasmid at *Xho*I. OPN was amplified by PCR again and ligated into the Topo vector with TOPO TA Cloning kit for sequencing (Invitrogen Life Technologies). It was then subcloned into pM vector (BD Sciences Clontech, Mountain View, USA) at the *Sal*I site and pBKCMV vector (Stratagene) at the *Xho*I site respectively. ApoD was subcloned into pVP16 and pcDNA6/HisC vectors respectively at the *Eco*RI-*Sal*I site by digestion from pTRG vector.

BacterioMatch two-hybrid assays. The human breast plasmid cDNA library in pTRG plasmid (Stratagene) was screened for proteins that interact with OPN using BacterioMatch Two-Hybrid System reporter strain. The prey (cDNA library constructs) and bait recombinant plasmids (pBT-OPN) were co-transformed into BacterioMatch two-hybrid reporter strain competent cells. Transformed cells were spread directly on LB-agar plates containing chloramphenicol (34 μg/ml), carbenicillin (500 μg/ml), kanamycin (50 μg/ml) and tetracycline (12.5 μg/ml) (CTCK plates). To verify the specificity of protein-protein interactions, the carbenicillin-resistant colonies from the initial library screen were directly patched onto X-gal indicator plates [LB-agar supplemented with kanamycin (50 μg/ml), tetracycline (12.5 μg/ml), chloramphenicol (34 μg/ml), X-gal (80 μg/ml) and 0.2 mM B-galactosidase inhibitor (phenylethyl B-D-thiogalactoside)] using sterile toothpicks. Library plasmids of positive isolates were transformed into and recovered from *Escherichia coli* strain Topo. These were co-transformed into BacterioMatch two-hybrid reporter strain competent cells with isolated target plasmids plus an additional bait plasmid (pBT-OPN). Then the putative positive pTRG clones were analyzed by restriction digest. Unique inserts were sequenced and analyzed by comparison to the GenBank sequence data bank.

Mammalian MATCHMAKER two-hybrid assay. For the mammalian two-hybrid assay, the full-length cDNA coding sequence of the target gene (ApoD) was cloned into pVP16 vector in the Mammalian Matchmaker two-hybrid assay system (BD Sciences Clontech) by *Bam*HI and *Sal*I creating fusions to the Gal4 DNA-binding domain. The cDNA of the full coding sequence of OPN was inserted into pM vector. Recombinants were confirmed by DNA sequencing. Mammalian two-hybrid assays were conducted in Rama37 cells. CAT enzyme activity was detected with a CAT enzyme assay system (Promega Corp., Southampton, UK). Briefly, cell extract was performed by standard reaction with reporter lysis buffer. CAT enzyme activity was determined by the liquid scintillation counting (LSC) method with a 1219 Rackbeta liquid scintillation counter (LKB Wallac, Freiburg, Germany). B-galactosidase activity was assayed using a B-galactosidase enzyme assay kit (Promega) by standard spectrophotometric analysis after cell extraction using reporter lysis buffer following the manufacturer's instructions.

Co-immunoprecipitation. As an *in vitro* protein interaction assay approach, co-immunoprecipitation was carried out with OPN and [^{35}S]-Methionine-labeled ApoD proteins which were produced by Promega TNT *in vitro* transcription/translation kit. Pro-Found™ co-immunoprecipitation kit (Pierce Biotechnology, Inc) was used following the manufacturer's instructions exactly. Of specific mouse monoclonal OPN antibody (MPIIIB101) ((300 μg/μl) University of Iowa, Iowa, USA) (34), 300 μl was used for co-immunoprecipitation, performed using the ProFound™ co-immunoprecipitation kit (PIERCE Science UK Ltd, Cramlington, UK) following the manufacturer's instructions. After reaction, 10% SDS polyacrylamide gel electrophoresis was performed to separate the proteins. Radiolabeled proteins were then detected by autoradiography.

