Induction of fibroblast apolipoprotein E expression during apoptosis, starvation-induced growth arrest and mitosis

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Apolipoprotein E (apoE) mediates the hepatic clearance of plasma lipoproteins, facilitates cholesterol efflux from macrophages and aids neuronal lipid transport. ApoE is expressed at high levels in hepatocytes, macrophages and astrocytes. In the present study, we identify nuclear and cytosolic pools of apoE in human fibroblasts. Fibroblast apoE mRNA and protein levels were up-regulated during staurosporine-induced apoptosis and this was correlated with increased caspase-3 activity and apoptotic morphological alterations. Because the transcription of apoE and specific pro-apoptotic genes is regulated by the nuclear receptor LXR (liver X receptor) α , we analysed LXR α mRNA expression by quantitative real-time PCR and found it to be increased before apoE mRNA induction. The expression of ABCA1 (ATP-binding cassette transporter A1) mRNA, which is also regulated by LXR α , was increased in parallel with apoE mRNA, indicating that LXR α probably promotes apoE and ABCA1 transcription during apoptosis.

Fibroblast apoE levels were increased under conditions of serum-starvation-induced growth arrest and hyperoxia-induced senescence. In both cases, an increased nuclear apoE level was observed, particularly in cells that accumulated lipofuscin. Nuclear apoE was translocated to the cytosol when mitotic nuclear disassembly occurred and this was associated with an increase in total cellular apoE levels. ApoE amino acid sequence analysis indicated several potential sites for phosphorylation. *In vivo* studies, using ³²P-labelling and immunoprecipitation, revealed that fibroblast apoE can be phosphorylated. These studies reveal novel associations and potential roles for apoE in fundamental cellular processes.

Key words: apolipoprotein E, apoptosis, fibroblast, growth-arrest, mitosis, phosphorylation, senescence.

INTRODUCTION

Apolipoprotein E (apoE) is a ~34 kDa glycoprotein that plays an important role in lipoprotein metabolism and cellular lipid transport. ApoE is expressed at high levels in macrophages, hepatocytes and astrocytes, and is detectable in most peripheral tissues [1]. As a component of triacylglycerol-rich lipoproteins, liver-derived apoE mediates endocytosis of lipoproteins via the LDL (low-density lipoprotein) receptor and the LRP (LDL-receptor-related protein) [2]. In humans, three apoE isoforms exist, E2, E3 and E4, which differ in their cysteine/arginine composition at positions 112 and 158 [3]. The apoE2 isoform (when associated with type III hyperlipoproteinaemia) and apoE4 isoform are associated with increased risk of atherosclerosis [3]. The complete lack of apoE expression in apoE gene knockout (apoE^{-/-}) mice results in severe atherosclerosis and neurological abnormalities, including memory and learning defects [4,5].

Although apoE plays a beneficial role in lipid mobilization and redistribution during neuronal growth and repair [1,6], the apoE4 isoform and its 22 kDa N-terminal peptide promote A β amyloid-induced neurotoxicity [7,8]. ApoE4 appears to destabilize lysosomes [8], which is known to induce apoptosis under a variety of circumstances [9,10]. Animal studies indicated that apoE is essential for amyloid deposition in the APP(V717F) (with a Val⁷¹⁷ → Phe mutation) transgenic mouse model of Alzheimer's disease [11]. The formation of both neuritic and cerebrovascular

plaques occurred in these mice when crossed with animals that carried the normal mouse apoE gene, but not in those crossed with apoE^{-/-} mice [12]. Interestingly, expression of human apoE3, but not apoE4, protected against the age-dependent neurodegeneration seen in apoE^{-/-} mice [13]. While the effects of the apoE4 isoform on neuronal integrity relate to the increased risk of Alzheimer's disease, and to the poor outcome after head trauma and stroke [14], the mechanisms responsible for these associations are not completely understood. A better understanding of apoE expression and function in normal and pathological conditions is required in order to provide further insight into disease mechanisms.

Early studies failed to detect apoE in human fibroblasts [15]. Recent data indicate, however, that apoE may be synthesized by fibroblasts under specific circumstances. For example, fibroblasts derived from injured frog nerves produce apoE [16], and immortalized fibroblasts increase apoE mRNA expression after growth arrest induced by serum starvation [17]. The aim of the present study was to investigate the potential presence of apoE in human fibroblasts and determine its intracellular location and conditions under which its expression may be altered. Several novel findings are described, including the identification of a nuclear pool of apoE, the up-regulation of apoE levels during apoptosis, serum-starvation-induced growth arrest and mitosis. We also demonstrate that fibroblast apoE has the capacity to be phosphorylated.

Abbreviations used: ABCA1, ATP-binding cassette transporter A1; AMC, 7-amino-4-methylcoumarin; apoE, apolipoprotein E; apoJ, apolipoprotein J; DAPI, 4,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; EMEM, Eagle's minimum essential medium; ER, endoplasmic reticulum; FCS, foetal calf serum; LDL, low-density lipoprotein; LRP, LDL-receptor-related protein; LXR, liver X receptor; PTP, protein tyrosine phosphatase; RT, reverse transcription; RXR, retinoid X receptor; SB, saponin buffer; SP1, specificity protein 1; SREBP, sterol-regulatory-element-binding protein.

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EXPERIMENTAL

Cell-culture conditions

Human foreskin fibroblasts from a normal subject (AG01518) and a patient with inclusion-cell disease (GM01586) were obtained at passage eight from the Coriell Institute (Camden, NJ, U.S.A.) and were grown under standard culture conditions at 37 °C in EMEM (Eagle's minimum essential medium), supplemented with 10 % FCS (foetal calf serum), 2 mM glutamine, 50 units/ml penicillin G and 50 mg/ml streptomycin in a humidified atmosphere containing 5 % CO₂ and 8 % O₂. The cells were subcultivated by trypsinization at a 1:4 ratio and transferred to 35-mm-diameter plastic Petri dishes (Costar) and cultures containing approx. 5×10^4 cells were established. Cells were also grown on 22-mm-diameter sterile coverslips under identical conditions for use in microscopy studies. Cells were routinely used from passages 14 to 20. For lipofuscin loading, AG01518 fibroblasts were exposed to normobaric hyperoxia (cultivation in 5 % CO₂ and 40 % O₂) for 6 months. As a consequence, the cells accumulated varying amounts of lipofuscin, with some cells highly loaded with the pigment and others containing only moderate amounts [18]. Where indicated, AG01518 fibroblasts were also grown in medium containing only 0.2 % or no FCS for up to 14 days in order to arrest cell growth [17]. To induce apoptosis, cells were rinsed in PBS and were incubated for up to 8 h in the presence of 0.1 μ M staurosporine (Sigma) in serum-free EMEM. Staurosporine was stored as a 0.2 mM stock in ethanol and was diluted in medium immediately before addition to cells.

