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

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

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

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24   the design, interpretation and troubleshooting of experiments. TCH wrote the paper with  
25   assistance from DBS.

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**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

## 56 INTRODUCTION

57 Liver sinusoidal endothelial cells (LSEC) play important roles in regulating liver function. LSEC  
58 line capillaries of the microvasculature and possess fenestrae to facilitate filtration between the  
59 liver parenchyma and sinusoid by serving as a selectively permeable barrier (7, 23). This role is  
60 augmented by high endocytic uptake rates, making LSEC effective scavengers for molecules  
61 such as albumin, acetylated low density lipoproteins, hyaluronan and antigens in the bloodstream  
62 (22, 23, 26, 34, 40, 43). Furthermore, LSEC have a phenotype unique from traditional vascular  
63 endothelial cells, such as pan-endothelial marker CD31 localized only to endosomes in  
64 differentiated, unstimulated LSEC (18). Differentiated LSEC are capable of affecting resident  
65 liver cell proliferation, survival, or maintaining their quiescence. As such, loss of function may  
66 underlie various hepatic pathologies (7, 16, 24, 33, 50, 57).

67

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

73

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

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

90

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

106

## 107 **MATERIALS AND METHODS**

### 108 *Chemically Defined Culture Media*

109 Serum/growth factor-free base medium was made as described with modifications (27, 31). Low  
110 glucose DMEM (Invitrogen, Carlsbad, CA) was supplemented with 0.03g/L L-proline, 0.10g/L  
111 L-ornithine, 0.305 g/L niacinamide, 1 g/L glucose, 2 g/L galactose, 2 g/L BSA, 50 µg/mL  
112 gentamicin (Sigma-Aldrich, St. Louis, MO), 1 mM L-glutamine (Invitrogen), 5 µg/mL insulin-5  
113 µg/mL transferrin-5 ng/mL sodium selenite (Roche Applied Science, Mannheim, Germany).  
114 “Modified hepatocyte growth medium” (HGM) included 200 µM ethanolamine and  
115 phosphoethanolamine, 100 nM ascorbic acid, 110 nM hydrocortisone (Sigma-Aldrich), 20  
116 µg/mL heparin (Celsus Laboratories, Cincinnati, OH) and 50 ng/mL VEGF (R&D Systems,  
117 Minneapolis, MN). Additional treatments included 1% Chemically Defined Lipid Concentrate  
118 (~8µM final concentration) (Invitrogen 11905031) or 50 µM OA, FFA-free BSA,  
119 phosphatidylcholine (PC, 50 µM), and lysophosphatidylcholine (LPC 50 µM) (Sigma-Aldrich).  
120 For signaling studies, PI3K inhibitor LY294002 and MEK1/2 inhibitor PD0325901 (EMD  
121 Calbiochem, Gibbstown, NJ) were added to LSEC cultures 4 hours following seeding and  
122 maintained throughout the experiment. Inhibitors were reconstituted in DMSO (Sigma-Aldrich)  
123 to 20 mM. LY294002 was dosed at concentrations of 10 µM and 3 µM, while PD0325901 was  
124 used at 1 µM and 0.3 µM. Inhibitors were replenished once a day with fresh medium changes.

125

### 126 *LSEC Isolation and Culture*

127 Livers from approximately 180 to 250 gram male Fisher rats (Taconic, Hudson, NY) were used  
128 under the guidelines set forth by Massachusetts Institute of Technology’s Committee on Animal  
129 Care. Cells were isolated using a two-step collagenase perfusion (27, 47) using Liberase  
130 Blendzyme (Roche Applied Science) in place of collagenase. The liver was perfused initially at  
131 25 mL/min and reduced down to 15 mL/min flow rates in calcium free 10 mM HEPES (Sigma-  
132 Aldrich) buffer followed by 10 mM HEPES buffer with Blendzyme for cell isolation. The  
133 supernatant cell suspension from the perfusion was used to isolate LSEC at room temperature (6,  
134 27). Very briefly, supernatant suspensions were spun down at 50 x g for 3 minutes. Supernatants  
135 were spun at 100 x g for 4 minutes. Supernatants following the spin were pelleted at 350 x g for  
136 10 minutes and resuspended in 20 mL modified HGM without VEGF. The suspension was  
137 loaded over 25%/50% Percoll (Sigma-Aldrich)/PBS layers and centrifuged at 900 x g for 20