Adhesion assay. Cell adhesion assays were performed in 24-well cell culture plates. Media that cells grew in prior to the assays were collected as the conditioned media. Of conditioned media, 800 μl was aliquoted into each well. Of cells ($1x10^6$

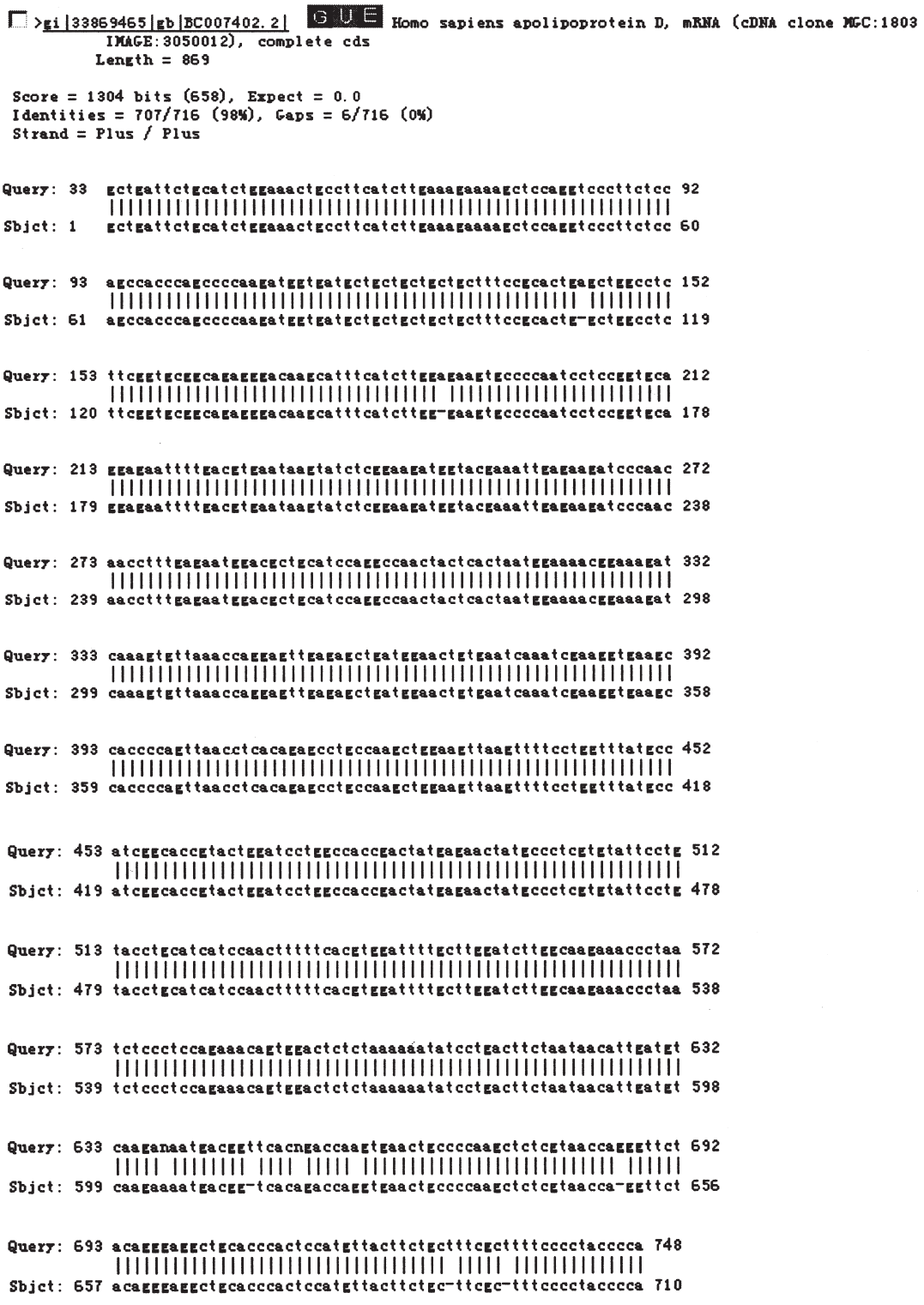

Figure 1. BLAST result of the DNA sequence of a sample isolated from human breast plasmid-based cDNA library against GenBank sequence database of NCBI, 98% identical to human apolipoprotein D (accession no. BC007402).

cells/ml) 200 μl was added to each well and incubated at 37˚C in a 5% CO_2 atmosphere for 30 min. The media were aspirated. Cells were washed once with 1ml of PBS. Of 0.25% Trypsin-EDTA (Sigma-Aldrich), 100 μl was aliquoted into each well and cells were incubated at 37˚C for 3 min. Of PBS, 400 μl was subsequently added to each well. The total 500 μl of cells in each well were collected into a 1.5 ml microcentrifuge tube. Cells were homogenized by gentle pipetting and then counted using a haemocytometer.