Assessment of apoptosis

A detailed morphological and biochemical analysis of apoptosis in staurosporine-treated AG01518 fibroblasts has been reported [19]. In the present study, apoptosis was determined by morphological changes, including cytoplasmic contraction, plasma-membrane blebbing (assessed by phase-contrast microscopy), and nuclear condensation and fragmentation after mounting slides in Vectashield medium supplemented with 1.5 μ g/ml DAPI (4,6-diamidino-2-phenylindole; Vector Laboratories, Burlingame, CA, U.S.A.). Cells were examined in a Microphot-SA fluorescence microscope (Nikon, Tokyo, Japan) and photographed using a digital video camera (Hamamatsu, Tokyo, Japan). Caspase-3-like activity was assessed in cell lysates after addition of 20 μ M acetyl-Asp-Glu-Val-Asp-AMC (where AMC is 7-amino-4-methylcoumarin; Pharmingen, San Diego, CA, U.S.A.) and measurement of the liberated fluorophore AMC at λ_{ex} , 380; λ_{em} , 435 nm. These markers of apoptosis have also been validated by the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) and annexin-V-binding techniques for detection of apoptosis in AG01518 fibroblasts [19].

Immunocytochemistry

Fibroblasts were fixed in 4 % paraformaldehyde in PBS for 20 min at 4 °C, rinsed in PBS, and permeabilized in 0.1 % SB [saponin buffer (0.1 % saponin and 5 % foetal bovine serum in PBS)] for another 20 min at room temperature (22 °C). The cells were then treated with 1:500 diluted murine anti-(human apoE) monoclonal antibody (catalogue number 0650-052-54; Biogenesis, Poole, Dorset, U.K.) in a humidifier at 4 °C overnight. After rinsing in 0.1 % SB, anti-mouse IgG FITC conjugate (1:100; Calbiochem) was added for 1 h at room temperature. The samples were rinsed again in 0.1 % SB, PBS and distilled water, and were mounted in Vectashield. Specimens were studied with a Microphot-SA fluorescence microscope using a blue excitation filter (450–490 nm) and a 520 nm barrier filter, and photographed using

a Hamamatsu digital video camera. Lipofuscin is a fluorescent cellular pigment that accumulates in lysosomes of senescent cells *in vitro* and *in vivo* [20]. Excitation with blue light results in a red–orange lipofuscin fluorescence [20]. Therefore it was possible to examine FITC and lipofuscin presence simultaneously in the present work.

Fibroblast apoE immunostaining fluorescence intensity was semi-quantitatively determined by analysing digital monochrome images that were originally collected using Adobe Photoshop 5 software and quantified using the public domain National Institutes of Health (NIH) Image program (<http://rsb.info.nih.gov/nih-image/>). For each condition, 30 cells were analysed, and the amount of fluorescence per cell was calculated as the product of mean pixel value by cell area as described previously [21].

Fibroblasts were also examined using an Olympus LSM-GB 200 laser scanning confocal microscope using an Argon laser (488 nm blue light and 543 nm green light) and a 60 $\times$ oil objective and numerical aperture of 1.3. Cells were immunostained as described above using anti-apoE monoclonal antibody, as well as monoclonal antibodies (all at 1:500 dilution) to PTPs (protein tyrosine phosphatases) 1B and 1D, which are ER (endoplasmic reticulum)-bound and soluble cytosolic proteins respectively (BD Biosciences, North Ryde, Australia). Cells were exposed to propidium iodide (5 μ g/ml) for 5 min during the final wash in PBS and mounted in Vectashield without DAPI. The confocal studies were conducted in order to assess possible accumulation of ER/cytosolic proteins into the nucleus during fixation and permeabilization. Where indicated, purified human apoE3 (Biodesign, Melbourne, Australia) was included with the anti-apoE monoclonal antibody (1:1 mass ratio, each 6.4 μ g/ml) to assess potential non-specific binding of the antibody.

Western blotting

Fibroblast apoE was detected by Western blot as previously described [22]. Cell protein was measured using the bicinchoninic acid method, and equal amounts of protein were mixed with Laemmli sample buffer and run on 12 % polyacrylamide/SDS gels using a Mini-Protean II system (Bio-Rad) and subsequently transferred on to nitrocellulose membranes (Bio-Rad). Loading equivalence and transfer efficiency were monitored by Ponceau S staining. The membranes were then incubated for 16 h at 4 °C with a rabbit anti-(human apoE) polyclonal antibody (A0077; Dako, Denmark) diluted 1:1000, followed by a 1 h incubation with horseradish-peroxidase-conjugated goat anti-rabbit IgG antibody (P0448; Dako). Blots were developed by using ECL[®] (enhanced chemiluminescence; Amersham Biosciences), and the membranes were exposed to X-ray film (Fuji), developed, scanned and signal intensity was quantified using NIH Image software [22]. Fibroblast nuclei were isolated using the EZ Prep Nuclei Isolation kit (Sigma), according to the manufacturer's instructions, counted using a haemocytometer, and the equivalent of 1×10^5 nuclei were run on 12 % polyacrylamide gels in parallel with nuclei-free cytosol and blotted as described above. The purity of nuclear preparations was assessed by staining with 0.5 % (w/v) Trypan Blue and examination by light microscopy. Nuclei isolated using this method contain an intact nuclear lamina, but are devoid of their nuclear membrane and associated ER-derived membranes. The nuclei-free cytosolic preparations contain the remainder of the cellular constituents, including ER, plasma membranes and organelles.