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

149

#### 150 *Live/Dead Assay*

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

154

#### 155 *Alamar Blue Metabolic Assay*

156 Metabolic activity of LSEC was assessed over the time period of 5 days using Alamar Blue  
157 (Invitrogen) reduction assays. Positive reference standards were first made by heating base  
158 modified HGM at 125 °C with 10% Alamar Blue until the entire reagent was oxidized and  
159 converted to a bright shade of red. On the days of analysis, 10% Alamar Blue reagent was  
160 introduced to each well and allowed to incubate at 37 °C, 5% CO<sub>2</sub> for 6 hours prior to screening  
161 in a SpectraMax E2 (MDS Analytical Technologies, Sunnyvale, CA) fluorescent plate reader.  
162 Reference standards were included on each plate as positive controls and served as a point of  
163 reference in interpreting results. Fluorescent measurements were read by exciting the samples at  
164 530 nm and reading the emission wavelengths at 590 nm. Samples were pooled across 3  
165 biological replicates (5 technical replicates) for a total of 15 data points. All data points were  
166 normalized to blank readings prior to relative comparison to control samples.

167

#### 168 *Acetylated-LDL Uptake Assay*

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

178

### 179 *Immunofluorescence Microscopy*

180 LSEC were cultured for up to 5 days on Thermanox coverslips coated with 10  $\mu\text{g}/\text{mL}$   
181 fibronectin. Samples were rinsed with PBS and fixed in 3% paraformaldehyde in PBS for 30  
182 minutes. Following fixation, samples were rinsed three times with PBS and permeabilized with  
183 0.1% Triton X-100 (Sigma-Aldrich) for one hour, excluding samples immunostained for CD31  
184 which were not permeabilized so as to evaluate only surface expression. Following  
185 permeabilization, samples were rinsed three times with 2% BSA in 0.1% Tween-20 in PBS  
186 (PBS-T). Samples were blocked with 5% goat or donkey serum (Jackson ImmunoResearch,  
187 West Grove, PA) in 2% BSA PBS-T for 1 hour before overnight incubation at 4 °C with primary  
188 antibodies for anti-rat CD32b/SE-1 (IBL America, Inc., Minneapolis, MN) at 1:100,  
189 CD31/PECAM-1 (Chemicon/Millipore, Temecula, CA) at 1:100, and PCNA (Abcam,  
190 Cambridge, MA) at 1:600. The following day, samples were rinsed 3 times in 2% BSA PBS-T  
191 before a 1 hour incubation step with secondary AlexaFluor 488/555 (Invitrogen) antibodies at  
192 1:250. Coverslips were then rinsed in 2% BSA PBS-T and stained with 1:500 Hoechst.  
193 Following incubation with secondary antibodies, samples were rinsed once in 2% BSA PBS-T  
194 prior to being treated briefly with nuclear Hoechst staining for 1 minute. Following Hoechst  
195 staining, samples were rinsed twice in normal PBS before being mounted onto glass slides with  
196 Fluormount and sealed with nail polish.

197

### 198 *Scanning Electron Microscopy (SEM)*

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



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

202

### 203 *Flow Cytometry*

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

216

### 217 *Western Blotting*

218 LSEC were harvested on day 5 of culture by incubating with cell lysis buffer (46) for 30 minutes.  
219 Cell lysis buffer consisted of 1% Triton X-100, 50mM  $\beta$ -glycerophosphate, 10 mM sodium  
220 pyrophosphate, 30 mM sodium fluoride (Sigma-Aldrich), 50 mM Tris (Roche Applied Science),  
221 150 mM sodium chloride, 2 mM EGTA, 1 mM DTT, 1 mM PMSF, 1% Protease Inhibitor  
222 Cocktail and 1% Phosphatase Inhibitor Cocktails (Sigma-Aldrich). Samples were spun down at  
223 12,000 rpm for 12 minutes at 4 °C and supernatants were reserved. Total protein content of  
224 sample lysates was determined using micro bicinchoninic acid kits (Thermo Fisher Scientific,  
225 Rockford, IL) before being loaded onto the NuPage Novex system (Invitrogen). Lysates were  
226 loaded with 6X reducing buffer (Boston BioProducts, Worcester, MA) in 4%-12% Bis-Tris gels  
227 (Invitrogen) and transferred to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA).  
228 Membranes were blocked with 5% BSA in PBS-T and incubated with antibodies for  $\beta$ -actin  
229 (1:5000), phosphoERK1/2 (1:5000), ERK1/2 (1:5000), phosphoAkt (1:1000), and Akt (1:5000)  
230 (Cell Signaling Technology, Beverly, MA) overnight at 4 °C. Membranes washed and then

