Dual action of apolipoprotein E-interacting HCCR-1 oncoprotein and its implication for breast cancer and obesity

Seon-Ah Ha [a], Seung Min Shin [a], Hyun Kee Kim [a], Sanghee Kim [a], Hong Namkoong [a], Youn Soo Lee [b], Hae Joo Kim [a], Sang Min Jung [a], Yu Sun Lee [a], Yeun Jun Chung [c], Yong Gyu Park [d], Sang Seol Jung [e], Jin Woo Kim [a, f, *]

[a] *Molecular Genetic Laboratory, College of Medicine, The Catholic University of Korea, Seoul, Korea*
[b] *Department of Clinical Pathology, College of Medicine, The Catholic University of Korea, Seoul, Korea*
[c] *Department of Microbiology, College of Medicine, The Catholic University of Korea, Seoul, Korea*
[d] *Department of Statistics, College of Medicine, The Catholic University of Korea, Seoul, Korea*
[e] *Department of Surgery, College of Medicine, The Catholic University of Korea, Seoul, Korea*
[f] *Department of Obstetrics and Gynecology, College of Medicine, The Catholic University of Korea, Seoul, Korea*

Abstract

Obese women have an increased risk for post-menopausal breast cancer. The physiological mechanism by which obesity contributes to breast tumourigenesis is not understood. We previously showed that *HCCR-1* oncogene contributes to breast tumourigenesis as a negative regulator of p53 and detection of HCCR-1 serological level was useful for the diagnosis of breast cancer. In this study, we found that the HCCR-1 level is elevated in breast cancer tissues and cell lines compared to normal breast tissues. We identified apolipoprotein E (ApoE) interacting with HCCR-1. Our data show that HCCR-1 inhibits anti-proliferative effect of ApoE, which was mediated by diminishing ApoE secretion of breast cancer cells. Finally, HCCR-1 induced the severe obesity in transgenic mice. Those obese mice showed severe hyperlipidaemia. In conclusion, our results suggest that HCCR-1 might play a role in the breast tumourigenesis while the overexpression of HCCR-1 induces the obesity probably by inhibiting the cholesterol-lowering effect of ApoE. Therefore, HCCR-1 seems to provide the molecular link between the obesity and the breast cancer risk.

Keywords: breast cancer ● obesity ● HCCR-1 oncogene ● apolipoprotein E

Introduction

Cells expressing HCCR-1 are tumourigenic in nude mice [1]. The functional role of HCCR-1 oncogene in tumourigenesis is manifested as a negative regulator of the p53 tumour suppressor [1]. Previous study showed that the HCCR-1 is overexpressed in breast cancer cell lines compared to the normal cells [2]. Serological studies revealed 86.8% sensitivity for HCCR-1 in breast cancer, which was higher than 21.0% for CA15–3 [2, 3]. The HCCR-1 assay has an advantage over CA15–3 in diagnosing breast cancer. These results indicate that HCCR-1 is an oncoprotein that is related to breast cancer develop-

ment [1, 2]. The HCCR-1 signalling is known to be regulated by the phosphatidylinositol 3'-kinase–AKT pathway [4].

Apolipoprotein E (ApoE) is a key regulatory protein in lipoprotein metabolism [5] and it is also a potent inhibitor of cell proliferation [6, 7]. Apo E is capable of inhibiting the growth of several tumour cell lines including breast carcinoma cells [7], melanoma cells [7] and Kaposi's sarcoma cells [6]. Recombinant human ApoE-3 inhibited human breast cancer and melanoma cell proliferation by competition for growth factor binding to proteoglycans and by an anti-adhesive activity of ApoE [7]. Despite intensive research, the anti-proliferative mechanism of ApoE is still largely unknown. In part, this is due to the emerging multiplicity of interactions of ApoE with other molecules, and by an increasingly complex cascade of ApoE-dependent signalling and metabolic pathways.

It is generally accepted that obese women have an increased risk for post-menopausal breast cancer [8–10]. Several hypotheses have been proposed to explain the association of obesity with

*Correspondence to: Jin Woo KIM,
Molecular Genetic Laboratory, College of Medicine,
The Catholic University of Korea, Seoul 137-040, Korea.
Tel.: 82-2-590-2389
Fax: 82-2-593-2389
E-mail: jinwoo@catholic.ac.kr

breast cancer [11]. One hypothesis is that the biological cause of the association between obesity and breast cancer is the elevated circulating oestrogens in obese post-menopausal women [12]. A second hypothesis is that obesity results in an increase in circulating insulin and insulin-like growth factor [13]. A newer hypothesis places adipocytes and their autocrine, paracrine and endocrine functions at centre stage. This hypothesis sets forth that obesity should be considered an endocrine tumour [11, 14]. There are still several other explanations as to the molecular mechanisms that physiologically link obesity with breast cancer risk.

In this study, we undertook the following aims: (*i*) to investigate whether HCCR-1 is elevated in breast cancer tissues compared to normal breast tissues, (*ii*) to identify HCCR-1 binding protein and (*iii*) to generate transgenic mice containing oncogene *HCCR-1* to analyse the phenotype and to characterize the role of HCCR-1 in cellular events.

Materials and methods

Tissues and cell lines

Human normal and cancer tissues were obtained during operation. All patients were subjected to the analysis with individual consent for the study. The use of tissue samples was approved by the Ethics Committee of our institution. Mammalian cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). BT-474, MCF-7 and MDA-MB-231 are human breast carcinoma cell lines from mammary gland.

Human embryonic kidney (HEK) 293 (ATCC CRL-1573) and COS-7 (ATCC CRL-1651) cells were obtained from the ATCC. HEK293/HCCR-1-V5 cell line [1], which stably expressed V5 tagged HCCR-1 in HEK293 cells, was maintained in DMEM (Gibco BRL, Rockville, MD, USA) containing 200 μg/ml G418, 10% FBS and 1% PenStrep (Gibco). COS-7 cell line was maintained in RPMI supplemented with 10% FBS and 1% PenStrep.