RT (reverse transcription)-PCR

Cells were harvested for total RNA using Tri Reagent (Sigma), according to the manufacturer's instructions, with the

Figure 1 consists of six panels labeled A through F. Panels A, B, C, D, and E are fluorescence micrographs showing the localization of GFP::AtNUP133 (green) and DAPI-stained nuclei (red) in Arabidopsis root cells. Panel A shows three cells with a scale bar in the bottom right. Panel B shows a single cell with a large nucleus. Panel C shows a cell with two distinct red spots. Panel D shows a cell with multiple red spots. Panel E shows a cell with several red spots. Panel F is a bright-field micrograph showing the same cells stained with toluidine blue, with an inset showing a higher magnification view of the cells.

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Figure 2 Effects of staurosporine-induced apoptosis on fibroblast morphology and apoE immunostaining

Fibroblasts were grown on coverslips and treated with 0.1 μ M staurosporine for 1 h where indicated. After fixation, the cells were immunostained using an anti-(human apoE) monoclonal antibody and a FITC-labelled secondary antibody, mounted in Vectashield medium containing DAPI and examined using a Nikon Microphot-SA fluorescence microscope. Shown are phase-contrast (A and B), DAPI-stained nuclei (C and D) and apoE FITC (E and F). Control treatment (A, C and E) and 1 h treatment with 0.1 μ M staurosporine (B, D and F). Bar, 25 μ m.

of isolated fibroblast nuclei confirmed that apoE was located in the nucleus under normal culture conditions (Figure 4B). In addition to the expected ~34 kDa apoE band, a fragment of approx. 22 kDa was detected in fibroblast nuclei (Figure 4B). This fragment may represent the apoE N-terminal domain that is known to be generated by enzymic cleavage. Additional faint bands were also present at higher molecular masses, which could be due to aggregation or covalent cross-linking of apoE via mechanisms which remain to be identified.

To better understand the factors that contributed to apoE induction during apoptosis, apoE mRNA levels were assessed by PCR methods. ApoE mRNA levels were rapidly increased in response to staurosporine and reached a maximum at 4 h, as assessed by regular PCR (Figure 4C). Quantitative real-time PCR experiments confirmed these data (Figure 4D). To confirm the induction of apoptosis in the time-course experiments, caspase-3-like activity was also assessed. The activation of caspase 3 is a relatively late event in the apoptotic cascade and plays an important role in the disassembly of cellular structures, including nuclear proteins [26]. Caspase-3 activity was significantly increased by 4 h and was markedly increased by 8 h (Figure 4D). This confirms our previous studies and indicates that increased apoE mRNA levels precede caspase-3 activation, while detectable

Figure 3 Immunocytochemical demonstration of increased fibroblast apoE expression during staurosporine-induced apoptosis

Fibroblasts were prepared as described in the legend to Figure 1 in the absence of staurosporine (A) or treated with 0.1 μ M staurosporine for 2, 4 and 8 h (B, C and D respectively). After fixation, the cells were immunostained and examined for apoE FITC immunofluorescence. Bar, 25 μ m.

increases in apoE protein expression occur at approximately the same time as caspase-3 activation (Figure 4).

ApoE transcription in macrophages and adipose tissue is regulated by the nuclear receptors LXR and RXR (retinoid X receptor) [27]. Fibroblast apoE transcription was potently induced by 22(R)-hydroxycholesterol and 9-*cis*-retinoic acid, which are ligands for LXR and RXR respectively (Figure 4C). This indicates that the control of apoE transcription in fibroblasts may be via pathways that are in common with other cell types. If increased apoE transcription in apoptosis was via an LXR pathway, one might expect other LXR-regulated genes to be transcribed during apoptosis. It was also possible that the LXR gene itself may be induced. These possibilities were assessed by analysing ABCA1 mRNA (ABCA1 transcription is regulated by LXR [28]) and LXR α mRNA by PCR methods. Both ABCA1 and LXR α mRNA levels were increased when fibroblasts were induced to undergo apoptosis (Figure 4E). Quantitative real-time PCR showed that LXR α mRNA levels were increased within 1 h of staurosporine treatment and continued to increase throughout the time course, with a 17-fold increase observed after 8 h (Figure 4F). ABCA1 mRNA expression was increased after 2 h and reached a maximum 7-fold induction at the 8 h time point (Figure 4F). Real-time PCR also indicated that apolipoprotein C-II mRNA levels, also regulated by LXR [29], were similarly increased (results not shown). The fact that LXR α mRNA levels increase before the onset of increased apoE mRNA expression and the observation that additional LXR-regulated genes are also induced suggests that LXR-mediated induction of apoE transcription contributes to the regulation of apoE levels during apoptosis.

Fibroblast apoE expression is increased with growth arrest/lipofuscin loading

A previous study showed that serum-starvation-induced growth arrest stimulated apoE transcription in primary and immortalized fibroblasts [17]. Since serum starvation can induce apoptosis

Figure 4 Expression of apoE, LXR α and ABCA1 levels and caspase-3-like activity during staurosporine-induced apoptosis

Fibroblasts were grown in 35-mm-diameter plastic Petri dishes and treated with 0.1 μ M staurosporine (stsp) for the times indicated. Whole-cell lysates (A) and nuclear lysates (B) were analysed for apoE by Western blotting. The sizes of the bands are indicated in kDa. ApoE mRNA levels were analysed by RT-PCR and compared with β -actin mRNA expression before and after treatment with 0.1 μ M staurosporine for up to 8 h, or with the LXR/RXR ligands, 22-hydroxycholesterol (22OH) and 9-*cis*-retinoic acid (9cRA), both at a concentration of 10 μ M, for 8 h (C). The relative increases in apoE expression and caspase-3 activation in response to 0.1 μ M staurosporine are shown (D): quantitative real-time PCR analysis of mRNA (●), Western blot integrated absorbance (■), immunofluorescence intensity (◇), and caspase-3 activation as assessed by hydrolysis of the caspase-3 substrate acetyl-Asp-Glu-Val-Asp-AMC to yield the fluorophore AMC (○). Expression of fibroblast ABCA1 and LXR α mRNAs after treatment with 0.1 μ M staurosporine was analysed by standard RT-PCR (E) and quantitative real-time PCR (F): LXR α mRNA (●) and ABCA1 mRNA (■).

