Lipids promote survival, proliferation, and maintenance of differentiation of rat liver sinusoidal endothelial cells *in vitro*

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October 21, 2011

Running Head: Lipids promote rat LSEC survival & differentiation *in vitro*

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Author contributions: TCH did all the experiments. TCH, DAL, LGG and DBS contributed to the design, interpretation and troubleshooting of experiments. TCH wrote the paper with assistance from DBS.

ABSTRACT

Primary rat liver sinusoidal endothelial cells (LSEC) are difficult to maintain in a differentiated state in culture for scientific studies or technological applications. Relatively little is known about molecular regulatory processes that affect LSEC differentiation because of this inability to maintain cellular viability and proper phenotypic characteristics for extended times *in vitro*, as LSEC typically undergo death and detachment around 48-72 hours even when treated with VEGF. We demonstrate that particular lipid supplements added to serum-free, VEGF-containing medium increase primary rat liver LSEC viability and maintain differentiation. Addition of a defined lipid combination, or even oleic acid (OA) alone, promotes LSEC survival beyond 72 hours and proliferation to confluency. Moreover, assessment of LSEC cultures for endocytic function, CD32b surface expression, and exhibition of fenestrae showed that these differentiation characteristics were maintained when lipids were included in the medium. With respect to the underlying regulatory pathways, we found lipid supplement-enhanced PI3K and MAPK signaling to be critical for ensuring LSEC function in a temporally-dependent manner. Inhibition of Akt activity before 72 hours prevents growth of SECs, whereas MEK inhibition past 72 hours prevents survival and proliferation. Our findings indicate that OA and lipids modulate Akt/PKB signaling early in culture to mediate survival, followed by a switch to a dependence on ERK signaling pathways to maintain viability and induce proliferation after 72 hours. We conclude that free fatty acids can support maintenance of liver LSEC cultures *in vitro*; key regulatory pathways involved include early Akt signaling followed by ERK signaling.

Keywords: unsaturated fatty acids, fenestrae, VEGF, CD32b, monoculture

INTRODUCTION

Liver sinusoidal endothelial cells (LSEC) play important roles in regulating liver function. LSEC line capillaries of the microvasculature and possess fenestrae to facilitate filtration between the liver parenchyma and sinusoid by serving as a selectively permeable barrier (7, 23). This role is augmented by high endocytic uptake rates, making LSEC effective scavengers for molecules such albumin, acetylated low density lipoproteins, hyaluronan and antigens in the bloodstream (22, 23, 26, 34, 40, 43). Furthermore, LSEC have a phenotype unique from traditional vascular endothelial cells, such as pan-endothelial marker CD31 localized only to endosomes in differentiated, unstimulated LSEC (18). Differentiated LSEC are capable of affecting resident liver cell proliferation, survival, or maintaining their quiescence. As such, loss of function may underlie various hepatic pathologies (7, 16, 24, 33, 50, 57).

LSEC are also targets or facilitators of infection and toxicological damage to liver (5). In addition to intrinsically vital contributions they make to proper liver tissue function *in vivo*, cultured LSEC are important to consider as essential non-parenchymal components of *ex vivo* tissue engineered models of liver physiology, which are of emerging importance in drug discovery and development (19, 29, 47, 51).

Despite this importance, much of LSEC biology remains unknown because they are difficult to maintain in a differentiated state for prolonged periods *in vitro*. Conventional endothelial culturing techniques are not as successful with LSEC; low serum concentrations (5%) can be toxic and cells die within 48 to 72 hours in serum-free monocultures even in the presence of VEGF (21, 31). Previously, attempts at serum-free LSEC culture resulted in cell viability maintenance from 6 up to 30 days with surviving cells maintaining endocytic uptake (20, 21, 31). Receptor mediated endocytic uptake is a characteristic feature of endothelial phenotype, but is insufficient for specific characterization of LSEC differentiation as large venule endothelial cells in the liver, as well as several vascular endothelial cells also exhibit this function (21, 28, 40, 41, 54). Another rat study was also able to prolong cell survival *in vitro* with use of multiple growth factors such as FGF, hepatocyte growth factor, and PMA within the context of hepatocyte-conditioned medium (31). Human LSEC cultures have been reported to be sustained for long periods, however, these LSEC were positively selected for, or had a higher expression of CD31,

a marker of LSEC dedifferentiation (14, 32). There are also controversies regarding phenotyping human LSEC, as there are reports of heterogeneous expression of surface markers used to characterize LSEC, such as von Willebrand Factor and immunological markers (21).

This study tested the hypothesis that an alternative approach emphasizing non-protein components could be beneficial in maintaining LSEC function in culture. Due to the location of the liver downstream of the intestinal tract and a center for lipid metabolism (10), we hypothesized that LSEC require lipids to maintain cell viability. We found that even in serum-free, minimal growth factor (*i.e.*, solely VEGF) media, free fatty acids (FFAs) were able to sustain LSEC culture. The addition of lipid supplements to serum-free media with 50 ng/mL VEGF allowed us to bypass the critical time point between 48 and 72 hours when most differentiated LSEC die *in vitro*. We identified oleic acid (OA) as a major contributing agent responsible for enhancing this survival. OA and lipids in culture could also eventually induce proliferation of cells with LSEC phenotype to confluency, although OA alone was insufficient for maintaining long-term confluent cultures. Furthermore, our results indicate that OA and lipids can maintain multiple LSEC phenotype markers simultaneously for at least 5 days in culture. Our findings indicate that OA and lipids influence early Akt/PKB signaling to mediate cell survival, while late ERK signaling is necessary in culture for viability and proliferation to persist.

MATERIALS AND METHODS

Chemically Defined Culture Media

Serum/growth factor-free base medium was made as described with modifications (27, 31). Low glucose DMEM (Invitrogen, Carlsbad, CA) was supplemented with 0.03g/L L-proline, 0.10g/L L-ornithine, 0.305 g/L niacinamide, 1 g/L glucose, 2 g/L galactose, 2 g/L BSA, 50 μg/mL gentamicin (Sigma-Aldrich, St. Louis, MO), 1 mM L-glutamine (Invitrogen), 5 μg/mL insulin-5 μg/mL transferrin-5 ng/mL sodium selenite (Roche Applied Science, Mannheim, Germany). "Modified hepatocyte growth medium" (HGM) included 200 μM ethanolamine and phosphoethanolamine, 100 nM ascorbic acid, 110 nM hydrocortisone (Sigma-Aldrich), 20 μg/mL heparin (Celsus Laboratories, Cincinnati, OH) and 50 ng/mL VEGF (R&D Systems, Minneapolis, MN). Additional treatments included 1% Chemically Defined Lipid Concentrate (~8μM final concentration) (Invitrogen 11905031) or 50 μM OA, FFA-free BSA, phosphatidylcholine (PC, 50 μM), and lysophosphatidylcholine (LPC 50 μM) (Sigma-Aldrich). For signaling studies, PI3K inhibitor LY294002 and MEK1/2 inhibitor PD0325901 (EMD Calbiochem, Gibbstown, NJ) were added to LSEC cultures 4 hours following seeding and maintained throughout the experiment. Inhibitors were reconstituted in DMSO (Sigma-Aldrich) to 20 mM. LY294002 was dosed at concentrations of 10 μM and 3 μM, while PD0325901 was used at 1 μM and 0.3 μM. Inhibitors were replenished once a day with fresh medium changes.

LSEC Isolation and Culture

Livers from approximately 180 to 250 gram male Fisher rats (Taconic, Hudson, NY) were used under the guidelines set forth by Massachusetts Institute of Technology's Committee on Animal Care. Cells were isolated using a two-step collagenase perfusion (27, 47) using Liberase Blendzyme (Roche Applied Science) in place of collagenase. The liver was perfused initially at 25 mL/min and reduced down to 15 mL/min flow rates in calcium free 10 mM HEPES (Sigma-Aldrich) buffer followed by 10 mM HEPES buffer with Blendzyme for cell isolation. The supernatant cell suspension from the perfusion was used to isolate LSEC at room temperature (6, 27). Very briefly, supernatant suspensions were spun down at 50 x *g* for 3 minutes. Supernatants were spun at 100 x *g* for 4 minutes. Supernatants following the spin were pelleted at 350 x *g* for 10 minutes and resuspended in 20 mL modified HGM without VEGF. The suspension was loaded over 25%/50% Percoll (Sigma-Aldrich)/PBS layers and centrifuged at 900 x *g* for 20

minutes. The interface between the Percoll layers were taken and resuspended with 1:1 modified HGM without VEGF before being spun down at 950 x *g* for 12 minutes. This LSEC enriched pellet was then resuspended into modified HGM with 25 ng/mL VEGF and 2% FBS (Hyclone/Thermo Fisher Scientific, South Logan, UT). Cells were counted using Sytox Orange exclusion and Hoechst 33342 (Invitrogen) staining on disposable hemacytometers (inCyto, Seoul, Korea). LSEC were then seeded onto 10 μg/mL fibronectin (Sigma-Aldrich) coated tissue culture plates at 400,000 cells/cm^2. Four to six hours following seeding, culture media were changed with serum-free modified HGM supplemented with VEGF. Additional conditions included supplementing 50 μM OA, 50 μM LPC, 50 μM PC, and 1% lipid concentrate to the culture over the course of 5 days at 37 °C and 5% CO_2. Media for all cultures were changed on a daily basis for all experiments.

Live/Dead Assay

LSEC viability was assessed using the Live/Dead Assay kit (Invitrogen L3224). LSEC were incubated for 1 hour with 2 μM Calcein AM and 4 μM Ethidium Bromide Homodimer in modified HGM. Cultures were washed with warm media prior to imaging.

Alamar Blue Metabolic Assay

Metabolic activity of LSEC was assessed over the time period of 5 days using Alamar Blue (Invitrogen) reduction assays. Positive reference standards were first made by heating base modified HGM at 125 °C with 10% Alamar Blue until the entire reagent was oxidized and converted to a bright shade of red. On the days of analysis, 10% Alamar Blue reagent was introduced to each well and allowed to incubate at 37 °C, 5% CO_2 for 6 hours prior to screening in a SpectraMax E2 (MDS Analytical Technologies, Sunnyvale, CA) fluorescent plate reader. Reference standards were included on each plate as positive controls and served as a point of reference in interpreting results. Fluorescent measurements were read by exciting the samples at 530 nm and reading the emission wavelengths at 590 nm. Samples were pooled across 3 biological replicates (5 technical replicates) for a total of 15 data points. All data points were normalized to blank readings prior to relative comparison to control samples.