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

236

237

### 238 *Image and Statistical Analysis*

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

## 245 RESULTS

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

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

259  
260 Live/Dead images of LSEC across conditions in both regular and FFA-free BSA were taken  
261 during five days of culture (Figure 2A, B). Massive cell death observed in the control concur  
262 with previously reported observations of LSEC demise beyond 48 hours in culture. While all  
263 conditions experienced cell number decline between days 2 and 3, lipid and OA treated cultures  
264 recovered and proliferated in both types of BSA, with statistically significant differences in  
265 population after day 3 compared to control ( $p < 5E-4$ ). (Figure 2C, D). Phase and live/dead  
266 staining indicate pronounced and distinct morphological changes for surviving LSEC. Lipid  
267 supplementation maintained LSEC to day 5. Cells grown with OA in normal BSA were viable  
268 after 4 days after isolation; however, FFA-free BSA did not synergize with OA to maintain cell  
269 viability. Lipid and OA conditions had persistently higher total live cell percentages compared to  
270 control, PC, and LPC conditions; PC and LPC did not offer any growth advantage for LSEC  
271 relative to control ( $p > 0.24$ ) (Figure 2). Live/Dead assay confirmed that LSEC with unhealthy  
272 granular appearance were dead and positive for ethidium bromide. PC and LPC cell cultures did  
273 not survive past day 2 in regular BSA (data not shown).

274

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

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

283

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

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

294

295 Another characteristic LSEC marker, CD32b, was used to corroborate phenotype.

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

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

309  
310 Proliferative capabilities were measured using PCNA and EdU (a BrdU analog) incorporation.  
311 OA and lipid treated cells stained positive for both PCNA and CD32b expression at day 5 while  
312 untreated cells did not (Figure 5A). Most cells were PCNA<sup>-</sup> in the control; those that were  
313 PCNA<sup>+</sup> were CD32b<sup>-</sup>. Day 5 cells had higher proportions CD32b<sup>+</sup>/EdU<sup>+</sup> cells in OA compared  
314 to control (Figure 6A, C). 1% lipid treated LSEC did not have statistically significant  
315 CD32b<sup>+</sup>/EdU<sup>+</sup> populations over the control. However, this is likely attributed to the culture  
316 achieving confluency by days 4 and 5 relative to the OA condition; we were able to obtain a  
317 greater number of overall and CD32b<sup>+</sup> events for 1% lipid samples than with any other  
318 condition. Even when debris is included we have statistically significant larger populations of  
319 distinct double positive cells following treatment. Combined with immunostaining observations,  
320 we can state that PCNA observed in untreated conditions most likely stems from contaminating  
321 cell types and/or dedifferentiated LSEC.

322

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

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

338 compared to control through day 5.

339

340 Inhibition studies were performed using PI3K inhibitor LY294002 and MEK1/2 inhibitor  
341 PD0325901. Inhibitors did not affect LSEC for the first 24 hours of incubation (Figure 8A, 9A)  
342 despite lower concentrations effectively reducing downstream Akt and ERK1/2 phosphorylation  
343 (Figure 9D). By day 2, 10  $\mu$ M PI3K inhibitor had adverse effects despite addition of OA or lipid  
344 (Figure 8B, 9A-C). Lower PI3K inhibitor concentrations (3  $\mu$ M) showed similar effects in  
345 unsupplemented medium, but cultures with OA or lipid survived while only the lipid condition  
346 continued to proliferate (Figure 8B, 9B, C). High MEK1/2 inhibitor concentrations only slightly  
347 affected OA conditions at day 2 by reducing attached cell number, although culture quality  
348 appeared similar to treatments without inhibitor. Lipid cultures did not appear to be perturbed by  
349 1  $\mu$ M MEK1/2 inhibitor by day 2. MEK1/2 inhibition prevented culture survival after day 4  
350 (Figure 8C,D, 9C). Lower MEK1/2 inhibitor concentration (0.3  $\mu$ M) did not vary from the high  
351 dose used ( $p \gg 0.05$  between all MEK1/2 inhibitor conditions at every time point), indicating  
352 LSEC may be more sensitive to changes downstream of MEK1/2 versus PI3K later in culture.  
353