[30], we assessed the potential contribution of apoptosis-related increases in apoE expression in growth-arrested AG01518 fibroblasts. We also wanted to know if the increased apoE mRNA levels previously described [17] were translated to detectable differences in apoE protein and, if so, in which intracellular compartment apoE was located. Figure 5 shows that there was a striking increase in the level of immunodetectable apoE when fibroblasts were grown in the presence of 0.2% (v/v) FCS for 2 weeks compared with cells grown in 10% FCS. This increased apoE expression was exacerbated when cells were grown in the complete absence of serum and was particularly pronounced in the nucleus (Figure 5E). Western blot and PCR analysis confirmed the increased expression of apoE in fibroblasts that were grown under serum-restricted or serum-free conditions for 2 weeks (Figures 6A and 6B) and the localization of apoE in both the nucleus and cytosol (Figure 6C). Importantly, apoptosis did not account for the increased apoE expression in serum-starved cells, as assessed by a lack of generalized caspase-3 activation (Figure 6D), and a lack of evidence for apoptotic nuclear and cellular morphological alterations (Figure 5). Unexpectedly, we noticed a marked heterogeneity in cellular expression of nuclear apoE, with the highest levels of apoE immunostaining predominantly located in cells that also contained lipofuscin (Figures 5C and 5E). These cells were also characterized by their well-defined nucleoli (Figures 5C and 5E).

We considered the possibility that lipofuscin accumulation itself could be associated with increased fibroblast apoE expression. To address this, AG01518 fibroblasts were cultured in 40% O₂ for 6 months in order to induce lipofuscin accumulation

[18]. This experimental approach generates a senescent fibroblast phenotype as assessed by several parameters, including telomere shortening [20]. We also examined fibroblasts derived from a patient with inclusion-cell disease, a condition which is known to result in lysosomal lipofuscin accumulation [19]. In both of these models of lipofuscin loading, apoE was clearly detected in the nucleus (Figure 7). Furthermore, the cells that were more heavily loaded with lipofuscin were the cells in which nuclear apoE immunostaining was most intense (Figure 7). There was no morphological evidence that the increased nuclear staining for apoE was associated with apoptosis in these cells.

Mitosis is another event that involves dramatic membrane remodelling. Under normal growth conditions, a few percent of fibroblasts undergo mitosis at any point in time (this varies depending on passage number and confluence). In subconfluent cultures, we noticed a striking increase in apoE immunoreactivity in dividing cells. This was clearly evident at stages of mitosis both before and after cytokinesis (Figure 8). Semi-quantitative estimates of immunostained fibroblasts indicated that apoE levels were increased up to 5-fold during mitosis (results not shown).

Phosphorylation of fibroblast apoE

Our data showed that apoE is present in the nucleus and in cytosolic compartments. We therefore analysed the apoE amino acid sequence for specific post-translational modifications found in nuclear and cytosolic proteins. Phosphorylation is widely recognized as an important post-translational modification that has the potential to regulate protein structure and function. Analysis of

Figure 5 Effects of serum starvation on fibroblast apoE expression and lipofuscin accumulation

Fibroblasts were grown on coverslips in the presence of 10% FCS (**A** and **B**) or 0.2% FCS (**C** and **D**), or in serum-free medium (**E** and **F**) for 2 weeks. After fixation, the cells were immunostained and examined for apoE FITC immunofluorescence (**A**, **C** and **E**; green fluorescence), lipofuscin accumulation (**A**, **C** and **E**; orange fluorescence) and nuclear morphology (**B**, **D** and **F**). Bar, 25 μ m.

apoE for consensus sequences for ten different protein kinases [31] revealed 13 potential phosphorylation sites (serine residues 76, 94, 129, 139, 175, 223 and 263, and threonine residues 8, 18, 42, 67, 83 and 194). Thr¹⁹⁴ is also known to be O-glycosylated in other cell types [32]. In order to assess the potential physiological relevance of our *in silico* analysis, fibroblasts were metabolically labelled with [³²P]P_i and the cell lysates were immunoprecipitated with an anti-apoE antibody. ³²P-labelled apoE was clearly detected as a band of approx. 34 kDa (Figure 9). The ³²P-labelled band had an identical migration pattern to ³⁵S-labelled immunoprecipitated apoE (Figure 9). The 34 kDa bands present in both of these samples were susceptible to thrombin digestion, as would be predicted for apoE (results not shown). These experiments demonstrate for the first time that apoE can be phosphorylated.

DISCUSSION

Our observation that human fibroblasts express low levels of apoE mRNA is consistent with a previous study [17]. We have shown that this mRNA is functional, since it is translated to apoE protein that is detected by immunocytochemical techniques and Western blotting. The amount of apoE present in fibroblasts grown

Figure 6 Expression of fibroblast apoE and caspase-3 activity during serum starvation

Fibroblasts were grown in 35-mm-diameter plastic Petri dishes in the presence of 10% FCS (**C**), 0.2% FCS (0.2) or serum-free (SF) conditions for the times indicated. Cell lysates were analysed for apoE by Western blotting (**A**) and compared with 14-day-old human monocyte-derived macrophage (M) lysate as an additional marker for apoE molecular mass (60 μ g of protein was loaded per well for fibroblasts, whereas 6 μ g was loaded for macrophages). ApoE mRNA levels were analysed by RT-PCR and compared with β -actin mRNA expression; LXR/RXR ligands (22OH, 22-hydroxycholesterol; 9CRA, 9-*cis*-retinoic acid) were added to control cells as a positive control for inducible apoE gene transcription (**B**), as described in the legend to Figure 4. Nuclear (Nuc) and cytosolic (Cyt) fractions derived from fibroblasts grown under serum-free conditions for 14 days were analysed by Western blotting and compared with purified apoE3 (apoE) as an additional marker for molecular mass (**C**). Caspase-3 activation as assessed by hydrolysis of the caspase-3 substrate acetyl-Asp-Glu-Val-Asp-AMC to yield the fluorophore AMC (**D**); the broken line indicates AMC fluorescence detected when fibroblasts were treated with 0.1 μ M staurosporine (stsp) for 8 h to induce apoptosis (see Figure 4D) and thereby emphasizes the minimal caspase-3 activity observed under normal culture conditions and during serum starvation.

Figure 7 ApoE expression in senescent/lipofuscin-loaded fibroblasts

Fibroblasts were grown under hyperoxic conditions (40% O₂) for 6 months (**A** and **B**). I-cell fibroblasts (**C** and **D**) were grown under standard growth conditions (see the Experimental section). After fixation, the cells were immunostained and examined for apoE FITC immunofluorescence (**A** and **C**; green fluorescence), lipofuscin accumulation (**A** and **C**; orange fluorescence) and nuclear morphology (**B** and **D**). Note that the cells that accumulated the most lipofuscin [the centre of (**A**) and left side of (**C**)] also stained strongly for nuclear apoE. Bar, 25 μ m.