Western blot analysis

Samples in 1× SDS sample buffer were subjected to electrophoresis in an SDS-PAGE and transferred to nitrocellulose by standard procedures. After washing with 0.05% Tween 20 in TBS, the membranes were incubated with primary antibodies, anti-V5 (Invitrogen, Carlsbad, CA, USA) or anti-VDAC1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies. After washing with 0.05% Tween 20 in TBS, immunoblots were incubated with horseradish-peroxidase-conjugated rabbit anti-mouse IgG or goat anti-rabbit IgG (KPL, Gaithersburg, MD, USA), and proteins were visualized using enhanced chemiluminescence detection kit according to the manufacturer's instructions (PIERCE, Rockford, IL, USA).

Fluorescence microscopy

COS-7 and HEK293/HCCR-1-V5 cells were transiently transfected using LipofectAMINE 2000 (Invitrogen). After 24–28 hrs of further incubation, 100 nM MitoTracker (Molecular Probes, Eugene, OR, USA) was added to the medium and washed three times with 2 ml phosphate buffer solution (PBS) before fixation. To stain V5 tagged HCCR-1, HEK293/HCCR-1-V5 cells were fixed with 100% methanol and incubated with mouse anti-V5 antibody (Invitrogen). They were washed with PBS, Alexa Fluor 488 goat anti-mouse IgG antibody (Molecular Probes) at a dilution of 5 μg/ml was applied for 1 hr. The cells were mounted using ProLong Gold Antifade Reagents (Molecular Probes). Fluorescent images were analysed and taken using a Bio-Rad MRC-1024MP laser scanning confocal microscope (Bio-Rad, Hercules, CA, USA).

Construction of expression vector and DNA transfection

Expression vector containing the coding region of *HCCR-1* or *ApoE* was constructed as follows. First, the *Sal*I fragment was isolated from the prokaryotic expression vector, pCEV-LAC, which contains the *HCCR-1* or *ApoE* cDNA. Then, pcDNA3.1-V5-His (Invitrogen) or pcDNA3.1-Myc-His was digested with *BamH* I and *Sal*I to make a compatible end with *Sal*I. A *Sal*I fragment containing the *HCCR-1* or *ApoE* coding sequence was inserted into the *Xho*I-digested pcDNA3.1. Lipofectamine 2000 (Gibco) was used to introduce the *HCCR-1* or *ApoE* expression vector into breast cancer cells. Selected transfectants were screened for the expression of HCCR-1 or ApoE by Western blot analysis.

Subcellular fractionation

The isolation of mitochondrial fractions of HEK293/HCCR-1 and HEK293/ApoE was carried out according to the mitochondria isolation kit (PIERCE). Briefly, cells were harvested by centrifuging at 850 *g* for 2 min. and the pellet was suspended with 800 μl of reagent A, and then incubated exactly 2 min. on ice. Next 10 μl of Reagent B was added to the suspended solution and incubated for 5 min. on ice with vortexing at maximum speed every minute. Then 800 μl of Reagent C was added to the solution and inverted the tube several times to mix. The solution was centrifuged at 700 *g* for 10 min. at 4°C, and the pellet was used for crude nucleic fraction. The supernatant was continuously centrifuged at 12,000 *g* for 15 min. at 4°C and transferred to a new tube for the post-mitochondrial supernatant fraction. The pellet was washed with 500 μl of Regent C, and used for isolation of the mitochondrial fraction. The isolated mitochondria were resuspended in 200 μl of freshly prepared 100 mM sodium carbonate pH 11.5 and incubated on ice for 30 min., and centrifuged at 100,000 *g* for 30 min. at 4°C. The supernatant was used as a mitochondrial matrix fraction. The pellet was resuspended with the same volume (200 μl) of 1× SDS loading buffer and used as a mitochondrial membrane fraction.

Haematoxylin-eosin staining

All paraffin blocks were processed and cut into 4-μm-thick sections. The sections were stained with routine haematoxylin and eosin and were studied to ascertain the morphology of the lesion.

Co-transfection and immunoprecipitation

Cells were transfected with pcDNA3.1 (Invitrogen) encoding *HCCR-1*-V5-His (Invitrogen) fusion protein and ApoE-Myc-His (Invitrogen). After 48 hrs,
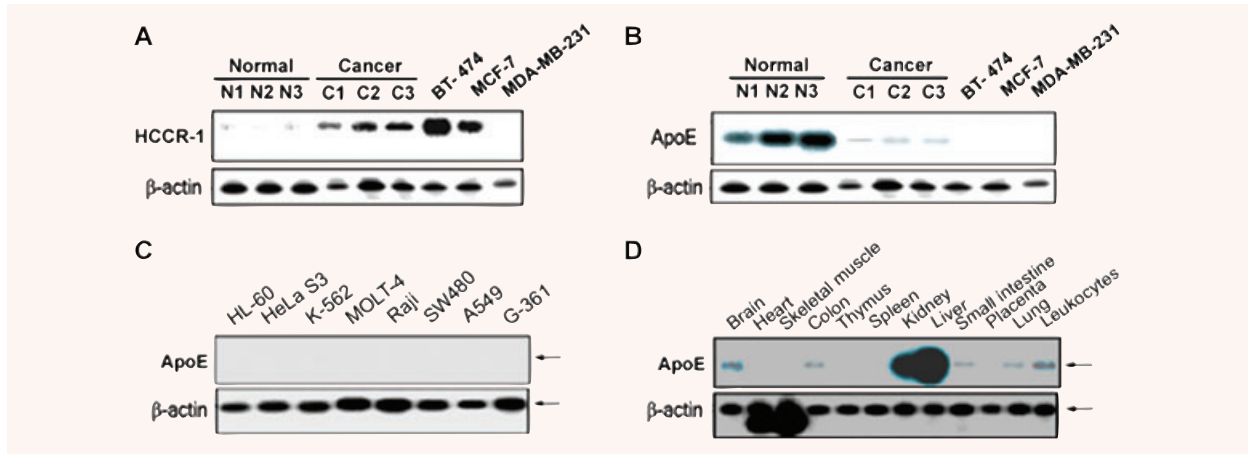
Fig. 1 HCCR-1 and ApoE expressions in human tissues. (**A, B**) Northern analyses of HCCR-1 (**A**) and ApoE (**B**) in human breast tissues and cell lines. Comparison of *HCCR-1* mRNA expressions in breast cancer cell lines (BT-474, MCF-7 and MDA-MB-231), and fresh primary breast cancer tissues (**C**) and their corresponding normal counterparts (N). Human β-actin cDNA was used as a control probe (bottom panel). (**C, D**) mRNA expression levels of ApoE in various normal human tissues and cancer cell lines. Northern-blots consisting of 8 human cancer cell lines (**C**) and 12 normal human tissues (**D**) were probed with radioactively labelled *ApoE* cDNA (top panel).