**DISCUSSION**

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

367  
368 A hallmark of LSEC is the presence of fenestrae, which were maintained in both OA and lipid  
369 samples on day 5. Of the few living cells remaining in the control, none were found to possess  
370 fenestrae, consistent with previous findings that fenestrae disappear within the first 48 hours of  
371 culture (7). Additional evaluation with CD32b phenotype marker validated findings that  
372 surviving LSEC in lipid or OA maintained differentiation by expressing this marker, one specific  
373 to liver sinusoidal endothelium (37). Along with CD32b expression, we also looked at the  
374 proliferative capacity of LSEC, since no previous studies have explicitly reported that  
375 differentiated rat LSEC can undergo proliferation. We successfully demonstrated that  
376 differentiated LSEC undergo proliferation, via nuclear PCNA expression and EdU incorporation,  
377 when treated with FFAs. Despite maintenance of several phenotypic characteristics in prolonged  
378 cultures, we did observe degradation of some markers. Although fenestrae arranged in sieve  
379 plates were observed, they were not abundant in OA and lipid treated cultures, and a large  
380 percentage of these LSEC no longer exhibited fenestrae by day 5. Many cells in the FFA-treated  
381 condition processed large transcytotic pores greater than 1  $\mu\text{m}$  in diameter. These may be the  
382 remnants of sieve plates that have degraded or fenestrae that have fused. We noticed that  
383 although LSEC still expressed CD32b, the presence was diffuse and overall fluorescent intensity  
384 was lower than for freshly isolated LSEC (Figure 5C). Other studies have reported sharper

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

389  
390 In LSEC we observed phospho-Akt/Akt levels decreased in FFA conditions as time progressed,  
391 while the inverse occurred with phospho-ERK/ERK, primarily by ERK1. From these  
392 observations and inhibitor studies, we believe low threshold levels of phospho-Akt are required  
393 for cell survival between days 2 and 3. At this point, high concentrations of PI3K inhibitor  
394 LY294002 abolished the beneficial effects that OA and 1% lipid have on LSECs, while lower  
395 concentrations did not affect cultures. Beyond 3 days, cells in low PI3K inhibitor could  
396 proliferate and recover albeit not to the level seen in uninhibited samples. Granular morphology  
397 appeared earlier at day 2 (as opposed to day 3 in control samples without inhibitor) in untreated  
398 samples with PI3K inhibitor. This may indicate that downstream signals of PI3K are closely  
399 associated with cell survival during this time. Past day 3, MEK1/2 inhibition was fatal to  
400 cultures, as LSEC did not survive or proliferate regardless of the concentration of MEK1/2  
401 inhibitor PD0325901 added to cultures. Interestingly, OA and lipid-treated cultured LSEC did  
402 not have a significant dependence on MAPK before this time, as 10  $\mu$ M inhibitor only slightly  
403 affected the number of cells in culture. At early time points, MEK1/2 inhibition also prevented  
404 LSECs from undergoing increased spreading seen with FFA treatments. As such, MAPK  
405 signaling may be partially responsible for the morphology change induced by FFAs before day 3,  
406 but required afterward for survival and proliferation.

407  
408 While it is understood that ECM, cell-cell contacts (37), and paracrine/autocrine signaling (17)  
409 are absolutely vital to achieve functional LSEC, consideration of the role of lipids is important  
410 given the results of this study. Effects of FFAs on LSEC can have several implications on liver  
411 pathophysiology. In general, lipids are crucial for survival for all mammalian cells as energy  
412 substrates, membrane lipid bases, and influencing cell signal processes (3). Concentrations of  
413 FFAs in circulation can vary dramatically depending on the metabolic state, but have been  
414 reported to be anywhere between 10  $\mu$ M to 1 mM in human plasma, though generally within the  
415 range of 200 to 600  $\mu$ M (25, 44, 45). Approximately 150  $\mu$ M total plasma FFA is taken up in the



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

429  
430 OA and other unsaturated FFAs have been reported to have numerous effects on metabolically  
431 active cells although the main mechanisms of OA and other FFA incorporation are still not fully  
432 understood. OA has been found to participate in crosstalk with EGFR and other pathways by  
433 affecting MAPK and PI3K (12, 13, 52, 53, 55, 56). However, much of the data from previous  
434 studies are contradictory in either stimulating or inhibiting these pathways dependent on the  
435 system being studied.

436  
437 Unsaturated FFAs have been found to be able to protect against oxidative stress by reducing lipid  
438 peroxidation and inhibiting the inflammatory pathway NF- $\kappa$ B which can lead to endothelial cell  
439 activation (9, 11, 15, 39). Thus, OA may prevent oxidative stress in LSEC that decreases ERK1/2  
440 activity (38, 48), thereby allowing cells to resume cell survival and proliferation after day 3. This  
441 would be in agreement with the results we observed in increased phospho-ERK1/2 activity.  
442 Furthermore, increased saturated to unsaturated fatty acid levels are strongly correlated with  
443 insulin resistance and decreased glucose production in the liver (33, 36). The introduction of  
444 more unsaturated FA into the system may facilitate insulin signaling and activation of phospho-  
445 Akt for cell survival in our early time points. While it is most likely that FFAs indirectly  
446 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.