Figure 8 ApoE expression in fibroblasts undergoing mitosis

Fibroblasts were grown on coverslips. After fixation, cells were immunostained and mounted in Vectashield medium containing DAPI. Phase-contrast microscopy was used to identify mitotic cells (**A** and **B**) that were clearly characterized by their DAPI-stained chromatin/nuclei (**C** and **D**). ApoE FITC immunofluorescence is also shown (**E** and **F**). Note the increased apoE expression during (**E**) and after (**F**) cell division. Bar, 25 μ m.

Figure 9 Phosphorylation of fibroblast apoE *in vivo*

Fibroblasts were cultured in the presence of [32 P]orthophosphate or [35 S]cysteine/methionine, and cell lysates were immunoprecipitated with anti-apoE antibody. The resulting radiolabelled apoE was run on 12 % polyacrylamide gels and analysed by phosphorimaging. [32 P]-labelled and [35 S]-labelled apoE are predominantly detected as $\sim$ 34 kDa bands with minor bands present at $\sim$ 30 kDa.

under standard conditions is low compared with that of other cells in which apoE is known to be expressed (such as macrophages). The level of apoE present in fibroblasts is close to the lower limits of detection by Western blotting. Our data (e.g. Figure 6A) indicate that apoE is present in fibroblasts at a level approx. 100-fold lower than in human monocyte-derived macrophages.

Fibroblast apoE was located both in the nucleus and in a cytosolic compartment. A previous study also detected apoE in a nuclear fraction from rat liver homogenate [33]. ApoE contains an 18-amino-acid signal peptide that would be expected to direct most apoE through the secretory pathway. This is a major transport pathway in cells expressing high levels of apoE. The route for apoE nuclear transport remains to be defined. Proteins with a molecular mass of less than approx. 40 kDa may passively diffuse through nuclear pores and bind to nuclear components and thereby accumulate in the nucleus [34]. ApoE may follow a similar route. Certain non-histone nuclear proteins are glycosylated and are proposed to originate from the ER and emerge packaged in vesicles from the *trans*-stack of the Golgi before translocation to the nucleus [35]. Golgi-derived apoE may also be redirected to the nucleus. Alternatively, a transient pool of apoE on the cell surface may be a source of nuclear apoE [36]. Consistent with this hypothesis, the nucleolar protein, nucleolin, is also expressed on the cell surface, from where it is internalized [37]. ApoE, which binds to cell-surface nucleolin [38], may be transported to the nucleus as an apoE–nucleolin complex. Similarly, the heparin-binding growth factor, midkine, is targeted from the cell surface to the nucleus via a nucleolin-mediated pathway [37,39]. Other heparin-binding proteins [e.g. aFGF (acidic fibroblast growth factor), bFGF (basic fibroblast growth factor), Schwannoma-derived growth factor, heparin-binding growth-associated molecule and HIV transactivator protein] similarly utilize cell-surface-derived heparan sulphate proteoglycans–LRP–nucleolin interactions in their trafficking to the nucleus [39–41]. Because apoE binds to heparan sulphate proteoglycans, LRP and nucleolin, it may be trafficked to the nucleus via a similar pathway.

The present study is the first to demonstrate apoE induction during apoptosis. Such up-regulation of apoE levels may be related to the facilitation of membrane remodelling during apoptosis. A similar function has been proposed for apoE in non-neuronal cells that regenerate in response to nerve injury [6]. Interestingly, the insect structural homologue of apoE, lipophorin III, is up-regulated in skeletal muscle and motor neurons that undergo apoptosis, where it is thought to facilitate membrane remodelling during eclosion of the adult tobacco hawk moth [42]. ApoE may have additional functions in apoptosis. Analogous to apoJ (apolipoprotein J; clusterin), apoE could mediate the clearance of apoptotic cellular debris via gp330 or LRP expressed on neighbouring cells [43]. Nuclear apoE may also play a role in the regulation of transcription during apoptosis as has been proposed for apoJ [44]. These possibilities will clearly require further study.

To understand the mechanisms that contribute to the increased apoE mRNA levels in apoptosis, we analysed LXR α and ABCA1 mRNA levels. LXR α regulates apoE and ABCA1 transcription [27,28] and is also implicated in the regulation of other genes involved in apoptosis [45]. Of potential significance, ABCA1 acts as a phosphatidylserine translocase, increasing phosphatidylserine levels in the exofacial leaflet of the plasma membrane, and may therefore play a role in membrane remodelling in apoptosis [46]. Our data showed that increased LXR α mRNA levels preceded increases in apoE and ABCA1 mRNAs, suggesting that LXR α regulates apoE and ABCA1 gene transcription in apoptosis. LXR α activation may be mediated by increased available cholesterol levels that are induced during apoptosis as a result of SREBP (sterol-regulatory-element-binding protein) cleavage [47]. SREBP cleavage stimulates transcription of the HMG (3-hydroxy-3-methylglutaryl)-CoA reductase and LDL receptor genes, thereby promoting the accumulation of cholesterol that may be required for cellular membrane remodelling during apoptosis [48]. Alternatively, during the extensive membrane remodelling that occurs during apoptosis, existing membrane cholesterol

may be mobilized and enter the regulatory pool from which LXR ligands are generated.

ApoE levels also increased in growth-arrested and lipofuscin-loaded/senescent fibroblasts. Under these conditions, the increased apoE expression was predominantly nuclear. The function and regulation of nuclear apoE expression remain to be determined. Growth-arrested fibroblasts and quiescent smooth muscle cells exhibit increased apoE mRNA [17,49]. This may be under the control of serum-responsive elements in the apoE promoter [17]. Apolipoprotein D mRNA levels are increased in senescent fibroblasts [50], potentially under the control of serum-responsive elements [17]. It is therefore plausible that serum-responsive elements in the apoE promoter are involved in its increased expression during growth arrest and senescence. We also noted that fibroblast nuclear apoE expression increased with passage number (results not shown) and that complete serum withdrawal for 2 weeks or more induced a senescent phenotype. This suggests that factors related to the regulation of senescence gene expression may contribute to the increased levels of apoE mRNA detected in our studies. Pertinent to this, the use of gene microarray technology recently revealed increased apoE gene expression in aging murine hippocampus [51].

