PIK3CA is implicated as an oncogene in ovarian cancer

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Ovarian cancer is the leading cause of death from gynecological malignancy and the fourth leading cause of cancer death among American women[1], yet little is known about its molecular aetiology. Studies using comparative genomic hybridization (CGH) have revealed several regions of recurrent, abnormal, DNA sequence copy number[2–4] that may encode genes involved in the genesis or progression of the disease. One region at 3q26 found to be increased in copy number in approximately 40% of ovarian[2] and other[5] cancers contains *PIK3CA*, which encodes the p110α catalytic subunit of phosphatidylinositol 3-kinase (PI3-kinase). The association between *PIK3CA* copy number and PI3-kinase activity makes *PIK3CA* a candidate oncogene because a broad range of cancer-related functions have been associated with PI3-kinase mediated signalling[6]. These include proliferation[7], glucose transport and catabolism[8], cell adhesion[9], apoptosis[10], RAS signalling[6] and oncogenic transformation[11–14]. In addition, downstream effectors of PI3-kinase, *AKT1* and *AKT2*, have been found to be amplified[15,16] or activated[17] in human tumours, including ovarian cancer. We show here that *PIK3CA* is frequently increased in copy number in ovarian cancers, that the increased copy number is associated with increased *PIK3CA* transcription, p110α protein expression and PI3-kinase activity and that treatment with the PI3-kinase inhibitor LY294002 decreases proliferation and increases apoptosis. Our observations suggest *PIK3CA* is an oncogene that has an important role in ovarian cancer.

We employed fluorescence *in situ* hybridization (FISH) to assess DNA sequence copy number of *PIK3CA* and several flanking loci in interphase nuclei from cell lines established from ovarian cancers, normal ovarian epithelium (NOE), breast cancers, melanomas and uncultured cells from primary and ascites ovarian tumours. In addition, we used FISH to assess the chromosomal locations of the added *PIK3CA* copies in metaphase spreads prepared from ovarian cancer cell lines. Ovarian cancer cell lines (7/9) exhibited increased *PIK3CA* copy number relative to a locus on 3p25 containing *D3S1293*, and 1 of 9 (OVCA420) showed increased copy number on both p and q arms of chromosome 3
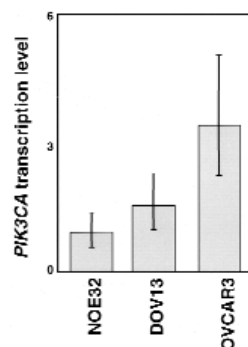

Fig. 2 Quantitative PCR analyses of *PIK3CA* transcription. We measured *PIK3CA* transcription for logarithmically growing cultures of NOE32, DOV13 and OVCAR3 using QPCR. Transcription levels were low in all three cell lines but were relatively higher in OVCAR3 than in NOE32 and DOV13. Assays were run in triplicate and with starting RT reactions of 100 ng and 200 ng. Results displayed are the averages of these measurements and are normalized to 1.0 for NOE32. Error bars show 95% confidence intervals for the measurements.

(Fig. 1*a*). DOV13 was the only ovarian cancer cell line that had normal *PIK3CA* copy number. *PIK3CA* relative copy number was also increased in cells from ascites ovarian tumours (5/5). Only 3 of 12 primary ovarian tumours were increased in *PIK3CA* copy number relative to 3p25; however, an additional 4 of the 12 tumours showed increased copy number for both 3p25 and 3q26. Thus, 7 of 12 tumours had increased *PIK3CA* copy number. *PIK3CA* was not increased in relative copy number in breast cancer and melanoma cell lines or in cultured NOE cells. FISH analyses of *PIK3CA* copy number in ovarian cancer cell lines CAOV3 and OVCA429 showed that *PIK3CA* was present in one or two copies on several different chromosomes (Fig. 1*b,c*). We also saw several copies of *PIK3CA* on one marker chromosome in a pattern similar to that observed in genes amplified during multiple bridge-breakage-fusion cycles (Fig. 1*c*), further supporting active selection of this part of the genome in ovarian cancers.