461

462

463

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638 **FIGURE CAPTIONS**

639 **Figure 1. Lipids in FFA form sustain long-term culture.** Phase contrast images of LSEC  
640 were taken at day 5 of culture in serum-free medium (modified HGM) with regular BSA (**A,B**)  
641 or fatty acid (FFA)-free BSA (**C,D**). LSEC were cultured with 50 ng/mL VEGF (control), plus  
642 50  $\mu$ M oleic acid (50  $\mu$ M OA), or 1% chemically defined lipid concentrate (1% lipid). Only  
643 conditions with OA or lipid appeared favorable for persistence of cell culture. Higher  
644 magnification images indicate a pronounced change in morphology in lipid treated conditions  
645 compared to untreated control cells which appeared more granular (**B,D**). Scale bars = 50  $\mu$ m  
646 (**A,C**), 20  $\mu$ m (**B,D**).

647  
648 **Figure 2. LSEC death at 48 hours is abrogated following treatment with FFA.** Live/Dead  
649 assays were performed on LSEC culture across several conditions (**A,B**), with cell number  
650 quantification by Cell Profiler (**C,D**). Samples were treated with calcein AM (green) for live  
651 cells and ethidium bromide homodimer (red) for dead cells. While all conditions experienced  
652 steep drops in total population by day 3, only OA or lipid treatments had significant live cell  
653 numbers ( $p < 5E-4$  compared to control), indicating lipid type importance. Abbreviations: PC =  
654 phosphatidylcholine, LPC = lysophosphatidylcholine, OA = oleic acid. Scale bar = 100  $\mu$ m.

655  
656 **Figure 3. OA and lipid supplement support phenotype and function in LSEC cultures.**  
657 Alamar Blue measurements were statistically higher at days 3 ( $p < 0.05$ ) and 5 ( $p < 0.005$ ) in 50  
658  $\mu$ M OA and 1% lipid supplement treatments over control for LSEC in regular BSA (**A**). Similar  
659 trends were also observed in FFA free BSA cultures (**B**). Alamar Blue reduction was statistically  
660 higher at day 3 for 50  $\mu$ M OA and 1% lipid supplement treatments over control, PC, and LPC. At  
661 day 5, only 1% lipid supplement treatment was statistically significant over control, PC, and  
662 LPC, indicating that the 50  $\mu$ M OA condition was insufficient to sustain long term cultures  
663 without the presence of other fatty acids. Most cells in the control condition did not survive past  
664 day 3; remaining cells did not co-stain for Hoechst (blue) and Di-I-Ac-LDL (red), while OA and  
665 lipid conditions consistently co-stained for both on day 5 (**C**). Contrast and brightness were  
666 adjusted for the entire image for Hoechst staining due to background fluorescence arising from  
667 the Thermanox coverslips. Abbreviations: PC = phosphatidylcholine, LPC =  
668 lysophosphatidylcholine, OA = oleic acid. Scale bars = 100  $\mu$ m (**C**), 2.5  $\mu$ m (**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  $\mu\text{m}$  for panels B-F and J-L. Scale bar in I represents  
678 10  $\mu\text{m}$  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  $\mu\text{m}$ .