A further novel observation in the present work was the up-regulation and redistribution of apoE during cell division. The redistribution of nuclear apoE to the cytosol is predictable during nuclear disassembly, but the factors that regulate apoE levels during mitosis remain to be determined. It is possible that apoE expression is increased in response to the transiently increased cholesterol levels demonstrated previously in dividing fibroblasts [52], or via transcriptional regulation that is co-ordinated with the cell cycle. Certain apoE promoter regulatory elements are recognized by specificity protein 1 (Sp1), which controls the expression of many genes important in cell-cycle regulation. Sp1 or other transcription factors may therefore regulate apoE expression in a cell-cycle-dependent manner.

The identification of apoE in nuclear and cytosolic compartments prompted us to examine the apoE amino acid sequence for potential novel functional domains. ApoE from all mammalian and rodent species examined contained consensus sequences for phosphorylation [12 ± 2 sites (mean $\pm$ S.D.); $n=15$ species]. We provide the first direct demonstration that apoE can be phosphorylated *in vivo*. Although the significance of this observation remains to be determined, possibilities include regulation of apoE nuclear import/export via the Yin–Yang pathway [53] or alteration of structural domains involved in apoE binding to lipids or receptors. Human apolipoprotein B and apolipoprotein A-I undergo phosphorylation *in vivo*, and it has been suggested that this may be a general phenomenon associated with a subclass of amphipathic lipid-binding proteins that includes vitellogenin, lipocortin-1, casein and myelin basic protein [54–56]. For apolipoprotein B-100, phosphorylation is suggested to alter lipid-binding affinity and regulate trafficking from the Golgi to a degradative intracellular pathway [57]. Phosphorylation may similarly affect apoE lipid affinity and degradation.

Studies in apoE^{-/-} mice demonstrate a crucial role for apoE in lipoprotein metabolism [4]. Several neurological abnormalities also exist in apoE^{-/-} mice, including decreased cholinergic activity, retardation in the acquisition of developmental milestones, and learning and memory impairments [5,58,59]. It is not clear if these latter abnormalities are related to dyslipidaemia or other, possibly unrecognized, functions of apoE. The nuclear localization of apoE may point to new functions (e.g. direct effects on gene transcription) that are analogous to those observed for other heparin-binding proteins that are targeted to the nucleus [39–41].

In summary, the present work highlights the potential importance of apoE in fundamental cellular processes related to lipid-membrane remodelling and senescence, and provides new challenges for understanding the roles that this protein plays in cell biology and pathophysiology. We show for the first time that human fibroblasts contain apoE in nuclear and cytosolic pools, that fibroblast apoE levels are increased during apoptosis via a mechanism that is potentially regulated by LXR α , that fibroblast apoE levels are also increased under conditions which induce growth arrest/senescence and during mitosis, and that apoE can be phosphorylated *in vivo*.

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REFERENCES

- Mahley, R. W. (1988) Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science* **240**, 622–630.
- Beisiegel, U., Weber, W., Ihrke, G., Herz, J. and Stanley, K. K. (1989) The LDL-receptor-related protein, LRP, is an apolipoprotein E-binding protein. *Nature (London)* **341**, 162–164.
- Rall, Jr, S. C., Weisgraber, K. H., Innerarity, T. L. and Mahley, R. W. (1982) Structural basis for receptor binding heterogeneity of apolipoprotein E from type III hyperlipoproteinemic subjects. *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4696–4700.
- Plump, A. S., Smith, J. D., Hayek, T., Aalto-Setälä, K., Walsh, A., Verstuyft, J. G., Rubin, E. M. and Breslow, J. L. (1992) Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell* **71**, 343–353.
- Gordon, I., Grauer, E., Genis, I., Sehaye, E. and Michaelson, D. M. (1995) Memory deficits and cholinergic impairments in apolipoprotein E-deficient mice. *Neurosci. Lett.* **199**, 1–4.
- Snipes, G. J., McGuire, C. B., Norden, J. J. and Freeman, J. A. (1986) Nerve injury stimulates the secretion of apolipoprotein E by nonneuronal cells. *Proc. Natl. Acad. Sci. U.S.A.* **83**, 1130–1134.
- Whitson, J. S., Mims, M. P., Strittmatter, W. J., Yamaki, T., Morrisett, J. D. and Appel, S. H. (1994) Attenuation of the neurotoxic effect of A β amyloid peptide by apolipoprotein E. *Biochem. Biophys. Res. Commun.* **199**, 163–170.
- Ji, Z. S., Miranda, R. D., Newhouse, Y. M., Weisgraber, K. H., Huang, Y. and Mahley, R. W. (2002) Apolipoprotein E4 potentiates amyloid β peptide-induced lysosomal leakage and apoptosis in neuronal cells. *J. Biol. Chem.* **277**, 21821–21828.
- Brunk, U. T., Dalen, H., Roberg, K. and Hellquist, H. B. (1997) Photo-oxidative disruption of lysosomal membranes causes apoptosis of cultured human fibroblasts. *Free Radical Biol. Med.* **23**, 616–626.
- Kagedal, K., Johansson, U. and Ollinger, K. (2001) The lysosomal protease cathepsin D mediates apoptosis induced by oxidative stress. *FASEB J.* **15**, 1592–1594.
- Bales, K. R., Verina, T., Dodel, R. C., Du, Y., Altstiel, L., Bender, M., Hyslop, P., Johnstone, E. M., Little, S. P., Cummins, D. J. et al. (1997) Lack of apolipoprotein E dramatically reduces amyloid β -peptide deposition. *Nat. Genet.* **17**, 263–264.
- Holtzman, D. M., Fagan, A. M., Mackey, B., Tenkova, T., Sartorius, L., Paul, S. M., Bales, K., Ashe, K. H., Irizarry, M. C. and Hyman, B. T. (2000) Apolipoprotein E facilitates neuritic and cerebrovascular plaque formation in an Alzheimer's disease model. *Ann. Neurol.* **47**, 739–747.
- Buttini, M., Orth, M., Bellosta, S., Akeefe, H., Pitas, R. E., Wyss-Coray, T., Mucke, L. and Mahley, R. W. (1999) Expression of human apolipoprotein E3 or E4 in the brains of ApoE^{-/-} mice: isoform-specific effects on neurodegeneration. *J. Neurosci.* **19**, 4867–4880.
- Corder, E. H., Saunders, A. M., Strittmatter, W. J., Schmechel, D. E., Gaskell, P. C., Small, G. W., Roses, A. D., Haines, J. L. and Pericak-Vance, M. A. (1993) Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science* **261**, 921–923.
- Zannis, V. I., McPherson, J., Goldberger, G., Karathanasis, S. K. and Breslow, J. L. (1984) Synthesis, intracellular processing, and signal peptide of human apolipoprotein E. *J. Biol. Chem.* **259**, 5495–5499.
- Saada, A., Dunaevsky-Hutt, A., Aamar, A., Reichert, F. and Rotshenker, S. (1995) Fibroblasts that reside in mouse and frog injured peripheral nerves produce apolipoproteins. *J. Neurochem.* **64**, 1996–2003.