Acetylated-LDL Uptake Assay

LSEC were grown on Thermanox coverslips (Nalgene Nunc, Rochester, NY) coated with 10 μg/mL fibronectin. On day 5, SECs were incubated for four hours with 10 μg/mL 1,1' dioctadecyl 3,3,3',3' tetramethylindo carbocyanine perchlorate labeled acetylated LDL (Di-I-Ac-LDL) (Biomedical Technologies, Inc., Stoughton, MA). Cells were washed several times with probe free modified HGM then rinsed with PBS. LSEC were fixed for 30 minutes in 3% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA), rinsed with PBS, mounted on glass slides with Fluormount (Sigma-Aldrich), and sealed with nail polish. Samples were compared with positive controls using human dermal microvascular endothelial cells (HDMVEC) (Lonza Inc., Allendale, NJ).

Immunofluorescence Microscopy

LSEC were cultured for up to 5 days on Thermanox coverslips coated with 10 μg/mL fibronectin. Samples were rinsed with PBS and fixed in 3% paraformaldehyde in PBS for 30 minutes. Following fixation, samples were rinsed three times with PBS and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for one hour, excluding samples immunostained for CD31 which were not permeablized so as to evaluate only surface expression. Following permeabilization, samples were rinsed three times with 2% BSA in 0.1% Tween-20 in PBS (PBS-T). Samples were blocked with 5% goat or donkey serum (Jackson ImmunoResearch, West Grove, PA) in 2% BSA PBS-T for 1 hour before overnight incubation at 4 °C with primary antibodies for anti-rat CD32b/SE-1 (IBL America, Inc., Minneapolis, MN) at 1:100, CD31/PECAM-1 (Chemicon/Millipore, Temecula, CA) at 1:100, and PCNA (Abcam, Cambridge, MA) at 1:600. The following day, samples were rinsed 3 times in 2% BSA PBS-T before a 1 hour incubation step with secondary AlexaFluor 488/555 (Invitrogen) antibodies at 1:250. Coverslips were then rinsed in 2% BSA PBS-T and stained with 1:500 Hoechst. Following incubation with secondary antibodies, samples were rinsed once in 2% BSA PBS-T prior to being treated briefly with nuclear Hoechst staining for 1 minute. Following Hoechst staining, samples were rinsed twice in normal PBS before being mounted onto glass slides with Fluormount and sealed with nail polish.

Scanning Electron Microscopy (SEM)

LSEC were grown on fibronectin-coated Thermanox coverslips. On days 3-5, LSEC were

rinsed with PBS and fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences) in PBS for 30 minutes. Samples were prepared following previously established protocols (27).

Flow Cytometry

Twenty-four hours before harvesting, 10 μM of 5-ethynyl-2'-deoxyuridine (EdU) (Invitrogen) was added to all conditions. Samples were detached with 0.025% Trypsin (Invitrogen) the following day, quenched with media containing 10% FBS, and immediately spun down at 1,600 rpm for 5 minutes. Cells were washed in PBS before being fixed in 2% paraformaldehyde in PBS for 15-30 minutes at room temperature. LSEC were centrifuged and resuspended in 1% BSA in PBS and incubated with primary CD32b antibody (1:100) prior to use of the Click-iT EdU kit, following manufacturer's instructions. Samples were analyzed on an Accuri-C6 (Accuri Cytometers, Inc., Ann Arbor, MI) flow cytometer and processed using FlowJo software (FlowJo, Ashland, OR). HDMVEC were used as a negative control population. Total and cellular events were captured with gates created using forward and side scatter data from HDMVEC populations. Following this, CD32b and EdU gates were designated using the double negative HDMVEC population.

Western Blotting

LSEC were harvested on day 5 of culture by incubating with cell lysis buffer (46) for 30 minutes. Cell lysis buffer consisted of 1% Triton X-100, 50mM β-glycerophosphate, 10 mM sodium pyrophosphate, 30 mM sodium fluoride (Sigma-Aldrich), 50 mM Tris (Roche Applied Science), 150 mM sodium chloride, 2 mM EGTA, 1 mM DTT, 1 mM PMSF, 1% Protease Inhibitor Cocktail and 1% Phosphatase Inhibitor Cocktails (Sigma-Aldrich). Samples were spun down at 12,000 rpm for 12 minutes at 4 °C and supernatants were reserved. Total protein content of sample lysates was determined using micro bicinchoninic acid kits (Thermo Fisher Scientific, Rockford, IL) before being loaded onto the NuPage Novex system (Invitrogen). Lysates were loaded with 6X reducing buffer (Boston BioProducts, Worcester, MA) in 4%-12% Bis-Tris gels (Invitrogen) and transferred to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA). Membranes were blocked with 5% BSA in PBS-T and incubated with antibodies for β-actin (1:5000), phosphoERK1/2 (1:5000), ERK1/2 (1:5000), phosphoAkt (1:1000), and Akt (1:5000) (Cell Signaling Technology, Beverly, MA) overnight at 4 °C. Membranes washed and then

incubated for 1 hour with horseradish peroxidase conjugated anti-mouse and anti-rabbit antibodies (Amersham/GE Healthcare Biosciences, Pittsburgh, PA) at 1:10,000 dilution in PBS-T with 5% blotting grade nonfat dry milk (Bio-Rad). Membranes were subsequently visualized using chemiluminescent ECL kits (Amersham/GE Healthcare Biosciences) on a Kodak Image Station (Perkin Elmer, Waltham, MA).

Image and Statistical Analysis

All experiments were repeated a minimum of three times with duplicate or triplicate samples. Fluorescent images were analyzed using Cell Profiler (Broad Institute, Cambridge, MA) and ImageJ (NIH, Bethesda, MD). Intact cell body counts from phase contrast were assessed at 100X magnification. Cells from a camera area of 1360 by 900 μm were counted from three biological replicates across seven days. Statistical significance was determined using ANOVA and Student's t-test (Microsoft Excel).

RESULTS

FFA lipids support cell survival past the first 48 hours in serum free media.

Isolated LSEC were plated and cultured using different lipid supplements of 50 μM OA or 1% lipid (a cocktail of saturated and unsaturated fatty acids) (Figure 1). Immunofluorescence staining of LSEC 24 hours after isolation indicated high purity of LSEC (Figure 5C). Distinct morphological changes were observed starting on day 3 in cultures with 50 μM OA or 1% lipid supplement, compared to control cultures (Figure 1A, B). Notably, LSEC cultured with 1% lipid underwent proliferation, and by day 5, the culture was at or near confluency. Both regular and FFA-free BSA were evaluated to account for potential variability of BSA-bound lipids. Medium supplemented with 50 μM OA yielded similar results as 1% lipid at day 5 in regular BSA. When FFA-free BSA was used, cells treated with 50 μM OA died after day 4 of culture (Figure 1C, D), although this was not observed with regular BSA. Untreated cells took on a granular appearance indicating that lipid moiety is a critical component for LSEC viability (Figure 1B, D). Granular morphology was also observed in LSEC cultured with PC and LPC (data not shown).

Live/Dead images of LSEC across conditions in both regular and FFA-free BSA were taken during five days of culture (Figure 2A, B). Massive cell death observed in the control concur with previously reported observations of LSEC demise beyond 48 hours in culture. While all conditions experienced cell number decline between days 2 and 3, lipid and OA treated cultures recovered and proliferated in both types of BSA, with statistically significant differences in population after day 3 compared to control ($p<5E-4$). (Figure 2C, D). Phase and live/dead staining indicate pronounced and distinct morphological changes for surviving LSEC. Lipid supplementation maintained LSEC to day 5. Cells grown with OA in normal BSA were viable after 4 days after isolation; however, FFA-free BSA did not synergize with OA to maintain cell viability. Lipid and OA conditions had persistently higher total live cell percentages compared to control, PC, and LPC conditions; PC and LPC did not offer any growth advantage for LSEC relative to control ($p>0.24$) (Figure 2). Live/Dead assay confirmed that LSEC with unhealthy granular appearance were dead and positive for ethidium bromide. PC and LPC cell cultures did not survive past day 2 in regular BSA (data not shown).

FFAs support metabolic and endocytic functionality in LSEC past day 3.

OA and lipid supplement supported significantly higher Alamar Blue reduction relative to control, in agreement with live/dead stain results (Figure 3A). These trends were also observed in FFA-free BSA cultures (Figure 3B). Endocytic capacity was measured using Di-I-Ac-LDL uptake as a functional assay for endothelial phenotype (Figure 3C). OA and lipid treatments sustained high endocytic uptake at day 5; cells positive for nuclear Hoechst were also strongly positive for Di-I-Ac-LDL. Many cells in the control did not remain after fixation; those that did remain stained positive for Hoechst but were negative for Di-I-Ac-LDL.

LSEC phenotype and proliferation are partially maintained with lipids in growth factor-reduced, serum-free media.

Following cell number reduction at day 3, LSEC phenotype was assessed. An important LSEC hallmark is the presence of fenestrae on cell surfaces. Using scanning electron microscopy we found both 50 μM OA and 1% lipid treated LSEC expressed numerous fenestrae at days 3-5 of culture (Figure 4), while control cells did not maintain fenestrae. Only about 5% of all FFA-treated cells expressed fenestrations in sieve plates. A larger percentage (10-15%) expressed large holes (Figure 4H, I) that are suspected to be sieve plate remnants. When the population was taken as a whole, porosity was well below the 10% observed for healthy LSEC in vivo (7), indicating that FFA alone does not maintain fenestrations at normal levels.

Another characteristic LSEC marker, CD32b, was used to corroborate phenotype. Immunostained coverslips revealed that cells treated with FFAs maintained CD32b expression at day 5 (Figure 5A). Control cells remaining in culture did not have colocalization of CD32b surface expression with nuclei; CD32b appeared as punctate staining which were most likely dead cell remnants. Non-viable adherent cells appeared less frequently in protocols with multiple rinse steps (e.g., Di-I-Ac-LDL uptake, Figure 3C; co-immunostaining, Figure 5; flow cytometry, Figure 6B). Although we stained for CD31, we did not observe CD31 expression on the cell surfaces of LSEC in FFA-treated conditions or remaining adherent cells in the control unless samples were permeabilized prior to staining (Figure 5B). $CD32b^+$ cells comprised a greater proportion of total cell populations in lipid treated LSEC in flow cytometry compared to controls (Figure 6C). The enhanced presence of $CD32b^+$ cells in OA and lipid is consistent with immunostaining results. CD32b staining was still present on day 5 cultures treated with lipid

(Figure 5C) and OA (not shown), but signal intensity was diminished compared to LSEC evaluated on day 1 following isolation.