We measured *PIK3CA* transcription, p110α expression and PI3-kinase activity in NOE and ovarian cancer cell lines with and without increased *PIK3CA* copy number to determine whether these events were linked. We used quantitative PCR (QPCR) to measure *PIK3CA* transcription levels for a short-term NOE culture (NOE32) and two ovarian cancer cell lines (DOV13 and OVCAR3). *PIK3CA* transcription levels were approximately threefold higher in
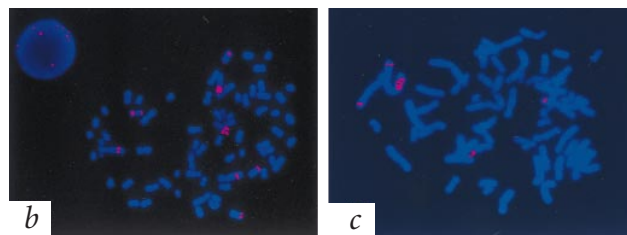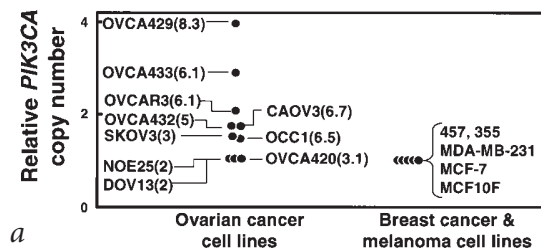

Fig. 1 Analysis of *PIK3CA* relative copy number. *a*, Relative copy number for *PIK3CA* measured using dual-colour FISH for 9 ovarian cancer cell lines, 1 normal ovarian epithelium culture, 3 breast cancer cell lines and 2 melanoma cell lines. Relative copy number was determined using FISH with P1 probe RMC03P019 (*D3S1293* located at 3p25) and BAC probe RMC03B3338A (*PIK3CA*). Numbers in parentheses are the average absolute copy numbers measured for *PIK3CA* for each line. Photomicrographs showing FISH with the BAC probe RMC03B3338A to the ovarian cancer cell line CAOV3 (*b*) and OVCA429 (*c*) are also shown. Hybridized probe was detected with Texas Red, therefore the hybridization domains appear red. The preparations were counterstained with DAPI so the nuclei and metaphase chromosomes appear blue.

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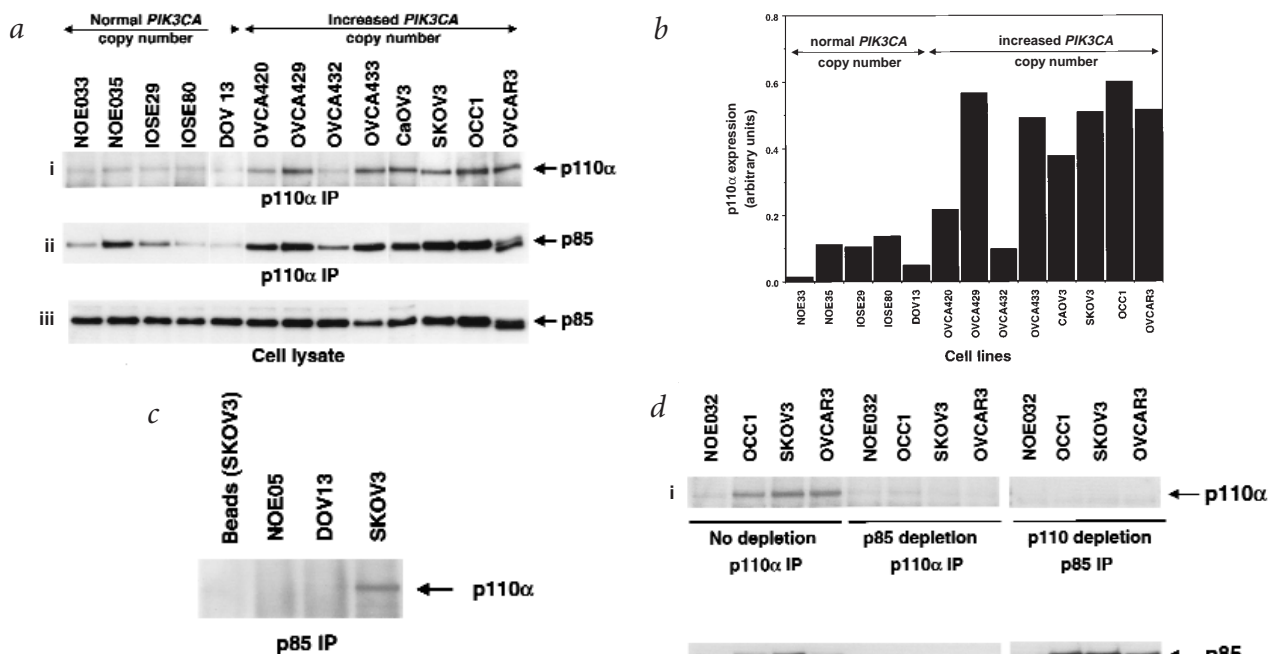
Fig. 3 Western-blot analysis of p85 and p110α expression. ***a***, p110α protein levels, p110α-associated p85 protein levels and total p85 protein levels in cultured ovarian cells normalized for total protein. The five samples to the left had normal relative *PIK3CA* copy number; the eight samples on the right had increased *PIK3CA* copy number. Cell lysates from logarithmically growing cells were immunoprecipitated using an antibody against the N terminus of PI3-kinase p110α (row i). Immunoprecipitates were separated by SDS PAGE and western blotted with an antibody to the C terminus of PI3-kinase p110α. The western blot from row i was stripped and probed with a rabbit polyclonal antibody against p85 (α and β), showing the levels of p85 that co-immunoprecipitated with p110α (row ii). Western blots of total cell lysates with an antibody against p85 (row iii) demonstrate that similar levels of p85 were present in all of the cells. ***b***, Densitometric analyses of p110α protein levels in the western blot in (*a*), row i. ***c***, Cell lysates from logarithmically growing cells normalized for actin levels were immunoprecipitated using an antibody against p85 (α and β). Immunoprecipitates were separated by SDS PAGE and western blotted with an antibody to the C terminus of PI3-kinase p110α. ***d***, p110α or p85 were depleted from cell lysates by incubation with an antibody against p85 or the N terminus of p110α. Due to limited availability of lysates, the amount of protein lysate used from the NOE cultures was less than that used from the tumour cell lines. The supernatants from the depletion were cleared by protein G sepharose for two cycles. The resultant supernatants were immunoprecipitated with antibodies against the N terminus of p110α or p85 as indicated. Immunoprecipitates were separated by SDS-PAGE and western blotted with an antibody against the C terminus of p110α (*d*, row i). The western blot from row i was stripped and probed with a rabbit polyclonal antibody against p85 (*d*, ii). Quantitative analysis of the blots (left, no p110α depletion, p110α-IP versus right, p110α depletion, p85-IP) showed that more p85 was depleted by anti-p110α in the cells with increased *PIK3CA* copy number than in NOE32 with normal *PIK3CA* copy number, as expected.