- 17 Do Carmo, S., Seguin, D., Milne, R. and Rassart, E. (2002) Modulation of apolipoprotein D and apolipoprotein E mRNA expression by growth arrest and identification of key elements in the promoter. *J. Biol. Chem.* **277**, 5514–5523
- 18 Terman, A. and Brunk, U. T. (1998) Ceroid/lipofuscin formation in cultured human fibroblasts: the role of oxidative stress and lysosomal proteolysis. *Mech. Ageing Dev.* **104**, 277–291
- 19 Terman, A., Neuzil, J., Kagedal, K., Ollinger, K. and Brunk, U. T. (2002) Decreased apoptotic response of inclusion-cell disease fibroblasts: a consequence of lysosomal enzyme missorting? *Exp. Cell Res.* **274**, 9–15
- 20 von Zglinicki, T. (2000) Role of oxidative stress in telomere length regulation and replicative senescence. *Ann. N.Y. Acad. Sci.* **908**, 99–110
- 21 Terman, A., Dalen, H. and Brunk, U. T. (1999) Ceroid/lipofuscin-loaded human fibroblasts show decreased survival time and diminished autophagocytosis during amino acid starvation. *Exp. Gerontol.* **34**, 943–957
- 22 Garner, B., Baoutina, A., Dean, R. T. and Jessup, W. (1997) Regulation of serum-induced lipid accumulation in human monocyte-derived macrophages by interferon- γ : correlations with apolipoprotein E production, lipoprotein lipase activity and LDL receptor-related protein expression. *Atherosclerosis* **128**, 47–58
- 23 Kielar, D., Dietmaier, W., Langmann, T., Aslanidis, C., Probst, M., Naruszewicz, M. and Schmitz, G. (2001) Rapid quantification of human ABCA1 mRNA in various cell types and tissues by real-time reverse transcription-PCR. *Clin. Chem.* **47**, 2089–2097
- 24 Rees, D., Sloane, T., Jessup, W., Dean, R. T. and Kritharides, L. (1999) Apolipoprotein A-I stimulates secretion of apolipoprotein E by foam cell macrophages. *J. Biol. Chem.* **274**, 27925–27933
- 25 Yuan, L., Yu, W. M., Yuan, Z., Haudenschild, C. C. and Qu, C. K. (2003) Role of SHP-2 tyrosine phosphatase in the DNA damage-induced cell death response. *J. Biol. Chem.* **278**, 15208–15216
- 26 Cohen, G. M. (1997) Caspases: the executioners of apoptosis. *Biochem. J.* **326**, 1–16
- 27 Laffitte, B. A., Repa, J. J., Joseph, S. B., Wilpitz, D. C., Kast, H. R., Mangelsdorf, D. J. and Tontonoz, P. (2001) LXRs control lipid-inducible expression of the apolipoprotein E gene in macrophages and adipocytes. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 507–512
- 28 Venkateswaran, A., Laffitte, B. A., Joseph, S. B., Mak, P. A., Wilpitz, D. C., Edwards, P. A. and Tontonoz, P. (2000) Control of cellular cholesterol efflux by the nuclear oxysterol receptor LXR α . *Proc. Natl. Acad. Sci. U.S.A.* **97**, 12097–12102
- 29 Mak, P. A., Laffitte, B. A., Desrumaux, C., Joseph, S. B., Curtiss, L. K., Mangelsdorf, D. J., Tontonoz, P. and Edwards, P. A. (2002) Regulated expression of the apolipoprotein E/C-I/C-IV/C-II gene cluster in murine and human macrophages: a critical role for nuclear liver X receptors α and β . *J. Biol. Chem.* **277**, 31900–31908
- 30 Helbing, C. C., Wellington, C. L., Gogela-Spehar, M., Cheng, T., Pinchbeck, G. G. and Johnston, R. N. (1998) Quiescence versus apoptosis: Myc abundance determines pathway of exit from the cell cycle. *Oncogene* **17**, 1491–1501
- 31 Blom, N., Gammeltoft, S. and Brunak, S. (1999) Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *J. Mol. Biol.* **294**, 1351–1362
- 32 Wernette-Hammond, M. E., Lauer, S. J., Corsini, A., Walker, D., Taylor, J. M. and Rall, Jr, S. C. (1989) Glycosylation of human apolipoprotein E: the carbohydrate attachment site is threonine 194. *J. Biol. Chem.* **264**, 9094–9101
- 33 Panin, L. E., Russkikh, G. S. and Polyakov, L. M. (2000) Detection of apolipoprotein A-I, B, and E immunoreactivity in the nuclei of various rat tissue cells. *Biochemistry (Moscow)* **65**, 1419–1423
- 34 Yoneda, Y. (2000) Nucleocytoplasmic protein traffic and its significance to cell function. *Genes Cells* **5**, 777–787
- 35 Polet, H. and Molnar, J. (1988) Demonstration that some of the nonhistone proteins, inducible to translocate into the nucleus, are glycosylated. *J. Cell Physiol.* **135**, 47–54
- 36 Fazio, S., Linton, M. F. and Swift, L. L. (2000) The cell biology and physiologic relevance of ApoE recycling. *Trends Cardiovasc. Med.* **10**, 23–30
- 37 Said, E. A., Krust, B., Nisole, S., Svab, J., Briand, J. P. and Hovanessian, A. G. (2002) The anti-HIV cytokine midkine binds the cell surface-expressed nucleolin as a low affinity receptor. *J. Biol. Chem.* **277**, 37492–37502
- 38 Semenkovich, C. F., Ostlund, Jr, R. E., Olson, M. O. and Yang, J. W. (1990) A protein partially expressed on the surface of HepG2 cells that binds lipoproteins specifically is nucleolin. *Biochemistry* **29**, 9708–9713