Proliferative capabilities were measured using PCNA and EdU (a BrdU analog) incorporation. OA and lipid treated cells stained positive for both PCNA and CD32b expression at day 5 while untreated cells did not (Figure 5A). Most cells were $PCNA^-$ in the control; those that were $PCNA^+$ were $CD32b^-$. Day 5 cells had higher proportions $CD32b^+$/ EdU^+ cells in OA compared to control (Figure 6A, C). 1% lipid treated LSEC did not have statistically significant $CD32b^+/EdU^+$ populations over the control. However, this is likely attributed to the culture achieving confluency by days 4 and 5 relative to the OA condition; we were able to obtain a greater number of overall and $CD32b^+$ events for 1% lipid samples than with any other condition. Even when debris is included we have statistically significant larger populations of distinct double positive cells following treatment. Combined with immunostaining observations, we can state that PCNA observed in untreated conditions most likely stems from contaminating cell types and/or dedifferentiated LSEC.

Temporal dependence of LSEC on PI3K and MAPK pathways observed at Days 3 and 5 in FFA-treated cultures.

Akt and ERK1/2 proteins were probed on days 3 and 5 by Western blotting, as significant phenotypic changes occurred at these times (Figure 7A, B). Signaling trends observed for Akt and ERK1/2 were consistent across biological replicates. Phospho-Akt/Akt ratios decreased dramatically by day 5 in OA and lipid treated LSEC. Day 3 total and phospho-ERK1/2 levels were similar for all conditions but increased by day 5 in OA and lipid treated LSEC. Phospho-ERK/ERK ratios remained relatively unchanged for ERK2 but increased by day 5 for ERK1, indicating ERK1 as the primary contributor to overall phospho-ERK/ERK in OA and lipid cultures. Despite no observable statistical significance for phospho-protein signals in Western blots, we found a temporal significance with regard to total signaling proteins present at days 3 and 5 compared to control conditions. Total Akt was statistically significant at days 3 ($p<0.05$) and 5 for OA ($p<0.05$) and day 5 lipid ($p<0.05$) conditions, while total ERK1 (p44) was statistically significant at day 5 ($p<0.05$) compared to control. Phospho-Akt levels remained relatively constant across all conditions and times, while total Akt increased in treated conditions

compared to control through day 5.

Inhibition studies were performed using PI3K inhibitor LY294002 and MEK1/2 inhibitor PD0325901. Inhibitors did not affect LSEC for the first 24 hours of incubation (Figure 8A, 9A) despite lower concentrations effectively reducing downstream Akt and ERK1/2 phosphorylation (Figure 9D). By day 2, 10 μM PI3K inhibitor had adverse effects despite addition of OA or lipid (Figure 8B, 9A-C). Lower PI3K inhibitor concentrations (3 μM) showed similar effects in unsupplemented medium, but cultures with OA or lipid survived while only the lipid condition continued to proliferate (Figure 8B, 9B, C). High MEK1/2 inhibitor concentrations only slightly affected OA conditions at day 2 by reducing attached cell number, although culture quality appeared similar to treatments without inhibitor. Lipid cultures did not appear to be perturbed by 1 μM MEK1/2 inhibitor by day 2. MEK1/2 inhibition prevented culture survival after day 4 (Figure 8C,D, 9C). Lower MEK1/2 inhibitor concentration (0.3 μM) did not vary from the high dose used ($p>>0.05$ between all MEK1/2 inhibitor conditions at every time point), indicating LSEC may be more sensitive to changes downstream of MEK1/2 versus PI3K later in culture.

DISCUSSION

To test our hypothesis on the requirement of lipids to maintain LSEC viability, we evaluated several different types of lipids in both regular and FFA-free BSA. FFA-free BSA permitted individual testing of lipids for effects on LSEC culture, since native albumin exists bound to a variety of FFAs (4). By day 5, we observed that LSEC cultured with FFAs maintained metabolic and endocytic activity, and proliferated to confluency. The particular form of lipids delivered to LSEC was important, since membrane lipids PC and LPC did not maintain viability. PC and LPC can facilitate cell signaling and stimulate proliferation in many cell types (2, 42), but did not maintain LSEC in culture. We observed that OA alone was insufficient for supporting long-term culture in FFA-free BSA, although OA could recapitulate the lipid supplement effects in regular BSA. In comparison, the 1% lipid supplement, a cocktail of saturated and unsaturated FFAs, was able to support LSEC viability regardless of the BSA used, affirming the necessity for a variety of FFAs to sustain survival and proliferation.

A hallmark of LSEC is the presence of fenestrae, which were maintained in both OA and lipid samples on day 5. Of the few living cells remaining in the control, none were found to possess fenestrae, consistent with previous findings that fenestrae disappear within the first 48 hours of culture (7). Additional evaluation with CD32b phenotype marker validated findings that surviving LSEC in lipid or OA maintained differentiation by expressing this marker, one specific to liver sinusoidal endothelium (37). Along with CD32b expression, we also looked at the proliferative capacity of LSEC, since no previous studies have explicitly reported that differentiated rat LSEC can undergo proliferation. We successfully demonstrated that differentiated LSEC undergo proliferation, via nuclear PCNA expression and EdU incorporation, when treated with FFAs. Despite maintenance of several phenotypic characteristics in prolonged cultures, we did observe degradation of some markers. Although fenestrae arranged in sieve plates were observed, they were not abundant in OA and lipid treated cultures, and a large percentage of these LSEC no longer exhibited fenestrae by day 5. Many cells in the FFA-treated condition processed large transcytotic pores greater that 1 μm in diameter. These may be the remnants of sieve plates that have degraded or fenestrae that have fused. We noticed that although LSEC still expressed CD32b, the presence was diffuse and overall fluorescent intensity was lower than for freshly isolated LSEC (Figure 5C). Other studies have reported sharper

declines in specific LSEC phenotype markers during culture, mostly associated with the dedifferentiation process, recently reported to involve Leda-1 (24, 37). We suspect that lipid-treated LSEC maintain a state of differentiation that allows them to persist and proliferate *in vitro*, but do not maintain physiological levels of CD32b antigen or fenestrations.

In LSEC we observed phospho-Akt/Akt levels decreased in FFA conditions as time progressed, while the inverse occurred with phospho-ERK/ERK, primarily by ERK1. From these observations and inhibitor studies, we believe low threshold levels of phospho-Akt are required for cell survival between days 2 and 3. At this point, high concentrations of PI3K inhibitor LY294002 abolished the beneficial effects that OA and 1% lipid have on LSECs, while lower concentrations did not affect cultures. Beyond 3 days, cells in low PI3K inhibitor could proliferate and recover albeit not to the level seen in uninhibited samples. Granular morphology appeared earlier at day 2 (as opposed to day 3 in control samples without inhibitor) in untreated samples with PI3K inhibitor. This may indicate that downstream signals of PI3K are closely associated with cell survival during this time. Past day 3, MEK1/2 inhibition was fatal to cultures, as LSEC did not survive or proliferate regardless of the concentration of MEK1/2 inhibitor PD0325901 added to cultures. Interestingly, OA and lipid-treated cultured LSEC did not have a significant dependence on MAPK before this time, as 10 μM inhibitor only slightly affected the number of cells in culture. At early time points, MEK1/2 inhibition also prevented LSECs from undergoing increased spreading seen with FFA treatments. As such, MAPK signaling may be partially responsible for the morphology change induced by FFAs before day 3, but required afterward for survival and proliferation.

While it is understood that ECM, cell-cell contacts (37), and paracrine/autocrine signaling (17) are absolutely vital to achieve functional LSEC, consideration of the role of lipids is important given the results of this study. Effects of FFAs on LSEC can have several implications on liver pathophysiology. In general, lipids are crucial for survival for all mammalian cells as energy substrates, membrane lipid bases, and influencing cell signal processes (3). Concentrations of FFAs in circulation can vary dramatically depending on the metabolic state, but have been reported to be anywhere between 10 μM to 1 mM in human plasma, though generally within the range of 200 to 600 μM (25, 44, 45). Approximately 150 μM total plasma FFA is taken up in the

liver, of which about 50 μM is comprised of OA (and is recapitulated in our experimental conditions) (25, 30). The liver is the primary organ responsible for lipid metabolism as 75% of the blood that enters into the liver arrives from the intestine which absorbs lipids from the gut or lipolysis from adipose tissue (10). Thus, FFAs are likely to have a profound influence on LSEC. Past studies have shown that polyunsaturated FFAs can protect hepatocytes from superoxide radicals (49), while bioactive lipids like sphingosine 1-phosphate provide oxidative protection to LSEC following liver injury (57). In contrast, studies also argue for the presence of lipids as precursors to chronic disease, apoptosis, steatosis, and insulin resistance/diabetes (1, 33, 35, 36). For example, caveolin-1 is important to lipid metabolism during liver regeneration, but may also implicate a role of pathogenesis in LSEC since it is upregulated in dedifferentiating cells (8, 10, 50). Moreover, we observed that cocultures of hepatocytes and LSEC induce hepatic cell death in lipid conditions, suggesting concentrations beneficial to LSECs can be lipotoxic for hepatocytes (data not shown).

OA and other unsaturated FFAs have been reported to have numerous effects on metabolically active cells although the main mechanisms of OA and other FFA incorporation are still not fully understood. OA has been found to participate in crosstalk with EGFR and other pathways by affecting MAPK and PI3K (12, 13, 52, 53, 55, 56). However, much of the data from previous studies are contradictory in either stimulating or inhibiting these pathways dependent on the system being studied.

Unsaturated FFAs have been found to be able to protect against oxidative stress by reducing lipid peroxidation and inhibiting the inflammatory pathway NF-κB which can lead to endothelial cell activation (9, 11, 15, 39). Thus, OA may prevent oxidative stress in LSEC that decreases ERK1/2 activity (38, 48), thereby allowing cells to resume cell survival and proliferation after day 3. This would be in agreement with the results we observed in increased phospho-ERK1/2 activity. Furthermore, increased saturated to unsaturated fatty acid levels are strongly correlated with insulin resistance and decreased glucose production in the liver (33, 36). The introduction of more unsaturated FA into the system may facilitate insulin signaling and activation of phospho-Akt for cell survival in our early time points. While it is most likely that FFAs indirectly modulate proteomic responses via metabolic pathways, we observed distinct changes in

447 phospho-protein signaling pathways. We could directly influence viability in OA and lipid
448 treated LSEC by inhibiting PI3K and MAPK pathways, showing a temporal shift in phospho-
449 protein signaling dependence from PI3K to MAPK.
450
451 Our results implicate the underlying importance of FFAs in the basic function of LSEC, as FFA
452 modulate LSEC phenotype, survival, and proliferation in the absence of serum. Changes in the
453 FFA profile due to shifts in systemic or dietary delivery to the liver can potentially result in
454 LSEC dysfunction, leading to oxidative stress and activation of inflammatory pathways.
455 Additionally, decreases in unsaturated FFA (and increase in saturated FFA) could lead to steatosis
456 and insulin resistance. As such, lipid balance in the liver is required to prevent onset of disease.
457 We demonstrated that LSEC monocultures can maintain their unique phenotype in culture
458 through at least 5 days of culture and were concomitantly proliferating. Our chemically defined
459 media system provides an *in vitro* platform to effectively move forward in understanding the
460 phenomena involved in LSEC biology.
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ACKNOWLEDGEMENTS

We would like to thank Laura Vineyard, Ryan Littrell, Rachel Pothier, and Yasuko Toshimitsu for performing rat liver perfusions; Lorenna Buck and Megan Palmer for help with flow cytometry; Tharathorn Rimchala for her advice in image quantitation and Jonathan Franks for SEM processing. We would also like to thank Abhinav Arneja, Benjamin Cosgrove, Shannon Alford, Kristen Naegle, Melody Morris, Sarah Kolitz and Ajit Dash for helpful discussions and suggestions. Financial Support: NSF EFRI-0735997 (D.A.L.), NSF Graduate Student Fellowship, NIH CA076541 (D.B.S.), NIH R01-GM069668 (D.A.L.)