Soft agar assay (or anchorage-independent growth assay). Soft agarose assays were performed as described previously (http://www.flemingtonlab.com). Briefly, 5,000 cells suspended in 2 ml DMEM growth medium were plated per well into

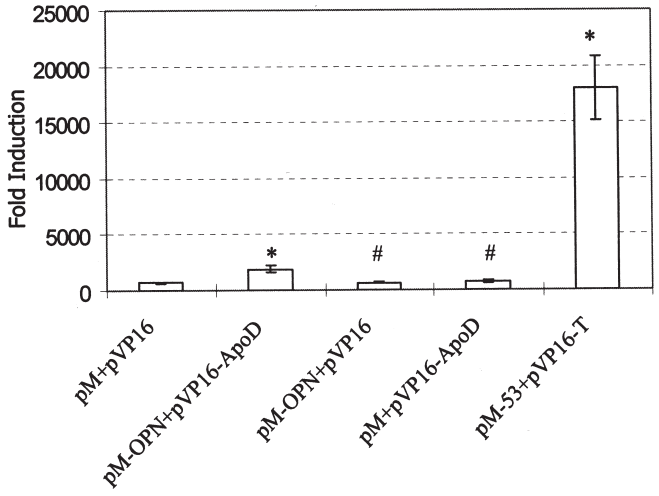

Figure 2. Mammalian two-hybrid assay to detect interactions between OPN and ApoD. Rama37 cells were transiently co-transfected using pVP16-ApoD or pVP16 plasmid with pM-OPN or pM plasmid. Co-transfection using pM-53 and pV16-T was used as positive control. A reporter plasmid (pG5-CAT) was included in all co-transfections. All co-transfections also contained pSV40-ß-galactosidase vector, which worked as a ß-galactosidase internal control reporter to normalize for transfection efficiency. Forty-eight hours after transfection, the transfected cells were collected and CAT enzyme assay was performed. Fold induction was performed for all the CAT assay results. Experiments were performed in triplicate. Values are means with S.D. from triplicate independent determinations. $^{*}p<0.001$ compared with cells co-transfected with pM and pVP16. $^{\#}p>0.05$ compared with cells co-transfected with pM and pVP16 (Student's t-test).

6-well tissue culture plates containing 1.0% agarose underlayers. Colonies were allowed to grow for 7 days. Then 50 μl of 1 mg/ml p-iodonitrotetrazolium violet (Sigma) was added to each well and mixed gently. The plate was placed in the 'Gene Genius™' bio-imaging system (Synoptics Ltd., Cambridge, UK) to count the separate colonies that had formed.

Invasion assay. Invasion assays were performed as previously described by Albini *et al* (35). Briefly, 12 μm-pore transwell filter chambers (Falcon, Oxford, UK) were used. The upper side of polycarbonate membranes were coated with Matrigel, a reconstituted basement membrane matrix (Becton Dickinson, Bedford, MA), diluted to 1 mg/ml. Cells ($1x10^5$) were seeded in the upper compartment of each transwell unit containing FCS-free medium with 0.1% BSA (Sigma). The medium plus BSA was introduced in the lower compartment with 10% FCS and 1 μg/μl fibronectin. After incubation for 48 h at 37˚C, media in the lower chamber of each well were removed. Matrigel and non-invaded cells from the inner side of the filter chambers were removed with a cotton bud. The membranes were then fixed in methanol for 5 min. Cells which had migrated through the Matrigel and membrane were attached to the lower surface of the membrane. They were stained with toludine blue and then washed by 100 μl 20% acetic acid. Their absorbances were read at 650 nm using a Thermo Max microplate reader (Molecular Devices, Sunnyvale, USA).