OVCAR3 than in NOE32 or DOV13 (Fig. 2). Thus, the increase in transcription level in OVCAR3 relative to NOE and DOV13 mirrored the *PIK3CA* copy number increase. We assessed expression levels of p110α and p85 (the regulatory subunit of PI3-kinase) by western-blot analysis in two short-term NOE cultures, two SV40-immortalized ovarian surface epithelial (IOSE) cell lines and nine ovarian cancer cell lines. p110α was more highly expressed in the cells with increased *PIK3CA* copy number (except for OVCA432) than in those without the copy number increase (Fig. 3*a*,*b*), whereas p85 was expressed equally in all cells (Fig. 3*a*, rows i and iii). p110α was also overexpressed relative to NOE in tumour cells purified from ovarian ascites fluid (5/5; data not shown). p110α is associated with p85 in cells in which it was highly expressed (Fig. 3*a*, row ii) as revealed by western-blot analysis of p85 co-immunoprecipitated using an antibody against p110α. The antibody used for analysis of p110α protein expression did react with proteins of the appropriate size that co-precipitated with p85 (Fig. 3*c*), attesting to its specificity. All of the p110α protein was associated with p85 protein (Fig. 3*d*), whereas most of the p85 protein was not associated with p110α. Thus, overexpression of p110α was associated with increased p110α-p85 heterodimer formation and thus may result in increased PI3-kinase activity. We compared lipid kinase activities in p110α immunoprecipitates from ovarian cancer cell lines with and without increased *PIK3CA* copy number. We found that the lipid kinase activities of precipitates from lines with increased *PIK3CA* copy number (OVCAR3, OCC1 and SKOV3) were higher than those

from lines with normal *PIK3CA* copy number (DOV13 and NOE; Fig. 4*a*). Non-immune serum and beads alone did not immunoprecipitate significant kinase activity (Fig. 4*b*). Addition of the specific PI3-kinase inhibitor LY294002 (10 μM) to the enzyme assay reduced kinase activity more than 80% (Fig. 4*b*), indicating that the activity was indeed due to immunoprecipitated PI3-kinase. These data indicate that increased expression of p110α in ovarian cancer cells leads to increased PI3-kinase activity.