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FIGURE CAPTIONS

Figure 1. Lipids in FFA form sustain long-term culture. Phase contrast images of LSEC were taken at day 5 of culture in serum-free medium (modified HGM) with regular BSA **(A,B)** or fatty acid (FFA)-free BSA **(C,D)**. LSEC were cultured with 50 ng/mL VEGF (control), plus 50 μM oleic acid (50 μM OA), or 1% chemically defined lipid concentrate (1% lipid). Only conditions with OA or lipid appeared favorable for persistence of cell culture. Higher magnification images indicate a pronounced change in morphology in lipid treated conditions compared to untreated control cells which appeared more granular **(B,D)**. Scale bars = 50 μm **(A,C)**, 20 μm **(B,D)**.

Figure 2. LSEC death at 48 hours is abrogated following treatment with FFA. Live/Dead assays were performed on LSEC culture across several conditions **(A,B)**, with cell number quantification by Cell Profiler **(C,D)**. Samples were treated with calcein AM (green) for live cells and ethidium bromide homodimer (red) for dead cells. While all conditions experienced steep drops in total population by day 3, only OA or lipid treatments had significant live cell numbers ($p<5E-4$ compared to control), indicating lipid type importance. Abbreviations: PC = phosphatidylcholine, LPC = lysophosphatidylcholine, OA = oleic acid. Scale bar = 100 μm.

Figure 3. OA and lipid supplement support phenotype and function in LSEC cultures. Alamar Blue measurements were statistically higher at days 3 ($p<0.05$) and 5 ($p<0.005$) in 50 μM OA and 1% lipid supplement treatments over control for LSEC in regular BSA **(A)**. Similar trends were also observed in FFA free BSA cultures **(B)**. Alamar Blue reduction was statistically higher at day 3 for 50 μM OA and 1% lipid supplement treatments over control, PC, and LPC. At day 5, only 1% lipid supplement treatment was statistically significant over control, PC, and LPC, indicating that the 50 μM OA condition was insufficient to sustain long term cultures without the presence of other fatty acids. Most cells in the control condition did not survive past day 3; remaining cells did not co-stain for Hoechst (blue) and Di-I-Ac-LDL (red), while OA and lipid conditions consistently co-stained for both on day 5 **(C)**. Contrast and brightness were adjusted for the entire image for Hoechst staining due to background fluorescence arising from the Thermanox coverslips. Abbreviations: PC = phosphatidylcholine, LPC = lysophosphatidylcholine, OA = oleic acid. Scale bars = 100 μm **(C)**, 2.5 μm **(D)**.

669

670 **Figure 4. Maintenance of fenestrations in FFA cultures.** LSEC cultures were evaluated for
671 fenestrations at 3, 4 and 5 days following isolation in the presence or absence of lipid
672 supplementation. At day 3 most cells in the control condition were dead (arrows) or had no
673 visible fenestrations (A, D, G, J) while OA (B, E, H, K) and lipid (C, F, I, L) treated cultures
674 displayed some cells with fenestrations arranged in sieve plates (arrowheads). Some cells
675 displayed very large transcytotic pores (arrows). These fenestrations (arrowhead) and large pores
676 (arrows) were maintained in a fraction of the treated cells until day 5.
677 Magnifications: Scale bar in L represents 1 μm for panels B-F and J-L. Scale bar in I represents
678 10 μm for panels A, G-I).

679

680 **Figure 5. LSEC differentiation marker CD32b, proliferation marker PCNA, and nuclear**
681 **Hoechst co-localize to same cells in FFA cultures.** 5-day-old LSEC cultures were imaged for
682 CD32b (green), nuclear PCNA antigen (red), and nuclear Hoechst dye (blue) **(A)**. Punctate
683 CD32b staining was observed in the control and did not co-localize with Hoechst. Broad, diffuse
684 CD32b staining was observed in OA and lipid cultures on cells positive for PCNA and Hoechst,
685 demonstrating that differentiated LSEC undergo proliferation at day 5 *in vitro*. Immunostaining
686 controls for absence of CD31 **(B)** and CD32b **(C)** signal degradation were performed. LSEC
687 were cultured for 24 hours prior to being stained with CD31 and Hoechst **(B)**. Samples that were
688 permeabilized were positive for CD31 while non-permeabilized LSEC did not stain positive for
689 CD31. LSEC culture were highly pure in LSEC population after 24 hours using CD32b staining
690 **(C)**. After several days in culture, LSEC increase in surface area while CD32b staining appears
691 to have decreased in overall intensity as compared to freshly isolated cells. This may indicate
692 that LSEC no longer are actively synthesizing new CD32b antigen. Contrast and brightness were
693 adjusted for the entire image due to background fluorescence arising from the Thermanox plastic
694 coverslips. Scale bar = 100 μm.

695

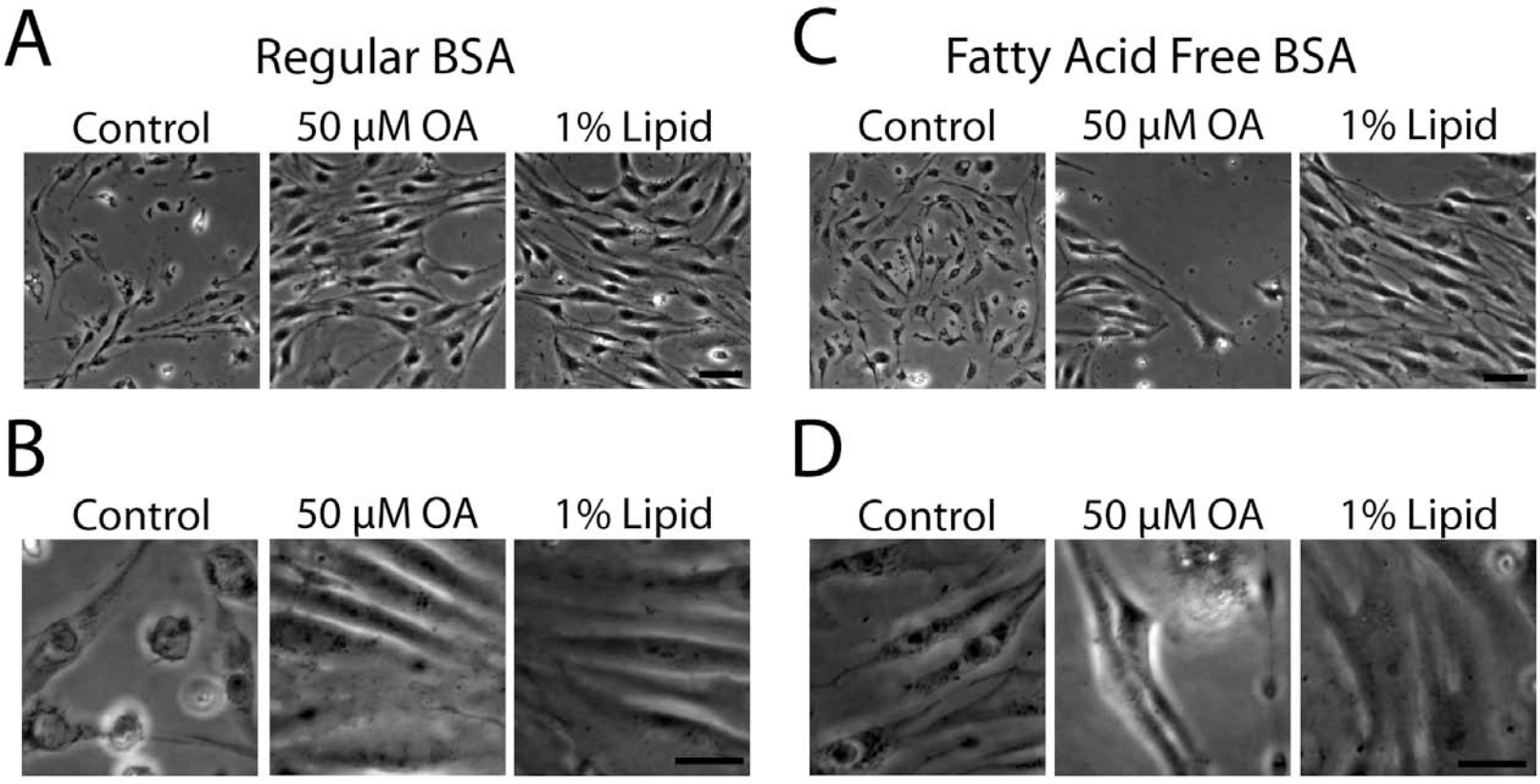
696 **Figure 6. OA and lipid supplement help promote proliferation and maintain differentiation**
697 **in long term LSEC culture.** Day 5 total events were captured by flow cytometry and gated for
698 CD32b and EdU using a double negative HDMVEC **(A)**. Total event (cellular + debris) and
699 cellular event counts were tallied and presented as fold number over control, showing