the cells were harvested and lysed with lysis buffer. The lysates were pre-cleared with preimmune serum (mouse) and protein A-Sepharose at 4°C for 30 min. Aliquots (1 mg) of precleared cell lysates were incubated with a 1:500 dilution of anti-V5 (Invitrogen) or 1:250 dilution of anti-Myc (Invitrogen) mAb and 40 ml of a 1:1 slurry of protein A-Sepharose beads (Amersham Biosciences, Uppsala, Sweden) in PBS for 16 hrs at 4°C. The immune complexes were subjected to SDS-PAGE and Western blot analysis with a 1:1000 dilution of anti-Myc or 1:3000 dilution of anti-V5 antibodies in TBS.

Detection of ApoE in culture supernatants by sandwich ELISA

To study the kinetics of ApoE production during proliferating phase of breast cancer cells, 10^6 cells were layered in flasks in DMEM for 8 days. The ApoE concentration was determined by sandwich ELISA using a commercially available kit (MBL ApoE4/Pan-ApoE ELISA kit, MBL Co., Woburn, MA, USA). MBL ELISA Kit measures Human ApoE4 or Pan-ApoE by sandwich ELISA. The assay uses affinity purified polyclonal antibody against ApoE and monoclonal antibody against ApoE4.

Production of transgenic mice

Transgenic mice were generated using standard pronuclear microinjection as previously described [15]. For microinjection, the fragment of transgene, HCCR-1, was separated free from the vector backbone of pcDNA3.1-V5-His by *Nru I* and *Xmn I* double digestion. The injected fragments of CMV-HCCR-1-bGH were isolated and microinjected into the pronuclei of one cell-stage fertilized embryos from C57BL/6N (Charles River, Japan). Then 20–25 injected DNA fertilized eggs that survived microinjection were implanted into the oviducts of one pseudopregnant recipient CD-1 (Charles River, Japan) mice.

Statistical analysis

Analyses were performed using the Statistical Analysis System (SAS, version 8.2, SAS Institute, Cary, NC, USA). Body weight and serum levels of ApoE, total cholesterol, HDL, LDL, triglyceride, leptin and insulin were presented as mean ± S.D. in each seven mice and differences between/among groups were compared by t-test, ANOVA and Dunnett's multiple comparison. Statistical significance was determined by $P < 0.05$.

Results and discussion

Expression pattern of HCCR-1 and ApoE is mutually exclusive in breast tissues

We examined the expression patterns of HCCR-1 or ApoE in human normal tissues, cancer tissues and cell lines. Northern blot analysis revealed an increased expression of *HCCR-1* in primary human breast cancer tissues and breast cancer cell lines compared to normal breast tissues (Fig. 1A). In contrast to the overexpression of HCCR-1 in breast cancer tissues and cell lines, expression of ApoE was little or absent in breast cancer tissues and cell lines (Fig. 1B). Thus, the expression of ApoE and HCCR-1 seems to be opposite. We also examined the expression of ApoE in other cancer cell types. ApoE mRNA was not detected in promyelocytic leukaemia cell HL-60, human cervical cancer cell HeLa, chronic myelogenous leukaemia cells K562, lymphoblastic leukaemia cell MOLT-4, Burkitt's lymphoma cell Raji, colon cancer cell SW480, lung cancer cell A549 and melanoma cell G361 (Fig. 1C). However, northern blot analysis with several normal

tissues revealed that normal liver and kidney tissues have an abundant expression of ApoE among 12 normal tissues tested (Fig. 1D).

Interaction of HCCR-1 with ApoE and co-localization of HCCR-1 and ApoE in the mitochondria

To understand the functional role of HCCR-1 at the molecular level, we performed a yeast two-hybrid screen and identified the ApoE protein [5], encoded by the gene *ApoE* (GenBank accession number BC003557.1), as an interacting partner for HCCR-1. ApoE is a key regulatory protein in lipoprotein metabolism [5] and it is also a potent inhibitor of cell proliferation [6, 7]. ApoE is capable of inhibiting the growth of several tumour cell lines including breast carcinoma cells, melanoma cells and Kaposi's sarcoma cells. Despite intensive research, the anti-proliferative mechanism of ApoE is still largely unknown. In part, this is due to the emerging multiplicity of interactions of ApoE with other molecules, and to an increasingly complex cascade of ApoE-dependent signalling and metabolic pathways.