MTT growth assay. MTT growth assay was performed as described previously (36). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma. In brief, 1500 cells of each cell type were seeded in quadruplicate into 96-well microplates, which were incubated at 37˚C in a 5% CO_2 humidified atmosphere (Sanyo Corp, Tokyo, Japan) for 24, 48, 72 and 96 h. Then 20 μl of 5 mg/ml MTT was added to each well, followed by 50 μl of 20% SDS (Sigma). Absorbance was measured at 570 nm with a Thermo Max microplate reader.

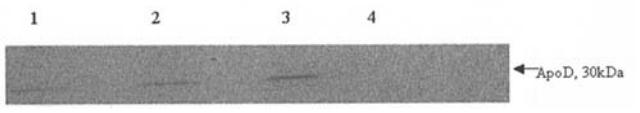

Figure 3. Co-immunoprecipitation of ^{35}S-labeled ApoD and OPN with monoclonal antibody for OPN showing interaction of ApoD with OPN. ^{35}S-labeled ApoD was incubated with nonradioactive OPN protein. Both ApoD and OPN protein were produced in the reticulocyte lysates by an expression vector. Lanes 1-3 showed that any resultant complexes were immunoprecipitated with OPN monoclonal antibody and analyzed on a 10% SDS-polyacrylamide gel. Lane 4 contains a negative control in which an empty pcDNA6/HisC vector instead of ApoD expression vector was used during incubation of *in vitro* transcription using TNT coupled reticulocyte system.

Results

ApoD was isolated as an OPN-interacting protein. From the plasmid-based Bacterio-Match two-hybrid screen in a human breast cDNA library, 86 clones were isolated that grew on selective CTCK plates. Plasmid molecules were isolated from each of the colonies and re-tested for their ability to interact with OPN. The 82 resulting positive colonies were sequenced. The DNA sequences of the interacting clones were examined against the GenBank database. Two apparent independent clones were determined to be nearly identical in length and sequence to the human apolipoprotein D cDNA (accession no: BC007402; GI number: 33869465) (Fig. 1).

Confirmation of the OPN-ApoD interaction in mammalian cells. To determine whether the OPN-ApoD interaction occurs in mammalian cells, we performed triplicate mammalian MATCHMAKER two-hybrid assay. Rama37 cells were transfected with combinations of pM, pM-OPN and pVP16, or pVP16-ApoD constructs. Co-transfection of Rama37 cells with pM-OPN, pVP16- ApoD enhanced the CAT activity by 2.85-fold compared with Rama37 transfected with empty pM and pVP16 vectors, or 2.7-fold compared with cells co-transfected with pM-OPN and pVP16 vector ($p<0.001$, Student's t-test) (Fig. 2). Co-transfection of Rama37 cells with pM-53 and pVP16-T resulted in a significantly increased CAT activity compared to the remaining transfectants ($p<0.001$, Student's t-test). These results indicate direct interaction between ApoD and OPN in Rama37 cells.

Co-immunoprecipation. Following the bacterial and mammalian two-hybrid assays, an additional co-immunoprecipitation binding assay was performed to confirm the specificity of binding. pBKCMV-OPN and pcDNA6/HIS C-ApoD constructs were expressed *in vitro* by TNT coupled reticulocyte lysate system (Promega Ltd.). Co-immunoprecipitation was performed using a specific monoclonal OPN antibody (MPIIIB101). Co-precipitated ^{35}S-labeled ApoD protein was detected by X-ray film (Fig. 3).