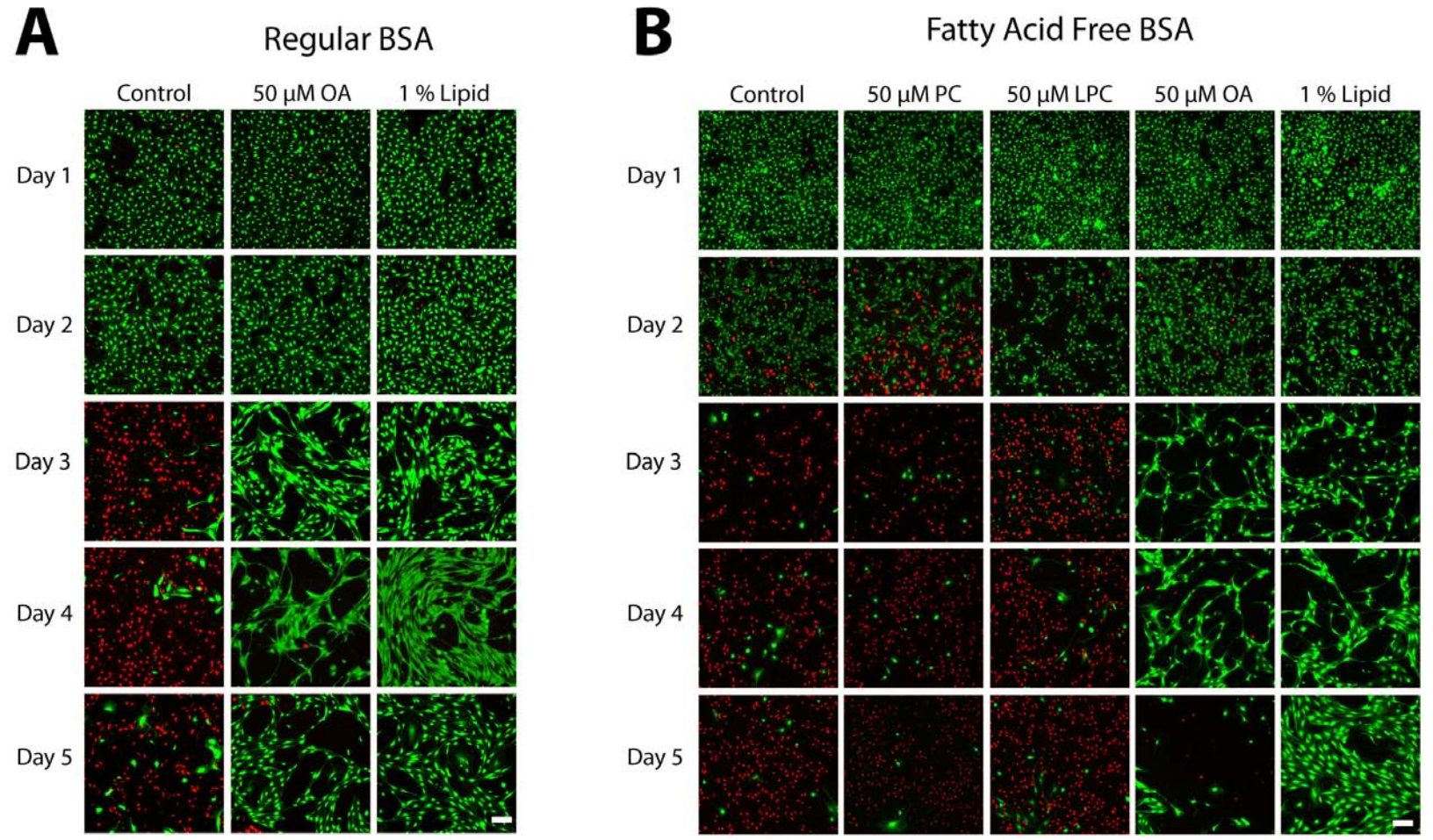
consistently 5-20 fold greater number of cellular events in OA and lipid conditions **(B)**. OA and lipid conditions maintained CD32b and were also EdU^+. LSEC with OA or lipid had statistically significant larger percentages of total events for $CD32b^+$, EdU^+, and dual $CD32b^+/EdU^+$ populations compared to control **(C)**. Overall CD32b expression in OA and lipid conditions were statistically significant from untreated cells ($p<0.01$, $p<0.001$).

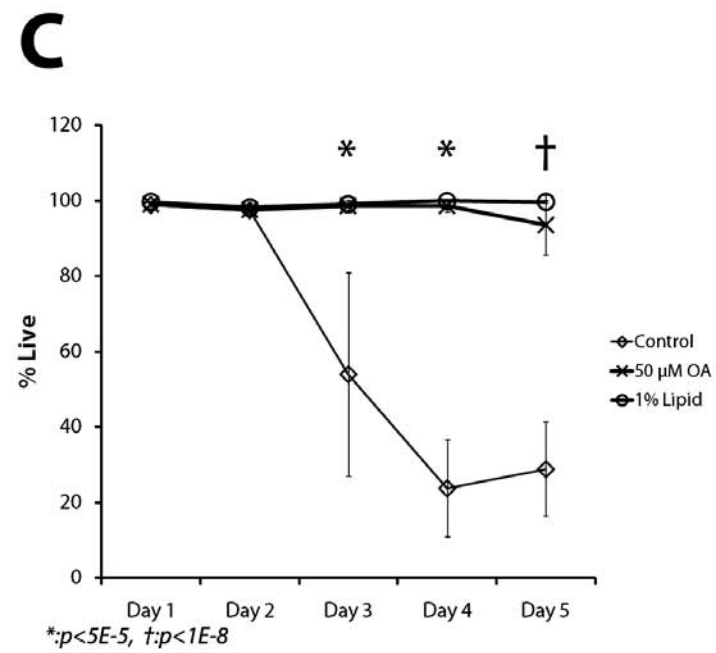
Figure 7. Lipid and oleic acid treated LSEC had higher phospho-ERK and phospho-Akt activity. Phospho- and total protein blots were performed for Akt and ERK at days 3 and 5 (representative shown **(A)**). Signaling trends observed for Akt and ERK1/2 were consistent across biological replicates. Replicates and pixel intensity data were analyzed using ImageJ and plotted against control after normalizing to β-actin values **(B)**. OA and lipid conditions had higher phospho-ERK1/total phospho-ERK1 ratios than in control. Phospho-Akt/total Akt ratios were lower in OA and lipid conditions than in control. Total Akt increased in OA and lipid conditions ($p<0.05$ from control at days 3 and 5), while total ERK1 increased at day 5 ($p<0.05$ from control).

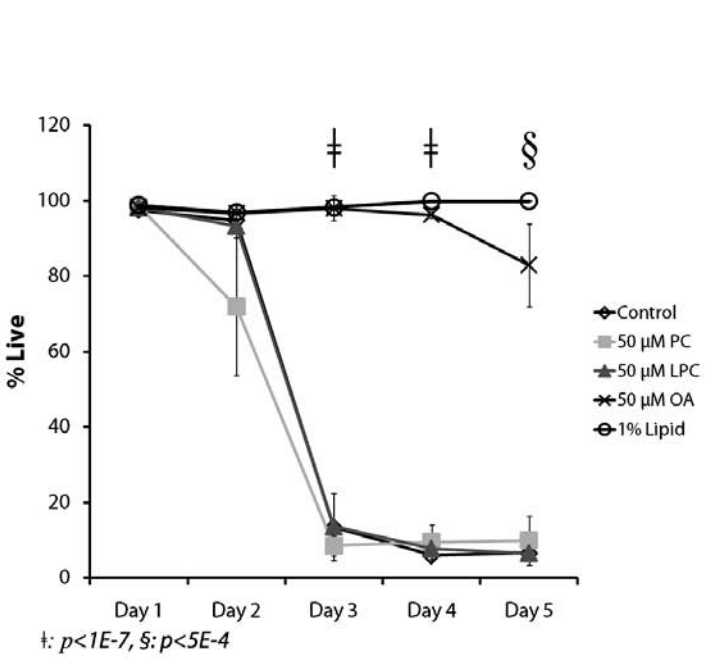
Figure 8. Oleic acid and lipid supplement support cultures through early maintenance of low threshold of phospho-Akt followed by late phosho-ERK signaling. Cells were cultured in identical conditions with PI3K inhibitor (LY294002 1 or 10 μM) or MEK1/2 inhibitor (PD0325901 0.3 or 1 μM) for 7 days. Drug inhibitors had no significant effects on cell cultures following the first day of drug inhibitor treatment (A). Significant cell integrity loss was observed in LSECs with 10 μM PI3K inhibitor, with less pronounced effects in 1 μM PI3K inhibitor by day 2, while MEK1/2 inhibitor started to affect oleic acid cultures but not 1% lipid treated SECs (B). SECs in oleic acid or 1% lipid were able to maintain culture viability by days 4 and beyond in culture despite low PI3K inhibitor concentrations (C,D). MEK1/2 inhibitor did eventually affect lipid treated SECs by day 4 (C), although many cells managed to survive in low MEK1/2 inhibitor concentrations. No SECs remained by day 7 of culture with MEK1/2 inhibitor, while low PI3K inhibited SECs treated with either oleic acid or 1% lipid recovered (D). Scale bar = 100 μm.

Figure 9. OA and lipid supplement support cultures through early maintenance of low threshold of phospho-Akt followed by late phospho-ERK signaling. Intact cell body counts show temporal difference in Akt and ERK signaling **(A-C)**. Low PI3K inhibitor delayed LSEC recovery in OA and lipid conditions while high concentrations prevented culture recovery as early as day 2. Following PI3K inhibition, OA condition was eventually unable to rescue the culture entirely, as the culture decline after day 5. Delays in intact cell loss were observed for low and high MEK inhibition until day 3 in lipid conditions, and LSEC did not recover at later times following MEK inhibition. Although control conditions contained many intact cell bodies, cells had granular morphology of dead cells observed in Figure 2. Western blots show PI3K and MEK1/2 inhibitors effectively reduce phosphoprotein signals within the first 24 hours of treatment **(D)**. Abbreviations: C = Control, OA = 50 μM oleic acid, L = 1% lipid, MEKi = MEK1/2 inhibitor PD0325901, PI3Ki = PI3K inhibitor LY294002.