The interaction of HCCR-1 and ApoE identified by yeast two-hybrid screen was further confirmed by immunoprecipitation studies *in vitro* (Fig. 2A). To do this, we transfected ApoE-Myc fusion construct into MCF-7 cells expressing HCCR-1 fused to V5 tag. The HCCR-1 protein was specifically co-precipitated with ApoE in the MCF-7 co-transfected cells (Fig. 2A). In the experiments performed above, we used Myc and V5 antibodies with cells overexpressing HCCR-1 and ApoE. Endogenous HCCR-1 in MCF-7 cells interacted with transfected ApoE in a co-immunoprecipitaion assay (data not shown). Together with yeast two-hybrid data, this result strongly supports that ApoE is physically interacting with HCCR-1.

We next performed subcellular fractionation experiments to investigate the localization of HCCR-1 and ApoE in MCF-7 cells (Fig. 2B). The stably expressed HCCR-1 protein in the MCF-7 cells was detected only in the mitochondria fraction (Fig. 2B; top panel), whereas ApoE was found only in the cytosol fraction in MCF-7 cells stably expressing ApoE (Fig. 2B; bottom panel). Intriguingly, however, subcellular fractionation assay with MCF-7 cells co-expressing HCCR-1 and ApoE revealed the co-localization of these two proteins in the mitochondria fraction, while trace amount of ApoE still remained in cytosol (Fig. 2B; bottom panel). These results indicate that when ApoE is co-expressed with HCCR-1 in MCF-7 cells, ApoE protein might be reshuffled to the mitochondria from the cytoplasm. Fluorescence images also revealed HCCR-1 (Fig. 2C; left-hand panel) and ApoE, in the mitochondria and the cytosol, respectively (Fig. 2C; right-hand panel).

ApoE plays a tumour suppressor role in breast cancer and HCCR-1 negatively regulates ApoE function in breast cancer

To verify the growth inhibitory role of ApoE, ApoE was transfected into breast cancer cells. The ApoE transfection induced about 87%

growth inhibition in MCF-7 cells compared with those transfected with vector alone at day 7 post-transfection (Fig. 3A). On the contrary, the growth rate of HCCR-1-transfected MCF-7 cells was increased by about 45% at day 7 post-transfection compared to controls (Fig. 3A). However, when ApoE was co-transfected into the *HCCR-1*-expressing MCF-7 cells, the growth was attenuated by about 66%. Likewise, ApoE-induced growth inhibition was reversed when HCCR-1 was overexpressed. This inhibitory effect of ApoE on the growth of breast cancer cells was associated with apoptotic processes such as DNA fragmentation (Fig. 3B). Therefore, these results suggest that HCCR-1 may inhibit anti-proliferative and/or possibly tumour suppressive effect of ApoE on breast cancer, thereby playing a causative role in breast cancer.

To investigate the molecular mechanism by which HCCR-1 inhibits anti-proliferative ability of ApoE on breast cancer cells, we evaluated the effect of HCCR-1 on ApoE secretion in MCF-7 cells. To do this, we compared the Pan-ApoE levels secreted from ApoE-transfected MCF-7 cells. ApoE secreted into the culture media was measured by ApoE4/Pan-ApoE sandwich ELISA kit. After 24 hrs of incubation with DMEM, the medium was harvested and the Pan-ApoE concentration was determined. We found that Pan-ApoE was secreted in small amounts (28.4 ± 0.1 ng/ml) from wild-type cells and it was increased when ApoE was transfected into cells (43.0 ± 0.6 ng/ml) (Fig. 3C) ($P < 0.05$). Synthetic siRNA (HCCR-1 siRNA-2) was designed using the HiPerformance algorithm (Qiagen GmbH, Germany). HCCR-1 siRNA-2 corresponding to nucleotides 579–600 within exon 5 of HCCR-1 enhanced Pan-ApoE secretion in ApoE-transfected MCF-7 cells and wild-type MCF-7 cells. In ApoE-transfected MCF-7 cells, Pan-ApoE secretion was significantly increased by HCCR-1 siRNA-2 (54.5 ± 0.2 ng/ml) compared to control, non-silencing siRNA (43.0 ± 0.3 ng/ml) ($P < 0.05$). It was also higher in wild-type MCF-7 cells transfected with siRNA-2 (42.4 ± 0.3 ng/ml) than those with control, non-silencing siRNA (28.3 ± 0.3 ng/ml) ($P < 0.05$) (Fig. 3D). Thus, this result suggests that inhibition of ApoE function by HCCR-1 is mediated by reducing the ApoE levels secreted from the breast cancer cells.