We investigated the biological effects of increased PI3-kinase activity in ovarian cancer cells by treating cultures established from NOE, IOSE and ovarian cancer cell lines with and without increased *PIK3CA* copy number with LY294002. Treatment with LY294002 produced a dose-dependent decrease in cell number as indicated by MTT dye conversion in OVCAR3 and SKOV3 cells compared with that for NOE, IOSE or DOV13 cells (Fig. 5*a*). We found (data not shown) that the NOE and DOV13 cells have the same sensitivity to LY294002 as other cell lines (for example, breast cancer cell lines) that do not have increased *PIK3CA* copy number or mutated *PTEN* (functionally equivalent to an increase in expression *PIK3CA* in our hands[18]). The fractions of apoptotic (TUNEL positive) cells at 72 hours after treatment with 3 μM and 10 μM LY294002 were increased in OVCAR3 cultures relative to the fractions of apoptotic cells in NOE or DOV13 cultures (Fig. 5*b*). We also found that LY294002-induced apoptosis was not associated with significant changes in the distributions of cells in the G1-, S- and G2M-phases of the cell cycle at this time point (Fig. 5*b*). These

Fig. 4 PI3-kinase activity. The ability to phosphorylate phosphatidyl inositol (PI) *in vitro* was measured for p110α protein immunoprecipitated from cellular lysates. Measurements were normalized to total actin levels. *a*, Autoradiograms of the *in vitro* kinase assay separated by thin layer chromatography for p110α immunoprecipitates. *b*, SKOV3 lysate shows an 80% decrease in ability to phosphorylate PI after treatment with the PI3-kinase inhibitor LY294002 (10 μM added to the enzyme assay). Precipitates made using normal serum or agarose beads alone show no kinase activity.
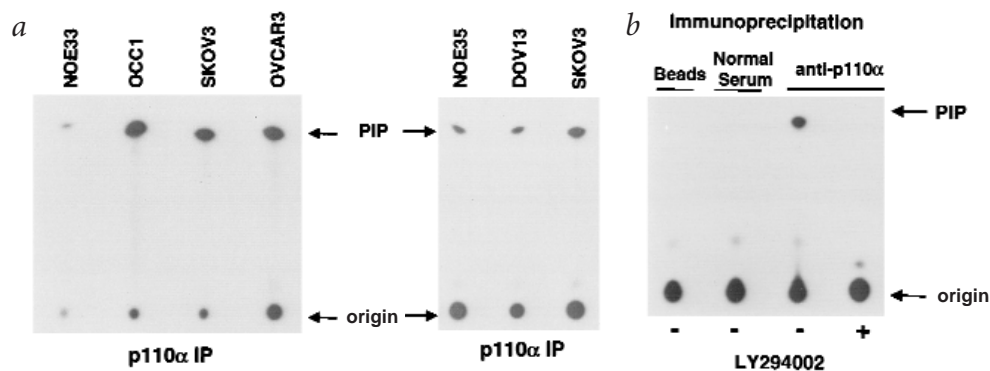
data suggest that the ovarian cancer cells with increased *PIK3CA* copy number have adapted to the higher PI3-kinase activity and respond with apoptosis when PI3-kinase is inhibited.

Increased PI3-kinase activity associated with increased *PIK3CA* copy number might contribute to ovarian cancer in several ways. One possibility is by increasing cell proliferation or increasing cell motility, for example, by acting synergistically with RAS (ref. 19). It is also possible that increased PI3-kinase activity contributes to ovarian cancer progression by reducing apoptosis. Several studies have linked PI3-kinase activation to increased cell survival through inhibition of apoptosis[9,10,20–22]. The influence of PI3-kinase activity on apoptotic death that occurs in cells separated from the extracellular matrix, termed anoikis[22], may be particularly significant in ovarian cancer due to the association between ovarian cancer incidence and number of cycles of ovulation[23] and the attending disruption of the stromal-epithelial organization. The recent observation that the loss of cell anchorage in keratinocytes resulted in a decrease in p53 levels[24] raises the possibility that a similar loss of p53 activity (and increased genomic instability) may occur in some cells disorganized during ovulation. Normally, such cells might be expected to lose PI3-kinase activity and be eliminated through anoikis[22]. However, abrogation of apoptosis in cells as a result of increased

PIK3CA copy number and the resulting increase in PI3-kinase activity might allow them to survive and to genetically evolve a more malignant phenotype. Our observation that increased copy number at 3q26 is an early event in ovarian cancer[2] is consistent with this model. If this model proves correct and if further studies show that PI3-kinase is activated in ovarian tumours as it seems to be in ovarian cancer cell lines, then inhibitors of PI3-kinase or downstream effectors become interesting as possible therapeutic agents against this devastating disease.