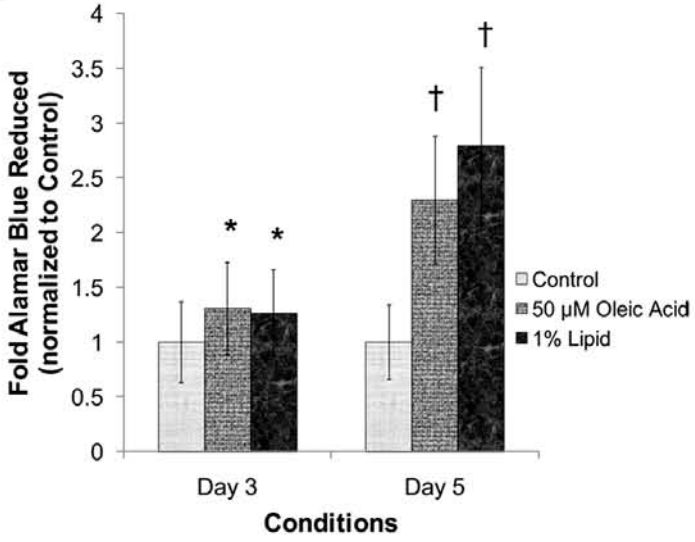

A
Regular BSA
Control
50 µM OA
1% Lipid
C
Fatty Acid Free BSA
Control
50 µM OA
1% Lipid
B
Control
50 µM OA
1% Lipid
D
Control
50 µM OA
1% Lipid


A
Regular BSA
Control
50 µM OA
1 % Lipid
Day 1
Day 2
Day 3
Day 4
Day 5
B
Fatty Acid Free BSA
Control
50 µM PC
50 µM LPC
50 µM OA
1 % Lipid
Day 1
Day 2
Day 3
Day 4
Day 5


C
120
100
80
60
40
20
0
% Live
*
*
†
Day 1
Day 2
Day 3
Day 4
Day 5
Control
50 µM OA
1% Lipid
*:p<5E-5, †:p<1E-8

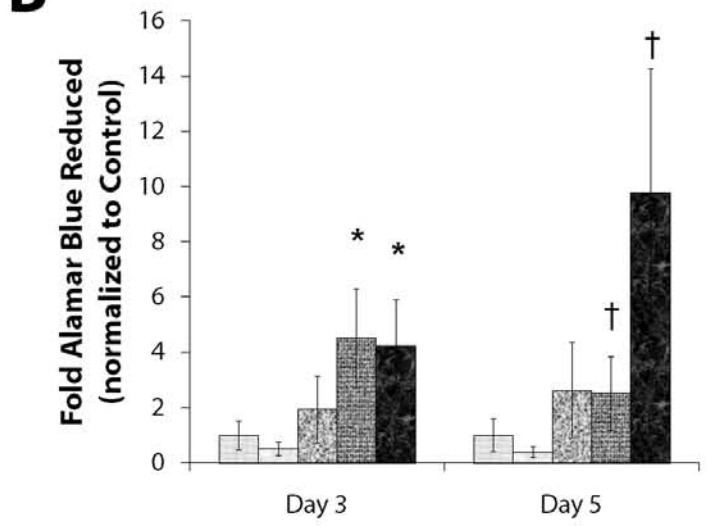

D
120
100
80
60
40
20
0
% Live
‡
‡
§
Day 1
Day 2
Day 3
Day 4
Day 5
Control
50 µM PC
50 µM LPC
50 µM OA
1% Lipid
‡: p<1E-7, §: p<5E-4

A



: p <0.05; †: p<0.005 from Control on Days 3 and 5

B



Control
50 µM PC
50 µM LPC
50 µM OA
1% Lipid

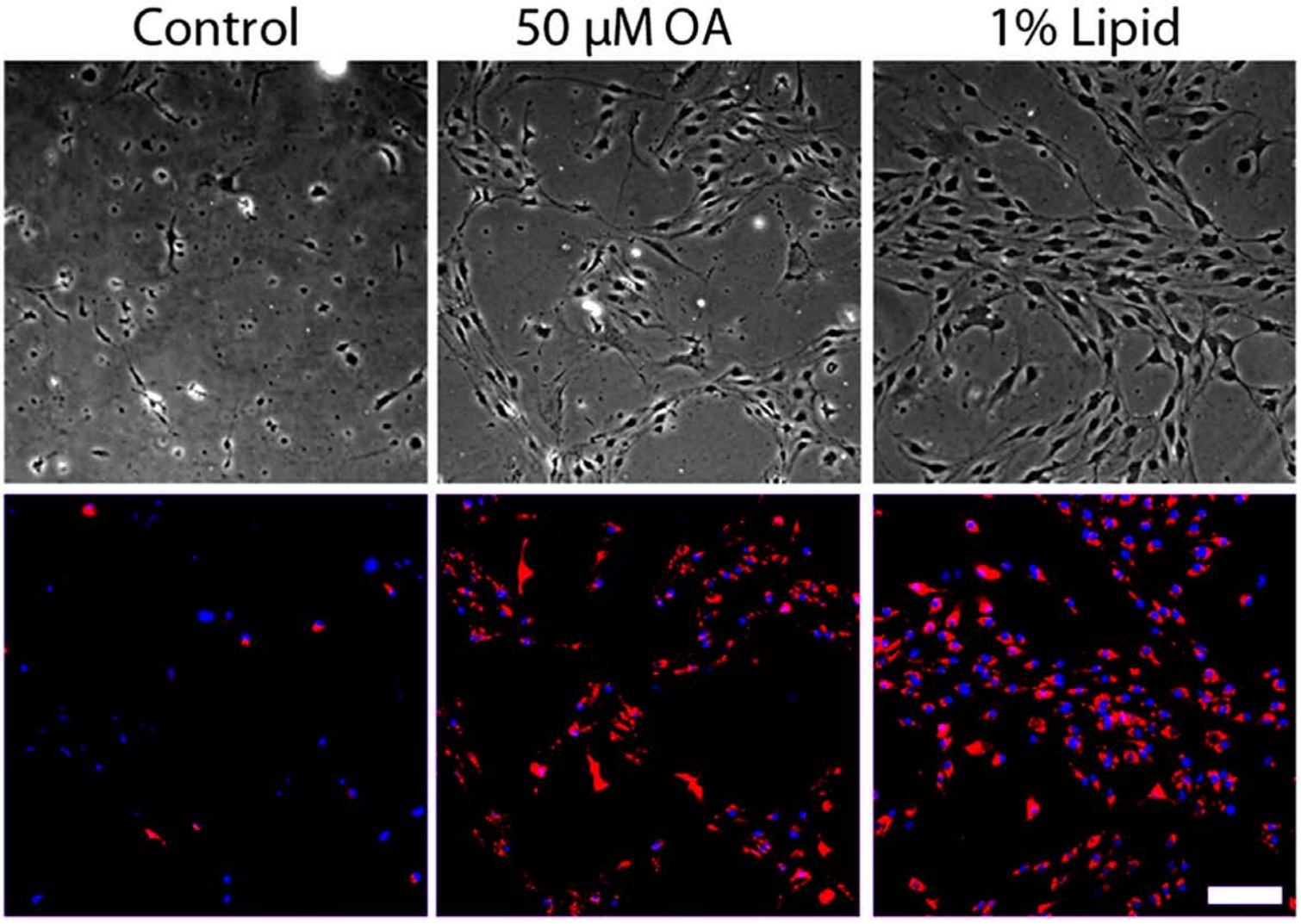
*** (Day 3)**

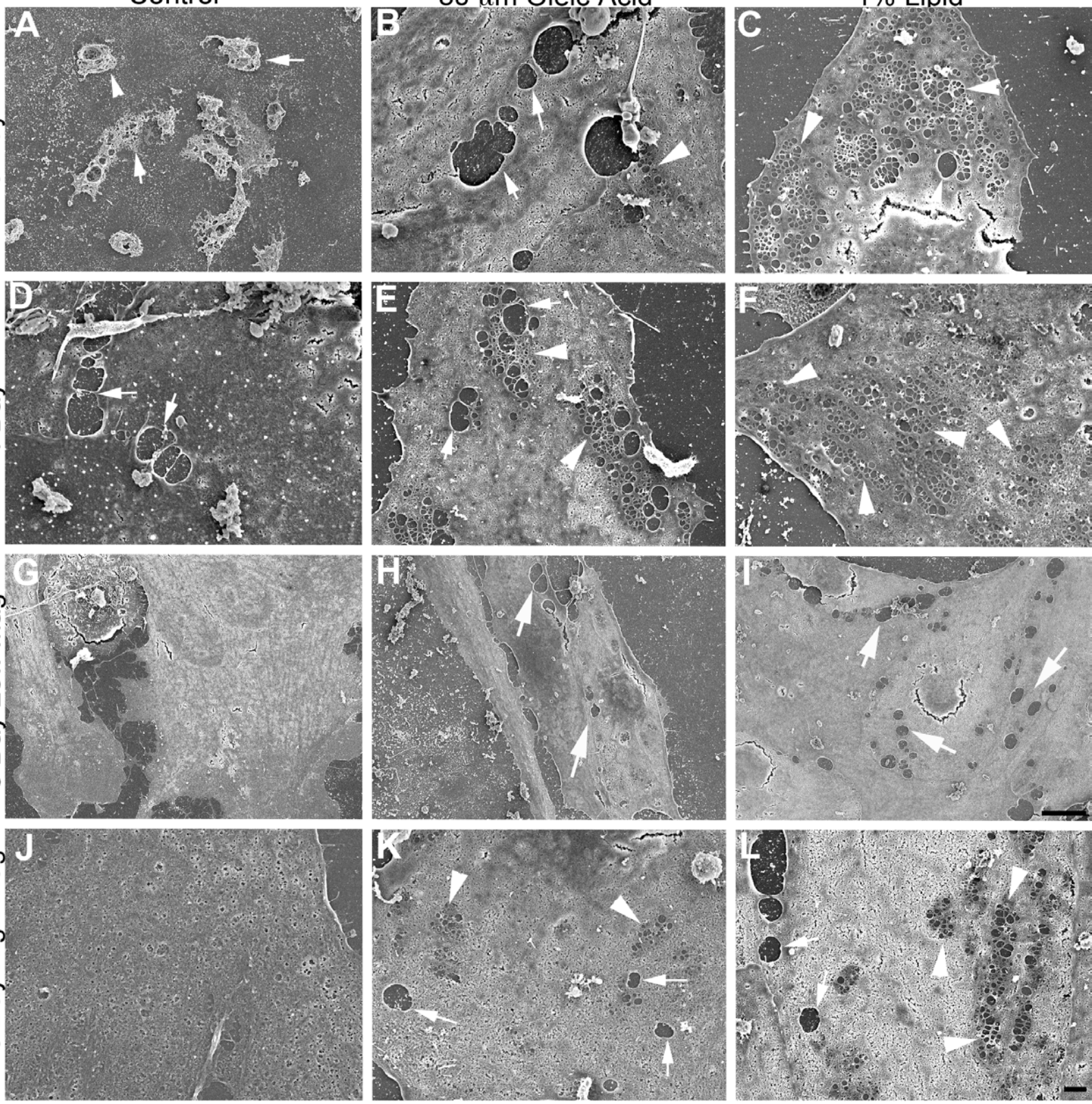
p-value	Control	50 µM PC	50 µM LPC
Lipid	<1E-7	<0.005	X
50 µM Oleic Acid	<1E-7	<0.005	<0.05