HCCR-1 interacting with ApoE induced the severe obesity in transgenic mice

To investigate whether overexpression of HCCR-1 exerts any role related to ApoE function, we generated transgenic mice (T/G) for HCCR-1. The overexpression of HCCR-1 in T/G induced the severe obesity in T/G. Obese mice were bred for more than five generations and weighed three times more than normal mice with the same age and gender (Fig. 4A). Of note, obesity was more severe in male T/G (Fig. 4A; left-hand panel) than female T/G (Fig. 4A; right-hand panel) of the same age. Obese male mice weighed 62.2 ± 3.7 g compared to normal male mice (24.5 ± 1.0 g) (Fig. 4A; left-hand panel) ($P < 0.0001$). There were also significant differences in weight between female obese mice (43.1 ± 1.3 g) and normal female mice (21.7 ± 1.5 g) (Fig. 4A; right-hand panel) ($P < 0.0001$).
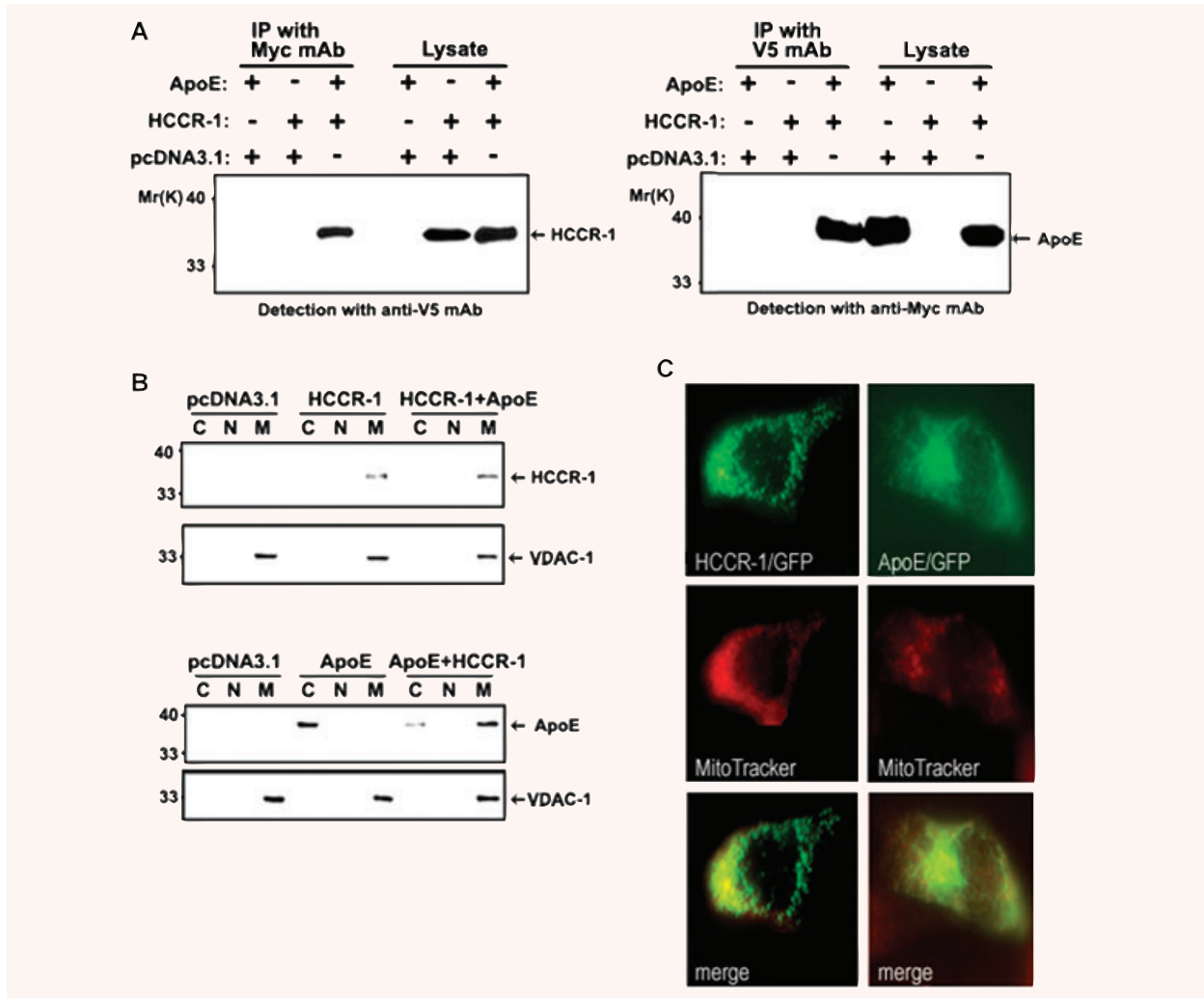
Fig. 2 Interaction of HCCR-1 with ApoE and their co-localization in the mitochondria. (**A**) Co-immunoprecipitation experiment of HCCR-1 and ApoE showing the direct interaction between HCCR-1 and ApoE. Cell lysate from MCF-7 cells expressing either HCCR-1-V5 or ApoE-Myc, or both was immunoprecipitated with mAb specific for Myc and HCCR-1 was detected by Western blotting using V5 mAb (left-hand panel). Likewise, ApoE interacting with HCCR-1 was detected by Western blotting using Myc mAb after co-immunoprecipitation with V5 mAb (right-hand panel). (**B**) Subcellular fractionation of HCCR-1 and ApoE in HEK293 cells. Cytosolic (C), nuclear (N) and mitochondrial (M) fractions were prepared from HEK293 cells transfected with HCCR-1, ApoE or both and analysed by SDS-PAGE and immunoblotting using antibodies, V5 and c-Myc for HCCR1 (top panel) and ApoE (bottom panel), respectively. The Voltage Dependant Anion Channel 1 (VDAC1) was used as a mitochondrial marker. (**C**) Fluorescence microscopy. Cells were transiently transfected with pEGFP-HCCR-1 (left-hand panel) and pEGFP-ApoE (right-hand panel) using LipofectAMINE 2000 (Invitrogen). Cells were incubated with 25 nM MitoTracker (Molecular Probes). Cells were then mounted using ProLong Gold Antifade Reagents. Fluorescent images were analysed by a Bio-Rad MRC-1024MP laser scanning confocal microscope (Bio-Rad).