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

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

705

706

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

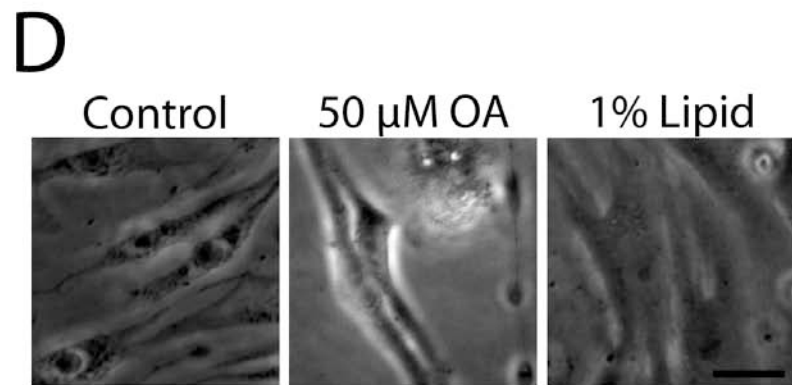
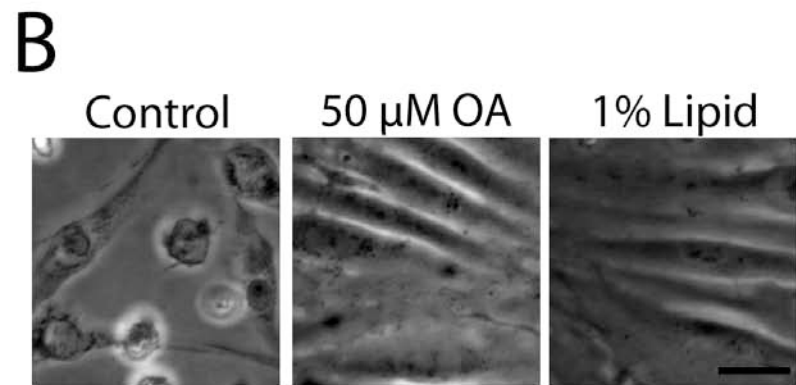
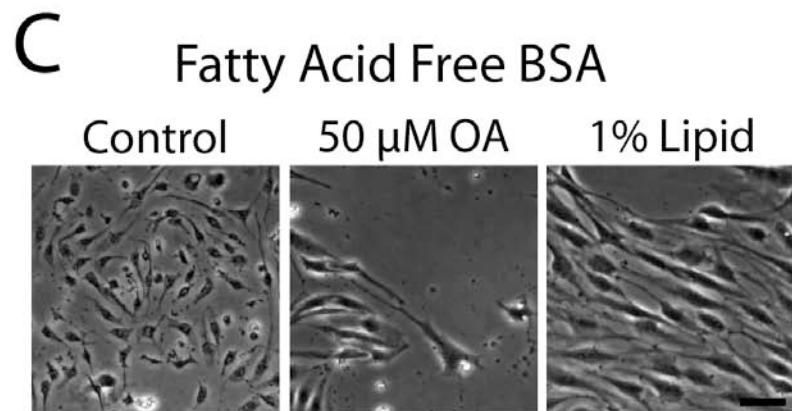
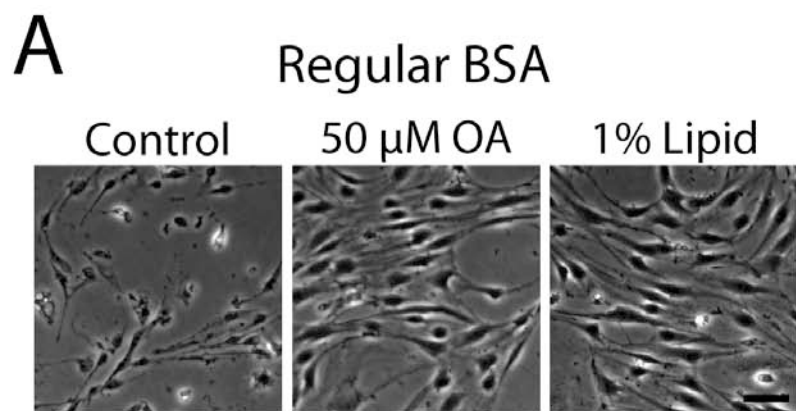
716

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

730

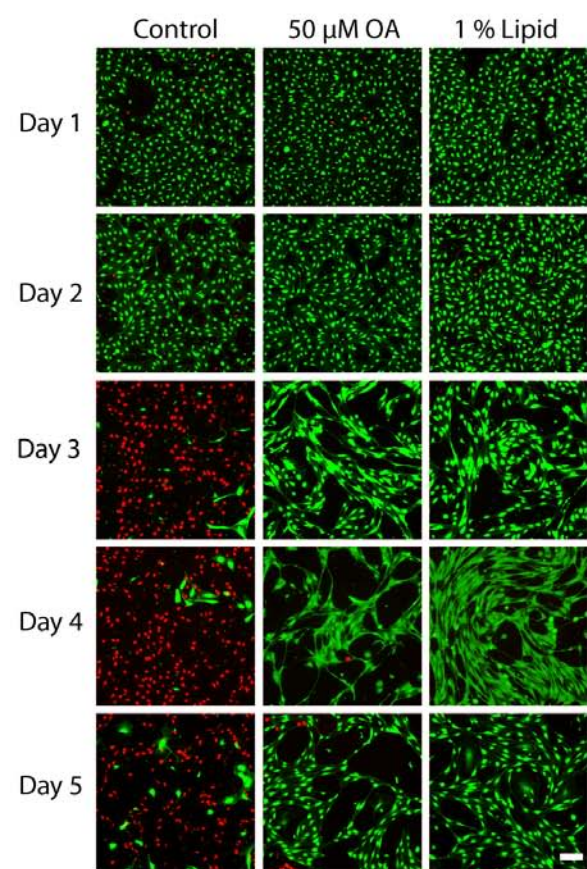
731 **Figure 9. OA and lipid supplement support cultures through early maintenance of low**  
732 **threshold of phospho-Akt followed by late phospho-ERK signaling.** Intact cell body counts  
733 show temporal difference in Akt and ERK signaling (A-C). Low PI3K inhibitor delayed LSEC  
734 recovery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