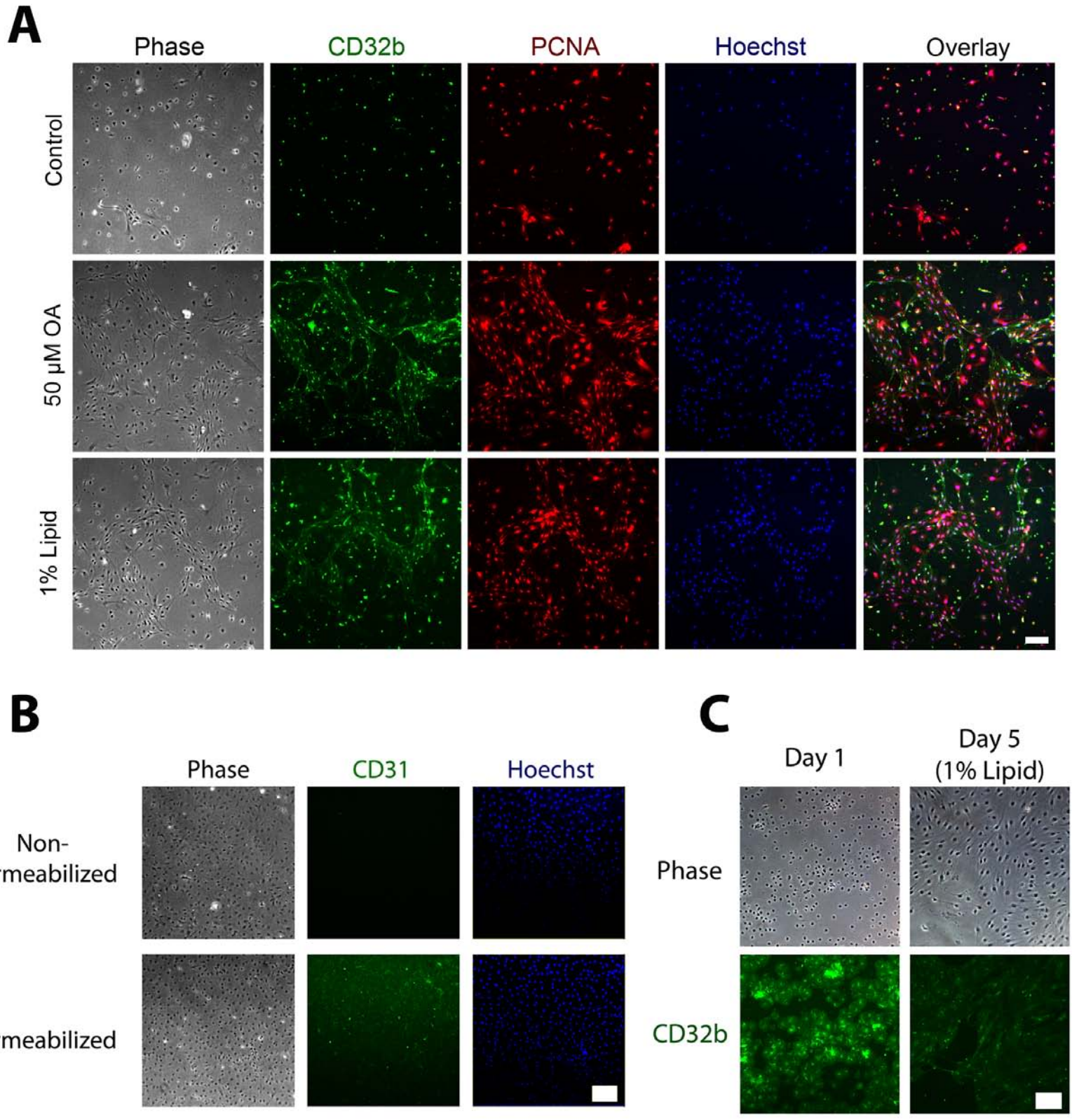
† (Day 5)

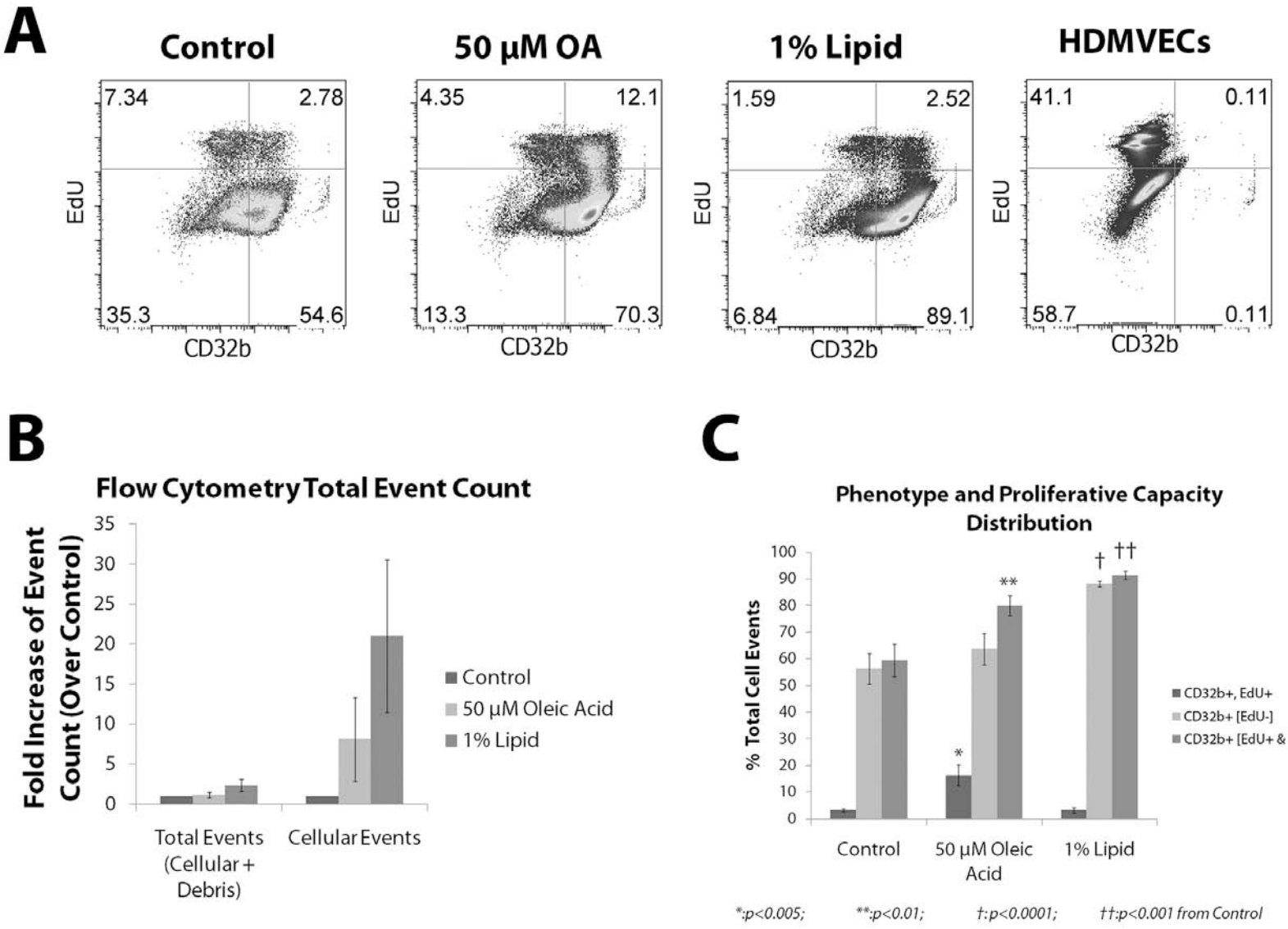
p-value	Control	50 µM PC	50 µM LPC
Lipid	<1E-7	<0.001	<0.01
50 µM Oleic Acid	<0.0005	<0.005	X