Obese mice showed pathological defects, including omentum, liver, pancreas and heart (Fig. 4B). In the transgenic male, the omentum showed a large volume of fat and hypertrophy of adipocytes (Fig. 4B; top panel). The liver showed diffuse microvesicular and macrovesicular fatty change in the hepatocytes (Fig. 4B; top panel). The pancreas of transgenic male revealed islet cell hyperplasia of Langerhans islands, which had increased number and size of them (Fig. 4B; top panel). The heart valves showed moderate myxoid change and hypertrophy (Fig. 4B; top panel). Compared with transgenic male, the transgenic female showed the mildly increased cell size and volume of omentum (Fig. 4B; second panel). In addition, the islet cell hyperplasia of pancreas and the myxoid change of heart valve appeared less pronounced (Fig. 4B; second panel). These defects were not observed in normal control male mice (Fig. 4B; bottom panel).
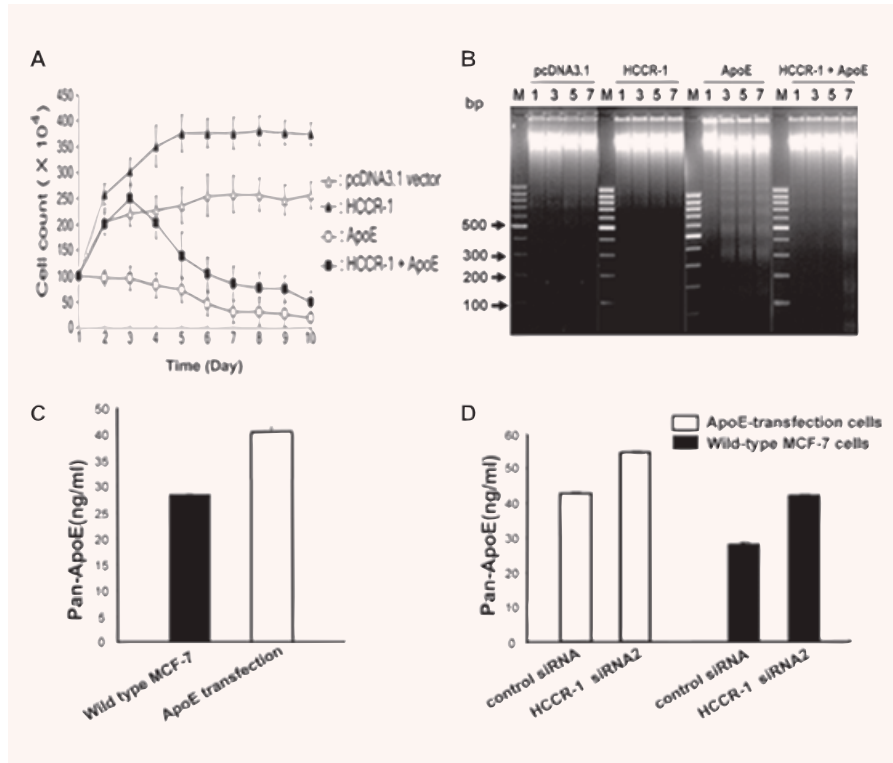
Fig. 3 HCCR-1 negatively regulates ApoE function in breast cancer. (**A, B**) Anti-proliferative effect of ApoE in breast cancer. (**A**) MCF-7 cells were transfected with HCCR-1, ApoE or both and vector as a negative control. The data are the number of viable cells grown for 10 days in culture and represent the mean ± S.D. of triplicate determinations. (**B**) Transfection with *ApoE* cDNA induces apoptosis in breast cancer cells. Electrophoretic analysis of DNAs from MCF-7 cells transfected with vector alone, HCCR-1, ApoE or both. Cells were cultured for 1, 3, 5 and 7 days, respectively, and their genomic DNAs were analysed on 2% agarose gel stained with ethidium bromide. (**C, D**) ApoE secretion by MCF-7 cells. ApoE was measured in MCF-7 cells by ELISA, as described in 'Materials and Methods.' Results are represented as mean ± S.D. of triplicate determinations. The ApoE concentration was determined by sandwich ELISA using a commercially available kit (MBL ApoE4/Pan-ApoE ELISA kit, MBL Co.).

The expression profile of HCCR-1 and ApoE was contrasting to one another on the Western blot consisting of several tissues derived from control and T/G (Fig. 4C). For example, ApoE was strongly expressed on tissues from control mice such as brain, lung, liver, kidney, intestine and omentum in which HCCR-1 expression is very low. On the other hand, HCCR-1 expression was up-regulated on the same tissues derived from T/G in which ApoE expression was severely decreased (Fig. 4C). Thus, this mutually exclusive expression pattern of HCCR-1 and ApoE reminiscent of the data shown in Fig. 1A and B might indicate that they are regulated differently but in a coordinated manner by signalling pathways, eventually leading to opposite biological consequences.

There is also accumulating evidence implicating the role for serum lipids in many cancers and other chronic diseases [16–18]. In general, low serum cholesterol levels and high serum triglycerides have been implicated as possible biological markers for increased susceptibility to cancers of the colon, lung, prostate and breast. High serum triglyceride levels were positively associated with breast cancer risk [16–18]. Based on this, we examined various serum lipid levels in obese mice. The levels of total cholesterol, HDL cholesterol, LDL cholesterol and triglyceride in HCCR-1 transgenic obese male mice were increased about 4.2, 4.0, 3.8 and 2.7 times more than normal control mice of same age, respectively (Fig. 4D). The levels of total cholesterol (184.8 ± 5.4 mg/dl), HDL cholesterol (152.9 ± 0.4 mg/dl), LDL cholesterol (46.7 ± 0.7 mg/dl) and triglyceride (21.9 ± 2.3 mg/dl) in HCCR-1 transgenic obese male mice were significantly higher than those in normal

male mice (total cholesterol 43.8 ± 1.0 mg/dl, HDL cholesterol 38.4 ± 1.0 mg/dl, LDL cholesterol 12.3 ± 0.6 mg/dl and triglyceride 8.0 ± 0.1 mg/dl, respectively) ($P < 0.05$) (Fig. 4D). But there were no significant differences in ApoE, leptin and insulin secretions between obese mice and normal control mice of same age (Fig. 4D). The levels of ApoE in obese male and female mice, and control mice were 0.9 ± 0.06 mg/dl, 0.9 ± 0.05 mg/dl and 1.4 ± 0.30 mg/dl, respectively (Fig. 4D). And the levels of leptin were 0.3 ± 0.02 ng/dl, 0.3 ± 0.03 ng/dl and 0.3 ± 0.01 ng/dl in obese male and female mice, and control mice, respectively (Fig. 4D). Serum insulin levels were not different between obese mice and normal control mice (Fig. 4D). The levels of insulin in obese male and female mice, and control mice were 2.3 ± 0.6 μIU/ml, 2.0 ± 0.3 μIU/ml and 2.0 ± 0.6 μIU/ml, respectively. Therefore, our *in vivo* data using T/G mice demonstrate that HCCR-1 might be a key regulator of lipid metabolism probably by controlling ApoE function such as cholesterol-lowering effect.