Methods

Cell lines and tumour samples. We obtained ovarian cell lines OVCA420, OVCA429, OVCA430, OVCA432, OVCA433 and DOV13 from R. Bast, IOSE from N. Auersperg and melanoma lines 355 and 457 from R. Taetle. OCC1 was developed in the Mills laboratory from ascites of an ovarian cancer patient. Other cell lines were obtained from the ATCC. All cell lines were grown in RPMI 1640 medium with 10% fetal bovine serum (FBS). We isolated NOE cells from fresh normal ovarian biopsy specimens and cultured them as described[25]. We fixed disassociated cells from these cultures in methanol-acetic acid solution (3:1) and dropped them onto microscope slides for FISH. We purified tumour cells from ascites of ovarian cancer patients as described[26]. We obtained touch preparations of serous ovarian carcinomas from the tissue bank maintained by P01 CA64602 and prepared them for FISH as described[27].
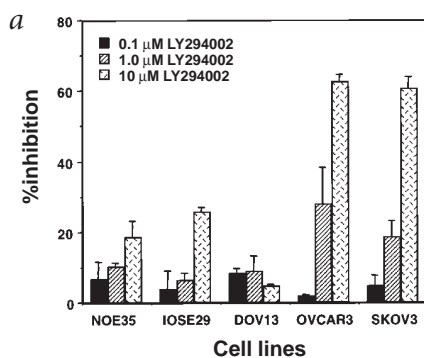

Fig. 5 Effect of LY294002 on cell proliferation and apoptosis. *a*, MTT dye reduction measured 96 h after initiation of culture in cultures treated with 0.01 μM, 0.1 μM and 10 μM LY294002 in DMSO. Results from several independent experiments are presented as percentage inhibition by LY294002 (=((OD without LY294002-OD with LY294002)/OD without LY294002)×100). Results are shown as mean±1s.e.m. Similar results were obtained with cells cultured in serum-free media, in the presence of 10 ng/ml EGF (data not shown), or in 10% fetal bovine serum (data not shown). *b*, Apoptosis measured using a TUNEL assay in which cells were stained for free 3′-hydroxyl ends of DNA fragments with FITC and for DNA content with propidium iodide (PI) and analysed using flow cytometry. Cells were incubated for 72 h with 0 μM, 3 μM or 10 μM LY294002 in 0.5% fetal bovine serum. The intensity of FITC fluorescence is shown on the Y-axis and PI fluorescence on the X-axis. Apoptotic cells appear as discrete populations with elevated FITC fluorescence.
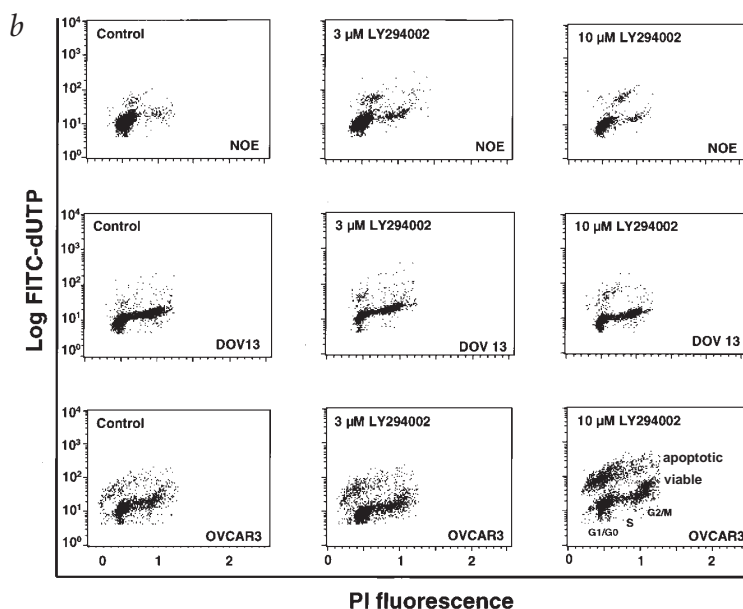
FISH probes. We selected clone RMC3B3338A from the Research Genetics BAC library using PCR with the *PIK3CA*-specific primers 5´−CACATCATG-GTGGCTGGACAAC−3´ and 5´−TGTGCAATTCCTATGCAATCGG−3´. We selected P1 clone RMC3P019 from the DuPont human genomic P1 library[28] using PCR with primers specific to *D3S1293*. We mapped *PIK3CA* to chromosome 3q26 by FISH. In addition, we linked it to *D3S3715* (lod>6) at 196.6 cM using the G3 radiation hybrid mapping panel and the Stanford server as described (http://www-shgc.stanford.edu/Mapping/rh/search.html). We labelled all probes by random primer extension using the BRL BioPrimer kit with digoxigenin-11-dUTP (Boehringer) or Texas-Red dUTP (NEN DuPont). Digoxigenin-labelled probes were detected using fluorescein-anti-digoxigenin (Vector Laboratories) as described[27].