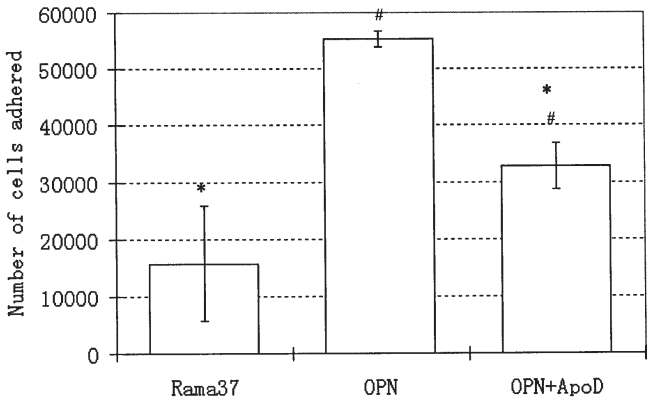

Figure 4. Adhesion potential of Rama37, Rama37-OPN and Rama37-OPN-ApoD cell lines. Cells were allowed to attach to plastic substratum for 30 min of incubation at 37˚C and then collected and counted. Experiments were carried out in triplicate. Student's t-test was performed to obtain p-values. Data are expressed as means ± S.D. *p<0.001 compared with Rama37-OPN cells, #p<0.001 compared with Rama37 cells.

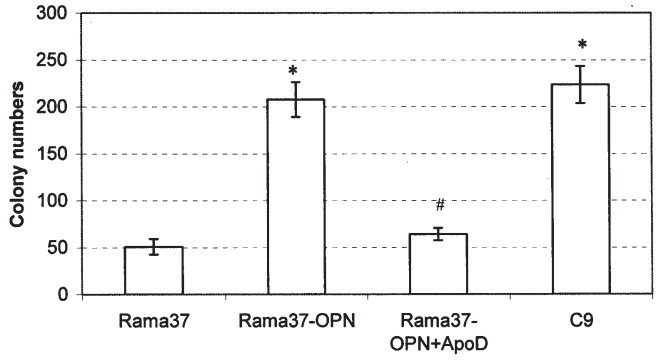

Figure 5. Effect of over-expression of ApoD on anchorage-independent growth of Rama37-OPN cells. Rama37-OPN, Rama37-OPN-ApoD and wild-type Rama37 cells were plated in growth media into a 6-well plate over a bottom layer of 1% agarose gel. C9-Met-DNA cells were included as a positive control. Colonies were stained with p-iodonitrotetrazolium violet and counted after 7 days of incubation. Experiments were carried out in triplicate. Student's t-test was carried out to obtain p-values. Results were expressed as the mean number of colonies of triplicate measurement. The data bars represent the standard deviation. *p<0.001 compared with Rama37 cells. #p>0.05 compared with Rama37 cells.

Adhesion assay. To test the effect of ApoD on adhesion, adhesion assays were performed with co-transfectants of ApoD and OPN, transfectant of OPN and non-transfected Rama37 cells. ApoD was shown to exert an inhibitory activity on the cell adhesive capacity of OPN-transfected Rama37 cells (Fig. 4). Overexpression of ApoD resulted in a 40.7% decrease compared to OPN-transformed Rama37 cells ($p<0.005$, Student's t-test).

Soft agar assay (anchorage-independent growth assay). Rama37 cells have previously been shown to have low cloning efficiency in soft agarose. The ability of OPN transfectant, ApoD and OPN co-transfectants and non-transfected Rama37 cells were compared. This property is considered to be reflective of malignancy (35,37). C9-Met-DNA cells were used as a positive control. The number of colonies of ApoD co-transfectants decreased about 70% relative to OPN transfectants but remained higher than non-transfected Rama37 ($p<0.001$, Student's t-test) (Fig. 5).