Obesity is associated with higher-grade breast cancers, especially in black women [19]. In addition, obesity affects changes in hormones, which influence breast cancer growth, including oestrogens, androgens, insulin-like growth factors and insulin [20]. Although attention has been reasonably focused on oestrogen as a potential mediator of the obesity influence on breast cancer [21], emerging evidence suggests that insulin levels also could play a role. Both higher fasting insulin levels and obesity independently predict a significantly increased risk of recurrence and decreased survival in a breast cancer cohort [13]. In

Fig. 4 HCCR-1 interacting with ApoE induced the severe obesity in T/G. (**A, B**) Phenotypic analyses of HCCR-1 T/G. (**A**) The obesity of T/G. T/G were generated using standard pronuclear microinjection. For microinjection, the fragment of transgene, CMV-HCCR-1-bGH, was microinjected into the pronuclei of one cell-stage fertilized embryos from C57BL/6N (Charles River, Japan). Obese T/G and non-T/G are shown together with their body weights represented as the mean ± S.D. of 7-times determinations. (**B**) Haematoxylin-eosin staining of omentum, liver, pancreas and heart taken from the male (top panel) and female TG (second panel) together with the control male non-TG (bottom panel). Original magnification × 200. (**C**) Expression levels of ApoE and HCCR-1 in various organs of obese T/G and control mice. To detect the ApoE and HCCR-1 expressions, mAbs (A1.4) raised against ApoE of human origin (Santa Cruz biotechnology) and HCCR-1 polyclonal Ab were used in the immunoblot consisting of multiple tissues taken from T/G and non-T/G such as brain, lung, liver, kidney, intestine and omentum. (**D**) Serum profiles of cholesterol, triglyceride, ApoE, leptin and insulin in obese and control mice. Results are expressed as mean ± S.D. of triplicate determinations.
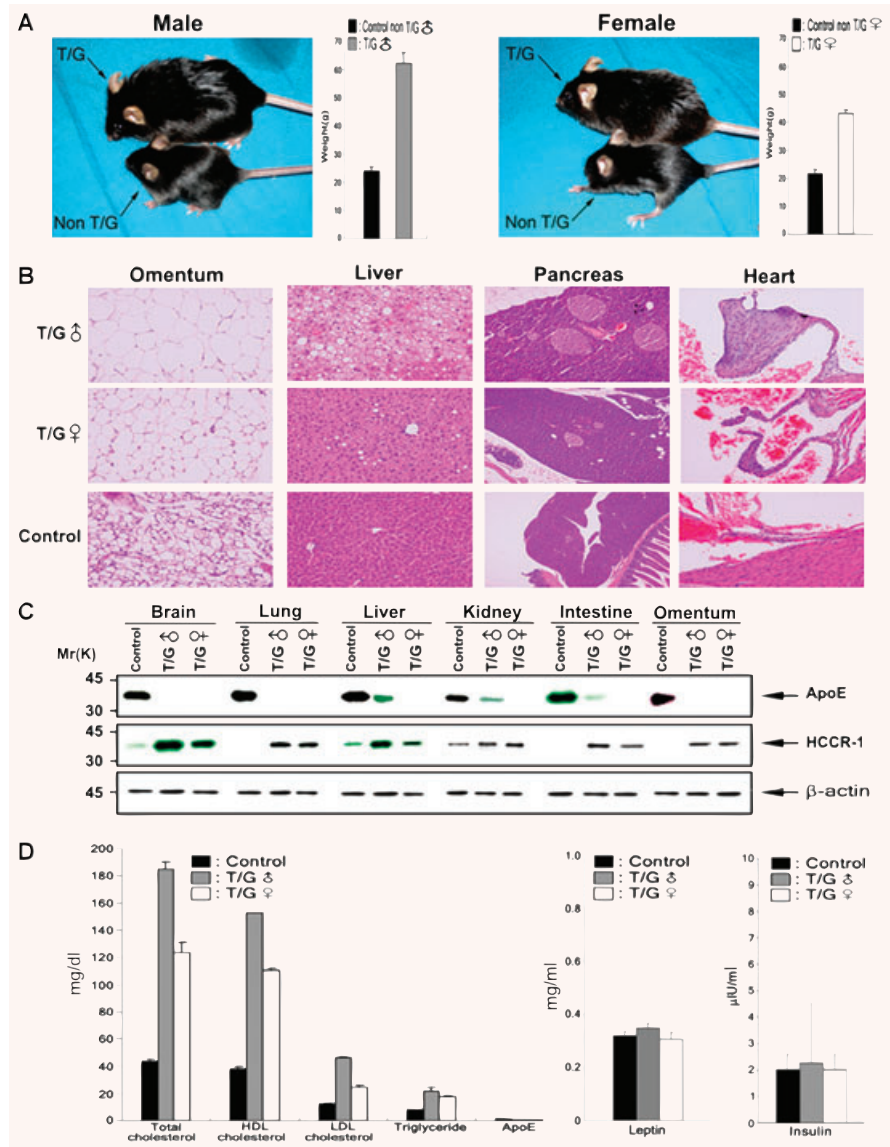
addition, a recent report found obesity was most strongly related to mortality in women with oestrogen receptor-negative breast cancers, suggesting that factors other than oestrogen mediate the effect [22, 23].