FISH. We stained interphase nuclei and metaphase chromosomes using dual-colour FISH as described[27]. Texas red and fluorescein signals were scored at ×63 using a double bandpass filter (Chroma Technology) so that both signals could be seen simultaneously. At least 100 cells were counted for each test/reference probe pair during interphase analyses of relative copy number. Signals that were too close to each other to be distinctly resolved, or those that seemed to be overlapping, were counted as one signal.

PIK3CA **transcription.** We assessed levels of *PIK3CA* transcription relative to *GAPD* in NOE32, DOV13 and SKOV3 using quantitative PCR (QPCR) as described[29]. Briefly, we isolated total RNA using Trizol reagent (Life Technologies) and treated with DNase I to remove contaminating genomic DNA and reverse transcribed it using Superscript II reverse transcriptase (Life Technologies). We amplified *PIK3CA* using primers F1, 5´−GTATGTC-TATCCGCCACATGTAG−3´, and R1, 5´−CACAGTCATGGTTGATTTTCA-GAG−3´. A TaqMan probe, 5´−FAM (6-carboxy fluorescein)-TCTTCACCA-GAATTGCCAAAGCACA−3´TAMRA (6-carboxy-tetramethylrhodamine), was included during QPCR. PCR primers and the TaqMan probe for *GAPD* were obtained (Perkin Elmer Applied Biosystems). All measurements were normalized to the *PIK3CA/GAPD* ratio for NOE32.

Protein expression and PI3-kinase activity. To assess expression of p110α, we immunoprecipitated cellular protein[30,31] with goat polyclonal antibody (2 μg) against the N terminus of p110α (Santa Cruz Biotechnology) and protein G-conjugated sepharose 4B (Pharmacia). Immunoprecipitated proteins were separated by 8% SDS PAGE and immunoblotted with a goat antibody against the N or C terminus of p110α (Santa Cruz Biotechnology) or with a rabbit antibody to p85 (α and β; Upstate Biotechnology). We determined the total expression of p85 by separating cellular protein (5 μg) from each cell lysate[30] using 8% SDS PAGE and immunoblotting with the rabbit antibody to p85. HRP-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology) or HRP-conjugated protein-A sepharose (Amersham) was used as secondary reagent for ECL. We determined PI3-kinase activity in p110α immunoprecipitates from cell lysates normalized for actin levels as described[32].

Effect of LY294002. We assessed cell number and viability in cells plated in 96-well plates ($1.5×10^4$ cells/well), serum-starved overnight and then incubated for 96 h with LY294002 (CalBiochem) dissolved in DMSO (or DMSO alone) with 0.5% fetal bovine serum (Sigma). DMSO was maintained at 0.5% in all wells. Cell number and viability were assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye reduction[33]. We assessed cellular apoptosis using a variation of the TUNEL assay[34]. Cells were cultured in 100-mm dishes until 50% confluent, starved overnight by serum depletion and then treated with LY294002 for 72 h. Both detached and adherent cells were then collected and fixed in 2% paraformaldehyde. We incorporated dUTP-FITC using an Apo-direct apoptosis detection kit (Pharmingen), counterstained for total DNA content with PI and evaluated for apoptosis and cell-cycle distribution using a FACScan (Becton Dickinson ImmunoSystems) flow cytometer.

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