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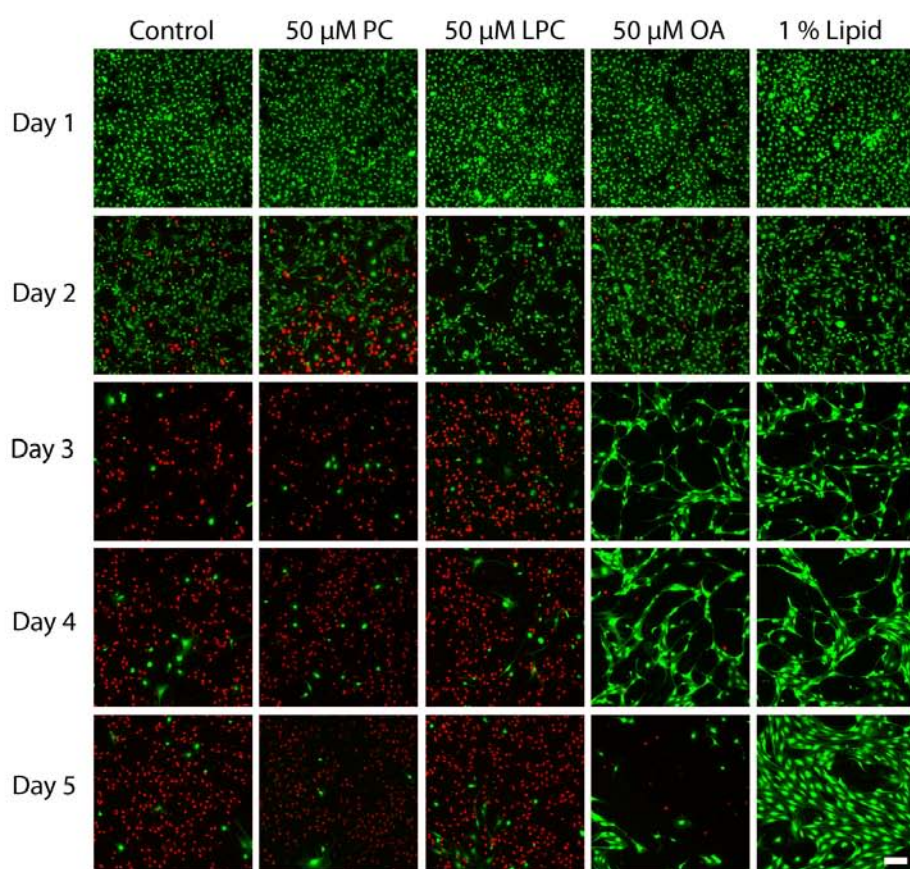
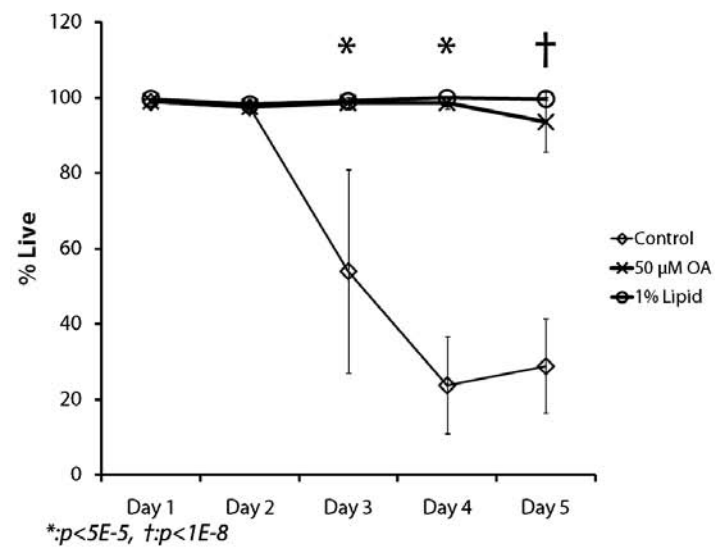
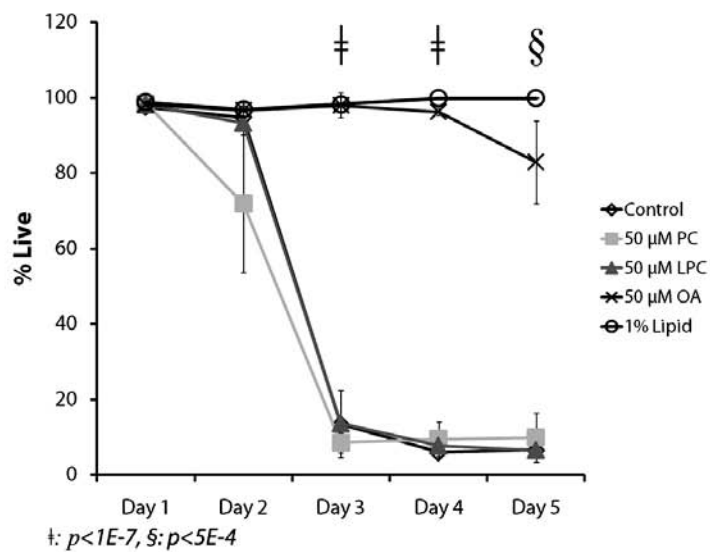