In this study, we found that the molecular mechanism by which HCCR-1 contributes to the breast cancer involves inhibiting the anti-proliferative role of the ApoE probably through the direct association. Our findings that HCCR-1 interacts with ApoE, a key regulator of lipid metabolism and HCCR-1 T/G exhibit the pathological defects related to the obesity provide the molecular evidences for the association of the obesity with a breast cancer.

In conclusion, HCCR-1 might play an important role in the breast tumourigenesis while the overexpression of HCCR-1 induces the obesity probably by inhibiting the cholesterol-lowering effect of ApoE. Considering the point that the obesity is becoming a cause of breast cancer, our studies on the functional relationship between HCCR-1 and ApoE shed a light on understanding the pathological relation of breast cancer and obesity, and ultimately might lead to developing therapeutic agents such as ones targeting ApoE-HCCR-1 interaction.

Acknowledgements

References

1. **Ko J, Lee YH, Hwang SY, *et al*.** Identification and differential expression of novel human cervical cancer oncogene HCCR-2 in human cancers and its involvement in p53 stabilization. *Oncogene.* 2003; 22: 4679–89.

2. **Jung SS, Park HS, Lee IJ, *et al*.** The HCCR oncoprotein as a biomarker for human breast cancer. *Clin Can Res.* 2005; 11: 7700–8.

3. **Lamerz R, Stieber P, Fateh-Moghadam A**. Serum marker combinations in human breast cancer (review). *In Vivo.* 1993; 7: 607–14.

4. **Cho GW, Shin SM, Namkoong H, *et al*.** The phosphatidylinositol 3-kinase/Akt pathway regulates the *HCCR-1* oncogene expression. *Gene.* 2006; 384: 18–26.

5. **Mahley RW**. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science.* 1988; 240: 622–30.

6. **Browning PJ, Roberts DD, Zabrenetzky V, *et al*.** Apolipoprotein E (ApoE), a novel heparin-binding protein inhibits the development of Kaposi's sarcoma-like lesions in BALB/c nu/nu mice. *J Exp Med.* 1994; 180: 1949–54.

7. **Vogel T, Guo NH, Guy R, *et al*.** Apolipoprotein E: a potent inhibitor of endothelial and tumor cell proliferation. *J Cell Biochem.* 1994; 54: 299–308.

8. **Huang Z, Hankinson SE, Colditz GA, *et al*.** Dual effects of weight and weight gain on breast cancer risk. *JAMA.* 1997; 278: 1407–11.

9. **van den Brandt PA, Spiegelman D, Yaun SS, *et al*.** Pooled analysis of prospective cohort studies on height, weight, and breast cancer risk. *Am J Epidemiol.* 2000; 152: 514–27.

10. **Lahmann PH, Hoffmann K, Allen N, *et al*.** Body size and breast cancer risk: findings from the European Prospective Investigation into Cancer And Nutrition (EPIC). *Int J Cancer.* 2004; 111: 762–71.

11. **Lorincz AM, Sukumar S.** Molecular links between obesity and breast cancer. *Endocr Relat Cancer.* 2006; 13: 279–92.

12. **van Landeghem AA, Poortman J, Nabuurs M, *et al*.** Endogenous concentration and subcellular distribution of estrogens in normal and malignant human breast tissue. *Cancer Res.* 1985; 45: 2900–6.

13. **Goodwin PJ, Ennis M, Pritchard KI, *et al*.** Fasting insulin and outcome in early stage breast cancer: results of a prospective cohort study. *J Clin Oncol.* 2002; 20: 42–51.

14. **Dizdar O, Alyamac E**. Obesity: an endocrine tumor? *Med Hypotheses.* 2004; 63: 790–2.

15. **Gordon JW, Ruddle FH.** Gene transfer into mouse embryos: production of transgenic mice by pronuclear injection. *Methods Enzymol.* 1983; 101: 411–33.

16. **Vatten LJ, Foss OP**. Total serum cholesterol and triglycerides and risk of breast cancer: a prospective study of 24,329 Norwegian women. *Cancer Res.* 1990; 50: 2341–6.

17. **Kumar K, Sachdanandam P, Arivazhagan R.** Studies on the changes in plasma lipids and lipoproteins in patients with benign and malignant breast cancer. *Biochemistry.* 1991; 23: 581–9.

18. **Agurs-Collins T, Kim KS, Dunston GM, *et al*.** Plasma lipid alterations in African-American women with breast cancer. *J Cancer Res Clin Oncol.* 1998; 124: 186–90.

19. **Chlebowski RT, Chen Z, Anderson G**. Ethnicity and breast cancer in the Women's Health Initiative: a unifying concept for unfavorable outcome in African American women. *Proc Am Soc Clin Oncol.* 2004; 23: 99s.

20. **Chlebowski RT, Aiello E, McTiernan A**. Weight loss in breast cancer patient management. *J Clin Oncol.* 2002; 20: 1128–43.

21. **Key TJ, Appleby PN, Reeves GK, *et al*.** Body mass index, serum sex hormones, and breast cancer risk in postmenopausal women. *J Natl Cancer Inst.* 2003; 95: 1218–26.

22. **Enger SM, Greif JM, Polikoff J, *et al*.** Body weight correlates with mortality in early stage breast cancer. *Arch Surg.* 2004; 139: 954–60.

23. **Chlebowski RT.** Obesity and early-stage breast cancer. *J Clin Oncol.* 2005; 23: 1345–7.