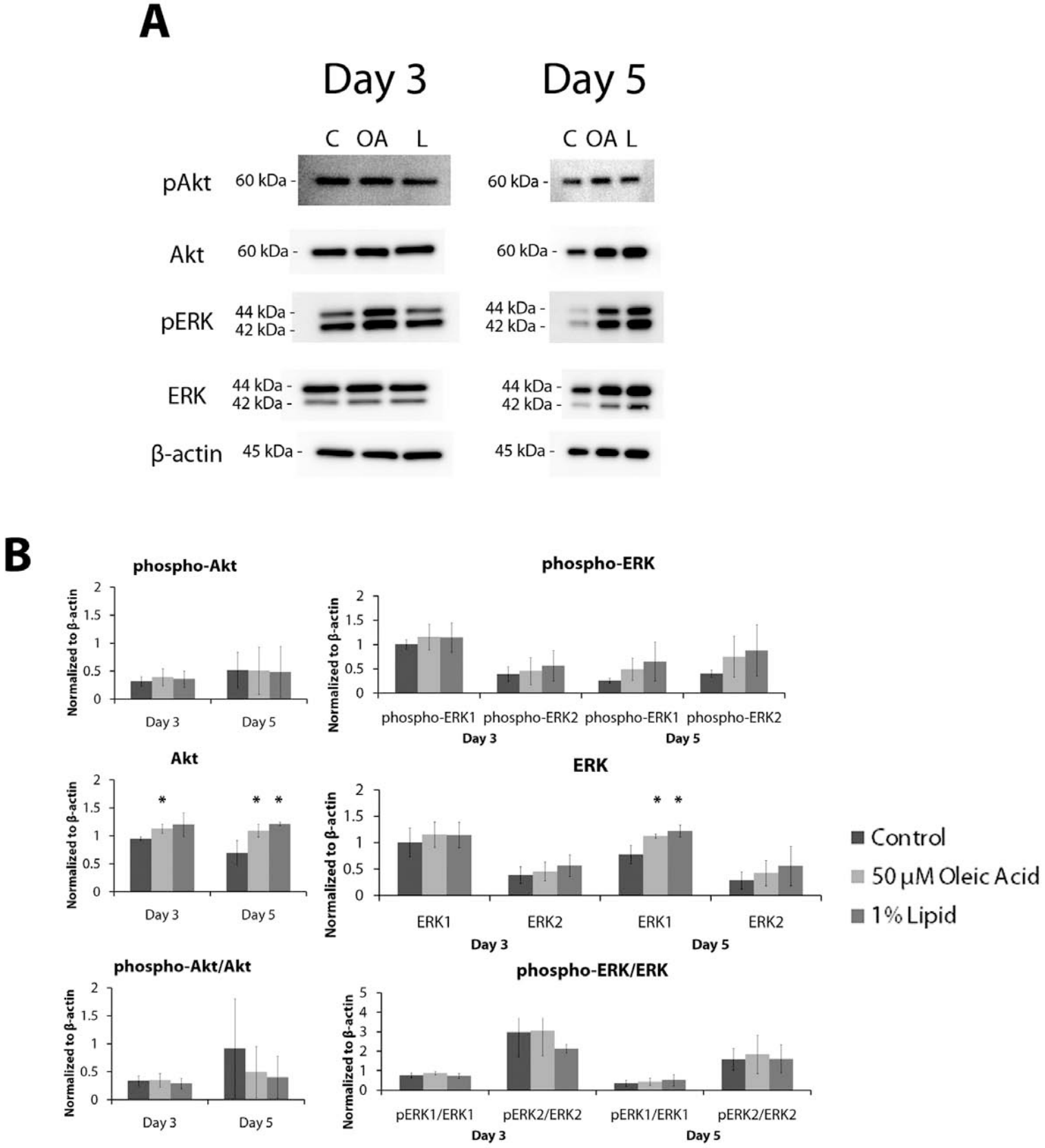
C



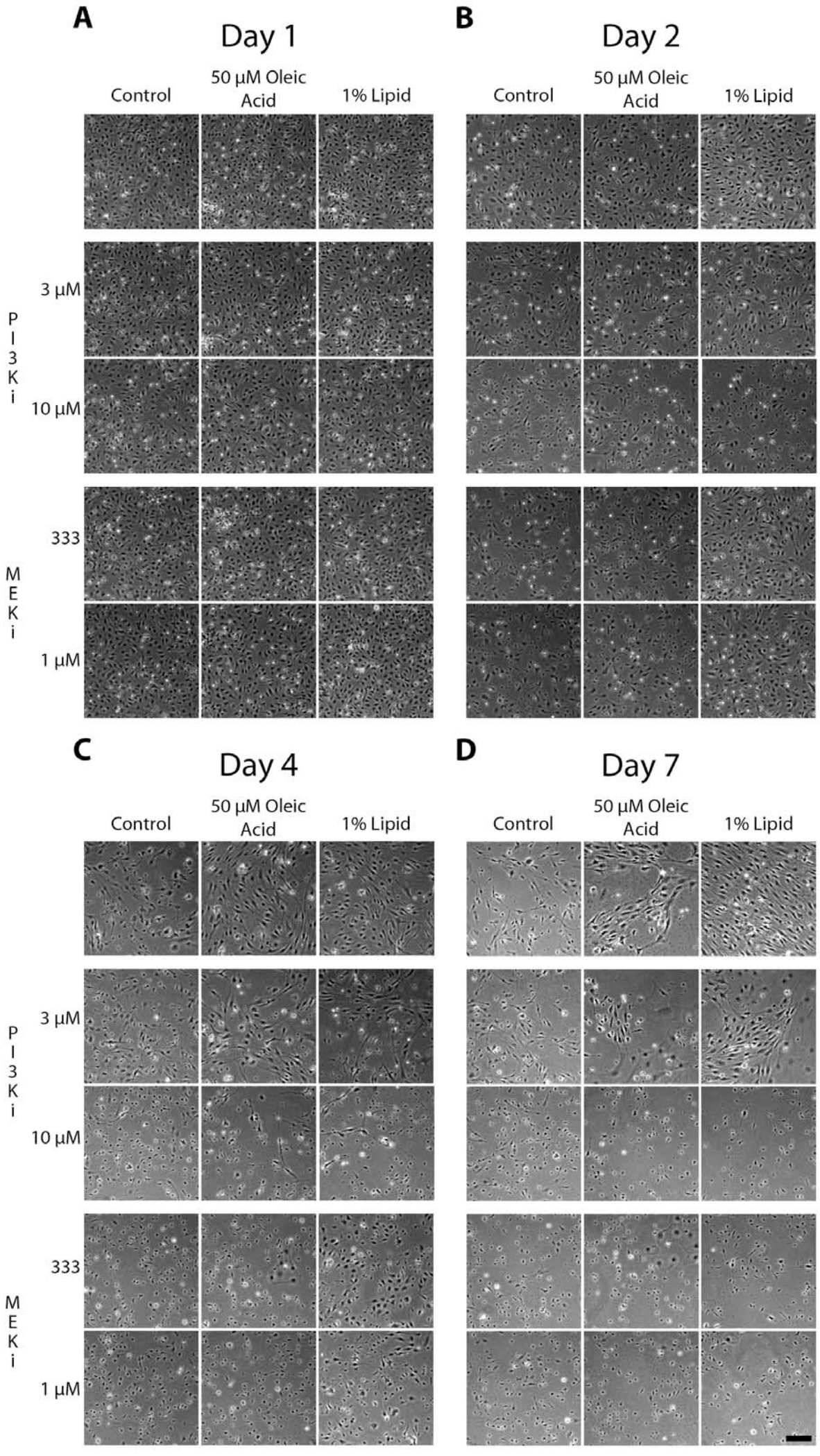

Control
50 μm Oleic Acid
1% Lipid
3 Day
4 Day
5 Day Low Mag
5 Day High Mag
A
B
C
D
E
F
G
H
I
J
K
L

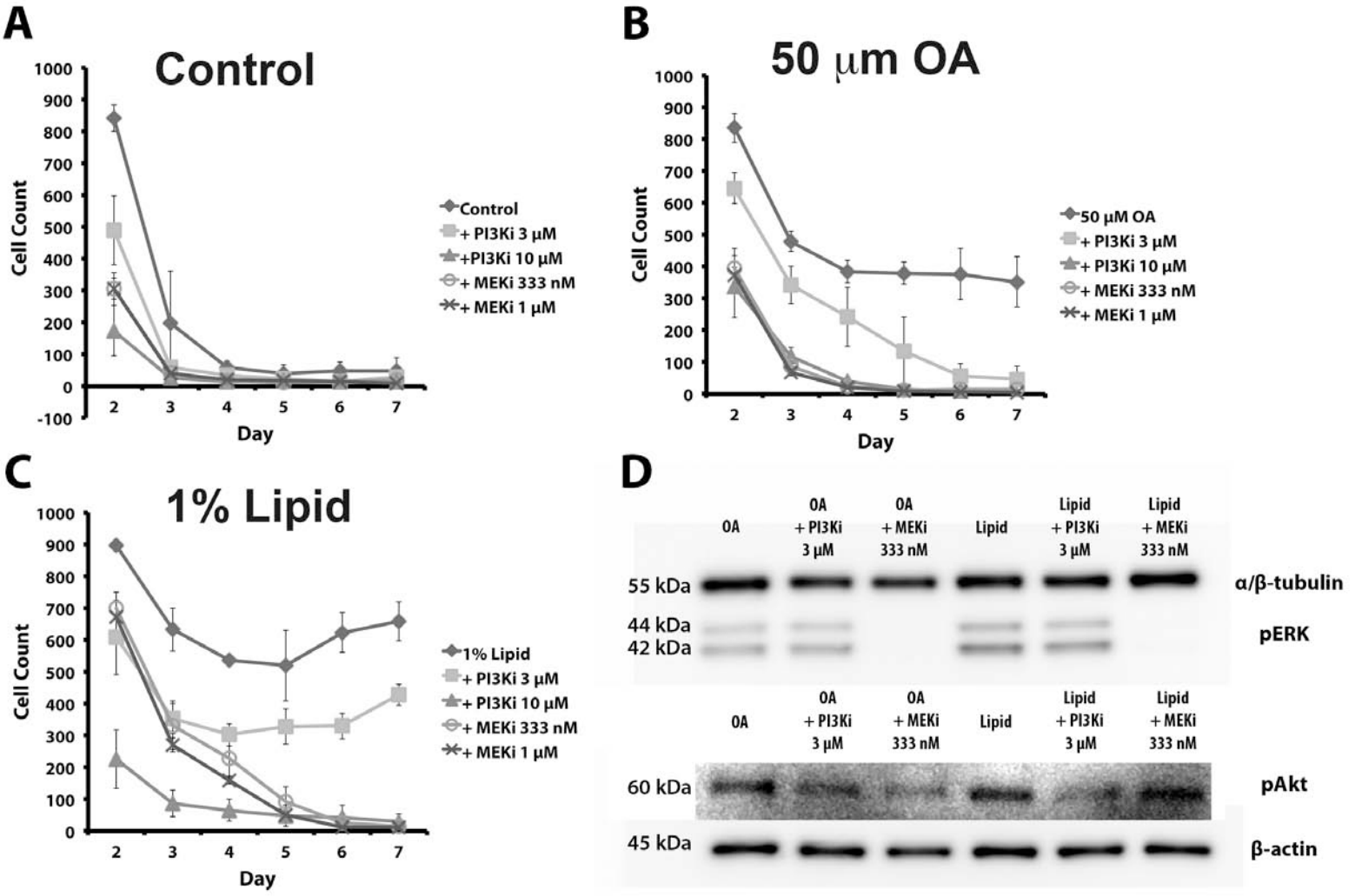

A
Phase
CD32b
PCNA
Hoechst
Overlay
Control
50 µM OA
1% Lipid
B
Phase
CD31
Hoechst
Non-
Permeabilized
Permeabilized
C
Day 1
Day 5
(1% Lipid)
Phase
CD32b


A
Control
50 µM OA
1% Lipid
HDMVECs
7.34
2.78
35.3
54.6
4.35
12.1
13.3
70.3
1.59
2.52
6.84
89.1
41.1
0.11
58.7
0.11
EdU
CD32b
B
Flow Cytometry Total Event Count
Fold Increase of Event Count (Over Control)
35
30
25
20
15
10
5
0
Total Events (Cellular + Debris)
Cellular Events
Control
50 µM Oleic Acid
1% Lipid
C
Phenotype and Proliferative Capacity Distribution
% Total Cell Events
100
90
80
70
60
50
40
30
20
10
0
*
**
†
††
Control
50 µM Oleic Acid
1% Lipid
CD32b+, EdU+
CD32b+ [EdU-]
CD32b+ [EdU+ & EdU-]
*:p<0.005;
**:p<0.01;
†:p<0.0001;
††:p<0.001 from Control



*: $p<0.05$ from Control on Days 3 and 5


A
Day 1
Control
50 μM Oleic Acid
1% Lipid
3 μM
10 μM
PI3Ki
333
1 μM
MEKi
B
Day 2
Control
50 μM Oleic Acid
1% Lipid
C
Day 4
Control
50 μM Oleic Acid
1% Lipid
3 μM
10 μM
PI3Ki
333
1 μM
MEKi
D
Day 7
Control
50 μM Oleic Acid
1% Lipid


A
Control
Cell Count
Day
Control
+ PI3Ki 3 µM
+PI3Ki 10 µM
+ MEKi 333 nM
+ MEKi 1 µM
B
50 µm OA
Cell Count
Day
50 µM OA
+ PI3Ki 3 µM
+ PI3Ki 10 µM
+ MEKi 333 nM
+ MEKi 1 µM
C
1% Lipid
Cell Count
Day
1% Lipid
+ PI3Ki 3 µM
+ PI3Ki 10 µM
+ MEKi 333 nM
+ MEKi 1 µM
D
OA
OA + PI3Ki 3 µM
OA + MEKi 333 nM
Lipid
Lipid + PI3Ki 3 µM
Lipid + MEKi 333 nM
55 kDa
α/β-tubulin
44 kDa
42 kDa
pERK
60 kDa
pAkt
45 kDa
β-actin