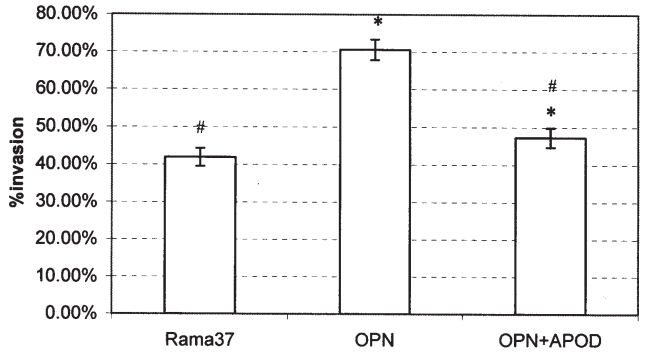

Figure 6. ApoD inhibits OPN-stimulated cell invasion in Rama37 cells. Effect of ApoD on OPN-induced cell invasion in Rama37 cells. Rama37, Rama37-OPN and Rama37-OPN-ApoD cells were added to upper chambers with or without Matrigel and allowed to invade a fibronectin-containing media in the lower chamber of each well for ~48 h under incubation at 37˚C. Penetrated cells were stained using methylene blue and then washed with 20% acetic acid. Samples were read on a multiwell plate reader to test absorbance at 650 nm. Absorbances of the group with Matrigel were divided by the corresponding group without Matrigel to get the percentage of migration. The error bar represents the standard deviation. Student's t-test was performed to obtain p-values. *p<0.001 compared with Rama37 cells. #p<0.001 compared with Rama37-OPN cells. Data represent means ± S.D. in triplicate.

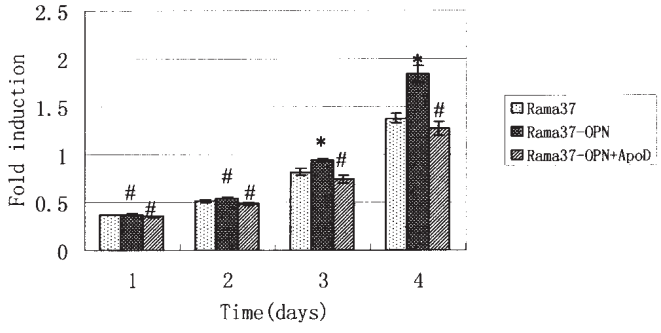

Figure 7. Effects of ApoD overexpression on proliferation of OPN-transfected Rama37 cells measured by an MTT growth assay. Rama37, Rama37-OPN and Rama37-OPN-ApoD cells were plated in 96-well plates at an initial density of 1500 cells/well. Cells were cultured for the indicated number of hours. Then MTT (20 μl of 5 mg/ml in PBS) was added to cells in each well. After 6 h, 50 μl 20% SDS solution was added to each well. After overnight incubation, the samples were read on a Thermo Max microplate reader to test absorbance at 570 nm of wavelength and background subtraction at 650 nm. Experiments were performed with an interval of 24 h over a period of 96 h and in quadruplicate. Error bar represents the standard deviation of the means. Student's t-test has been performed to obtain p-values. *p<0.001 compared with Rama37 cells, #p>0.05 compared with Rama37 cells.

Invasion assay. A critical event in tumor invasion and metastasis is the ability of tumor cells to invade through the extra-cellular matrix, thus allowing tumor cells to move beyond the confines of the primary tumor environment. OPN has been previously confirmed to be able to significantly enhance

migration of Rama37 cells (38). After co-transfection by ApoD, the ability of OPN-transfected cells to invade through the filter coated with Matrigel decreased by approximately 33% compared to the OPN transfectant alone ($p<0.001$, Student's t-test) (Fig. 6).

MTT growth assay. Carcinogenesis is linked to the development of proliferative abnormalities (39). Neoplasia has been considered to be primarily a disturbance in the regulation of proliferation (40). In this experiment, growth rates of control cells (Rama 37) and two experimental cell lines, Rama37-OPN-ApoD and Rama 37-OPN, which were stably co-transfected by ApoD and OPN expression constructs or stably transfected by OPN expression vector alone, were compared and statistically analyzed. These results showed that ApoD reduced OPN-mediated cell proliferation by approximately 20% ($p<0.001$, Student's t-test) (Fig. 7).

Discussion

In the present study, ApoD was identified as a novel co-repressor protein of OPN. ApoD was initially isolated by McConathy and Alaupovic in 1973 (41). Subsequently, it was shown that ApoD is a negative tumor marker and positive prognostic indicator for breast cancer progression (41-49). In contrast to most apolipoproteins, human ApoD is relatively small: sequence studies indicate a translation product of 189 residues composed of a 20-residue leader sequence and a mature protein of 169 amino acids, with a calculated MW of approximately 18,500 (50). Because it is 18% glycosylated, at either or both of two asparagines: positions 65 and 98 (51), ApoD has an overall MW of 29,000-32,000 (50,52). Interestingly, as an important component of high-density lipoprotein, ApoD has no marked similarity to other apolipoprotein sequences. It has a high similarity to plasma retinal-binding protein and other members of the α_2 micro-globulin protein superfamily of carrier proteins, which are also known as lipocalins (51,53).