- 39 Shibata, Y., Muramatsu, T., Hirai, M., Inui, T., Kimura, T., Saito, H., McCormick, L. M., Bu, G. and Kadomatsu, K. (2002) Nuclear targeting by the growth factor midkine. *Mol. Cell. Biol.* **22**, 6788–6796
- 40 Sandgren, S., Cheng, F. and Belting, M. (2002) Nuclear targeting of macromolecular polyanions by an HIV-Tat derived peptide: role for cell-surface proteoglycans. *J. Biol. Chem.* **277**, 38877–38883
- 41 Liu, Y., Jones, M., Hingtgen, C. M., Bu, G., Larabee, N., Tanzi, R. E., Moir, R. D., Nath, A. and He, J. J. (2000) Uptake of HIV-1 tat protein mediated by low-density lipoprotein receptor-related protein disrupts the neuronal metabolic balance of the receptor ligands. *Nat. Med.* **6**, 1380–1387
- 42 Sun, D., Ziegler, R., Milligan, C. E., Fahrbach, S. and Schwartz, L. M. (1995) Apolipoprotein III is dramatically up-regulated during the programmed death of insect skeletal muscle and neurons. *J. Neurobiol.* **26**, 119–129
- 43 Bartl, M. M., Luckenbach, T., Bergner, O., Ullrich, O. and Koch-Brandt, C. (2001) Multiple receptors mediate apoJ-dependent clearance of cellular debris into nonprofessional phagocytes. *Exp. Cell Res.* **271**, 130–141
- 44 Yang, C. R., Leskov, K., Hosley-Eberlein, K., Criswell, T., Pink, J. J., Kinsella, T. J. and Boothman, D. A. (2000) Nuclear clusterin/XIP8, an X-ray-induced Ku70-binding protein that signals cell death. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 5907–5912
- 45 Alberti, S., Steffensen, K. R. and Gustafsson, J. A. (2000) Structural characterisation of the mouse nuclear oxysterol receptor genes LXR α and LXR β . *Gene* **243**, 93–103
- 46 Smith, J. D., Waelde, C., Horwitz, A. and Zheng, P. (2002) Evaluation of the role of phosphatidylserine translocase activity in ABCA1-mediated lipid efflux. *J. Biol. Chem.* **277**, 17797–17803
- 47 Wang, X., Zelenski, N. G., Yang, J., Sakai, J., Brown, M. S. and Goldstein, J. L. (1996) Cleavage of sterol regulatory element binding proteins (SREBPs) by CPP32 during apoptosis. *EMBO J.* **15**, 1012–1020
- 48 Higgins, M. E. and Ioannou, Y. A. (2001) Apoptosis-induced release of mature sterol regulatory element-binding proteins activates sterol-responsive genes. *J. Lipid Res.* **42**, 1939–1946
- 49 Majack, R. A., Castle, C. K., Goodman, L. V., Weisgraber, K. H., Mahley, R. W., Shooter, E. M. and Gebicke-Haerter, P. J. (1988) Expression of apolipoprotein E by cultured vascular smooth muscle cells is controlled by growth state. *J. Cell Biol.* **107**, 1207–1213
- 50 Provost, P. R., Marcel, Y. L., Milne, R. W., Weech, P. K. and Rassart, E. (1991) Apolipoprotein D transcription occurs specifically in nonproliferating quiescent and senescent fibroblast cultures. *FEBS Lett.* **290**, 139–141
- 51 Terao, A., Apte-Deshpande, A., Dousman, L., Moraity, S., Eynon, B. P., Kilduff, T. S. and Freund, Y. R. (2002) Immune response gene expression increases in the aging murine hippocampus. *J. Neuroimmunol.* **132**, 99–112
- 52 Fielding, C. J., Bist, A. and Fielding, P. E. (1999) Intracellular cholesterol transport in synchronized human skin fibroblasts. *Biochemistry* **38**, 2506–2513
- 53 Hart, G. W., Greis, K. D., Dong, L. Y., Blomberg, M. A., Chou, T. Y., Jiang, M. S., Roquemore, E. P., Snow, D. M., Kreppel, L. K., Cole, R. N. et al. (1995) O-linked N-acetylglucosamine: the “yin-yang” of Ser/Thr phosphorylation? Nuclear and cytoplasmic glycosylation. *Adv. Exp. Med. Biol.* **376**, 115–123
- 54 Davis, R. A., Clinton, G. M., Borchardt, R. A., Malone-McNeal, M., Tan, T. and Lattier, G. R. (1984) Intrahepatic assembly of very low density lipoproteins: phosphorylation of small molecular weight apolipoprotein B. *J. Biol. Chem.* **259**, 3383–3386
- 55 Beg, Z. H., Stonik, J. A., Hoeg, J. M., Demosky, Jr, S. J., Fairwell, T. and Brewer, Jr, H. B. (1989) Human apolipoprotein A-I: post-translational modification by covalent phosphorylation. *J. Biol. Chem.* **264**, 6913–6921
- 56 Swift, L. L. (1996) Role of the Golgi apparatus in the phosphorylation of apolipoprotein B. *J. Biol. Chem.* **271**, 31491–31495
- 57 Jackson, T. K., Salhanick, A. I., Elovson, J., Deichman, M. L. and Amatruda, J. M. (1990) Insulin regulates apolipoprotein B turnover and phosphorylation in rat hepatocytes. *J. Clin. Invest.* **86**, 1746–1751
- 58 Oitzl, M. S., Mulder, M., Lucassen, P. J., Havekes, L. M., Grootendorst, J. and de Kloet, E. R. (1997) Severe learning deficits in apolipoprotein E-knockout mice in a water maze task. *Brain Res.* **752**, 189–196
- 59 Gozes, I., Bachar, M., Bardea, A., Davidson, A., Rubinraut, S., Fridkin, M. and Giladi, E. (1997) Protection against developmental retardation in apolipoprotein E-deficient mice by a fatty neuropeptide: implications for early treatment of Alzheimer's disease. *J. Neurobiol.* **33**, 329–342