Human ApoD is distributed widely. Its mRNA has been detected in a number of tissues including kidney, liver, pancreas, spleen, intestine, placenta, adrenal gland, and fetal brain tissue (54). The broad distribution of ApoD suggests that it may play a general role in cellular metabolism. Moreover, many of the same cell types vary dramatically in their content of ApoD in different tissues, suggesting that the uptake or secretion of ApoD by cells is tissue-specifically regulated (55).

As an identified progesterone/pregnenolone binding protein, ApoD can also bind to several small hydrophobic molecules including cholesterol, heme-related compounds, arachidonic acid and the major pheromone found in human axillary secretions, 3-methyl-2-hexenoic acid (56). The biological function of ApoD has not, however, been well established. Ligands associated with ApoD vary depending on the conditions or on the tissue or organ (57-60). For example, ApoD could act as an acute phase protein in nerve regeneration bringing specific molecules to the sites of regeneration (61). In the spleen, it could evacuate toxic heme-related molecules generated by red blood cell destruction (60). In other tissues, such as adrenal glands and testes, it could be a steroid carrier (54).

Interestingly, ApoD has recently been shown to function in cell growth regulation. Simard *et al* (62) studied the effects of steroid hormones on the proliferation of cultured human prostate cancer cells and observed a corresponding steroid-induced secretion of apolipoprotein and the inhibition of cell growth. They also noted a higher concentration of ApoD in cultured cells that were well differentiated. Peitsch and Boguski (60) found that ApoD has a hydrophobic surface that surrounds the ligand-binding pocket of the protein. In addition, the hydrophobic ligand pocket, formed by the orthogonal ß-sheets of the protein, appears to bind bilirubin. Hence they additionally speculated that ApoD might be part of the antioxidant defense system in which the conversion of heme to biliverdin and then to bilirubin serves to protect cells and tissues from oxidant damage. If ApoD does carry an antioxidant, it may also help to protect lipo-proteins from oxidant damage (60). Analysis of the potential relationship between ApoD levels in breast carcinomas and clinical outcome of patients revealed that low ApoD is associated with a shorter relapse-free survival and poorer survival in breast carcinomas (48). It has also been reported that ApoD is an indicator of good prognosis and is independent of a number of other prognostic variables (48). Additionally, Sugimoto *et al* found that ApoD secretion is inversely correlated to cell proliferation and cell density in the absence as well as in the presence of androgens in both low-passage (LP; 20-29th) and high passage (HP; 111-117th) LNCaP human prostate cancer cells (63). High concentration of ApoD was found to lead to a progressive inhibition of cell proliferation towards basal levels (62,63). Rapid tumor cell proliferation was shown to be associated with decreases in serum lipid and lipoprotein levels in cancer patients who suffered recurrence within 3 years after study initiation compared to cancer patients without recurrence (64). As one of the few examples of proteins inhibited by estrogens, the secretion of ApoD could be decreased by 40-60% by exposure to estrogen (47). Therefore it appears that stimulation of ApoD secretion is always associated with inhibition of cell proliferation. However, the precise role of ApoD in carcinomas remains unclear.

Using bacterial and mammalian two-hybrid assays and subsequent co-immunoprecipitation, ApoD protein was identified and verified to be associated directly with OPN. In these invasion assays, ApoD significantly reduced invasiveness of OPN-mediated malignant cells. Cell invasion is often associated with malignant transformation and destruction of normal surrounding tissue. The invasiveness of tumor cells represents one of several important properties necessary for the formation of metastases (65). Invasiveness *in vitro* has been correlated with the biological behaviour *in vivo* (66). It has been shown that OPN stimulates Matrigel invasion and activates the ERK and AKT/PKB signaling pathways (67,68). It was also thought that OPN facilitated invasion by up-regulation of the expression of metastasis-related molecule matrix metalloproteinase 9 (MMP-9) by activating the NF-κB pathway (69).

ApoD was found to block OPN-mediated cell adhesion and proliferation. This is consistent with previous reports (44,47) and indicates that ApoD plays an important role in breast cancer progression and metastasis. Increased adhesion enables cells to attach to vessel walls after extravasation and migration to their chosen site of metastasis. In these observations, ApoD

also significantly reversed anchorage-independent growth of OPN-transformed cells. It has previously been shown that ApoD overexpression in breast cancer cells could promote cell differentiation and growth arrest, which may partly explain its anti-malignancy effects (43,47) and its reversion on anchorage-independent growth potential of OPN-transformed cells. However, the underlying mechanism remains to be identified. The functions of ApoD on OPN may be related to Vitamin D_3. A vitamin D-responsive element is located after the interferon regulatory factor-1 (IRF-1) binding sequence on the OPN promoter region. Vitamin D_3 strongly up-regulates ApoD mRNA levels in T-47D human breast cancer cells (44). The potency of this regulation is much stronger than steroid hormones such as androgens and progesterone. It is reasonable to conclude that ApoD may regulate the expression and regulation of OPN via one of the Vitamin D_3 signaling pathways. As Matkovits reported, vitamin D_3 could reduce expression of OPN significantly. In the absence of vitamin D_3, high levels of PTH (parathyroid hormone) may induce OPN and result in higher levels of OPN mRNA (70).

Prince and Butler also demonstrated that OPN expression could be blocked by Actinomycin D in response to 1,25-dihydroxyvitamin D_3 (71). Epidemiological studies have also suggested an association of 1,25-dihydroxyvitamin D_3 deficiency with an increased risk of various malignancies, including colon and breast cancers (72). A previous study has shown that 1,25-dihydroxyvitamin D_3 can suppress proliferation and induce the differentiation of various cell types, including epithelial cells (73). A possible mechanism is that 1,25-dihydroxyvitamin D_3 could potentiate TNF-induced cytotoxicity in human cancer cells (82). Vitamin D_3 could also reduce invasiveness (74) and metastasis (75) of breast cancer cells and act as an anti-angiogenesis agent (76). ApoD possibly functions as a downstream transcription factor of Vitamin D_3 and may also regulate the level of vitamin D_3 and its functions by other currently unknown pathways.

It has been demonstrated that inhibition of breast cancer cell growth in response to 1,25-dihydroxyvitamin D_3 involves cell cycle arrest and activation of apoptosis (77-79). Interestingly, Wang *et al* (80) found that p53 is involved in 1,25-dihydroxyvitamin D_3-induced growth arrest and treatment with p53 antisense oligodeoxynucleotides could reverse the 1,25-dihydroxyvitamin D_3-induced G1/S block and result in an accumulation of cells with S-phase DNA content. Up-regulation of p53 contributes significantly to the G1/S growth arrest induced by 1,25-dihydroxyvitamin D_3 in HL60 cells (a human promyelocytic cell line) (80). Escaleira *et al* (81) also demonstrated vitamin D_3 treatment displaying strong growth inhibition and arrest in G0/G1 phase in murine HC-11 mammary cells, however, Ha-ras transformation of HC-11 cells induced down-regulation of vitamin D receptor (VDR) expression which desensitized the cells to the growth inhibitory effects of vitamin D_3. If the HC-11 mammary cells were treated by DIP (dexamethasone, insulin and prolactin) (82) to induce differentiation of HC-11 cells, the VDR level could be up-regulated. Therefore, it was concluded that mammary VDR levels co-regulated with Ha-ras (81).

In conclusion, these results revealed a novel interaction. Namely, that ApoD could be a potential anti-cancer factor via its role as an OPN co-repressor. This study is the first report concerning the inhibitory effect of ApoD on OPN-mediated malignant transformation. Collectively, ApoD was established as a novel co-repressor of OPN. The mechanism is complicated and may involve interaction with multiple signaling networks, e.g. vitamin D_3, p53 and H-ras. Elucidation of the roles for this interaction in the transcriptional regulation of OPN may provide a novel potential gene therapeutic tool for human carcinomas.

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