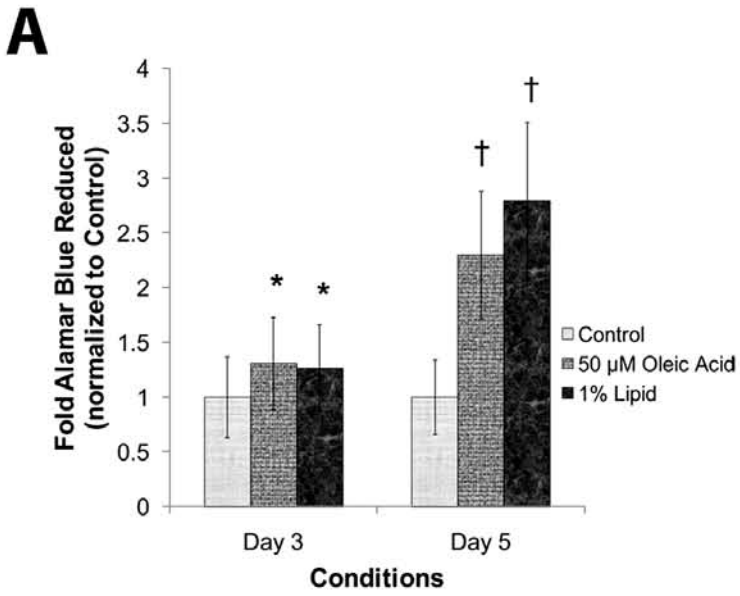
**A**

Regular BSA

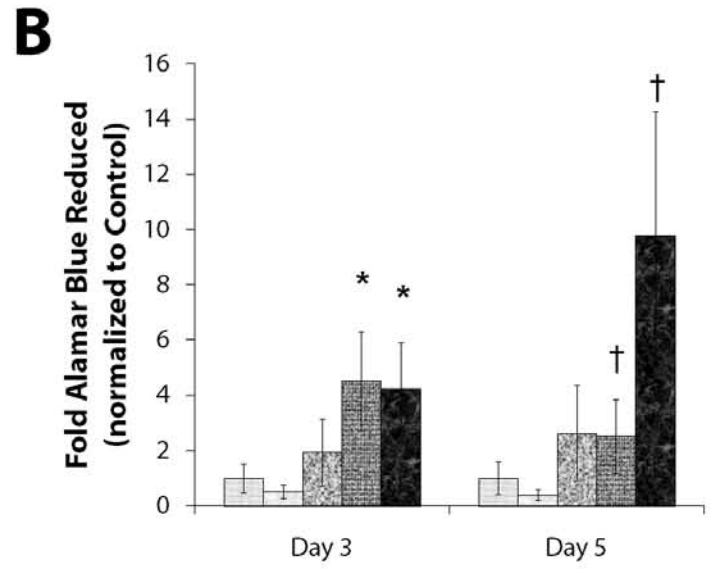
**B**

Fatty Acid Free BSA

**C****D**



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

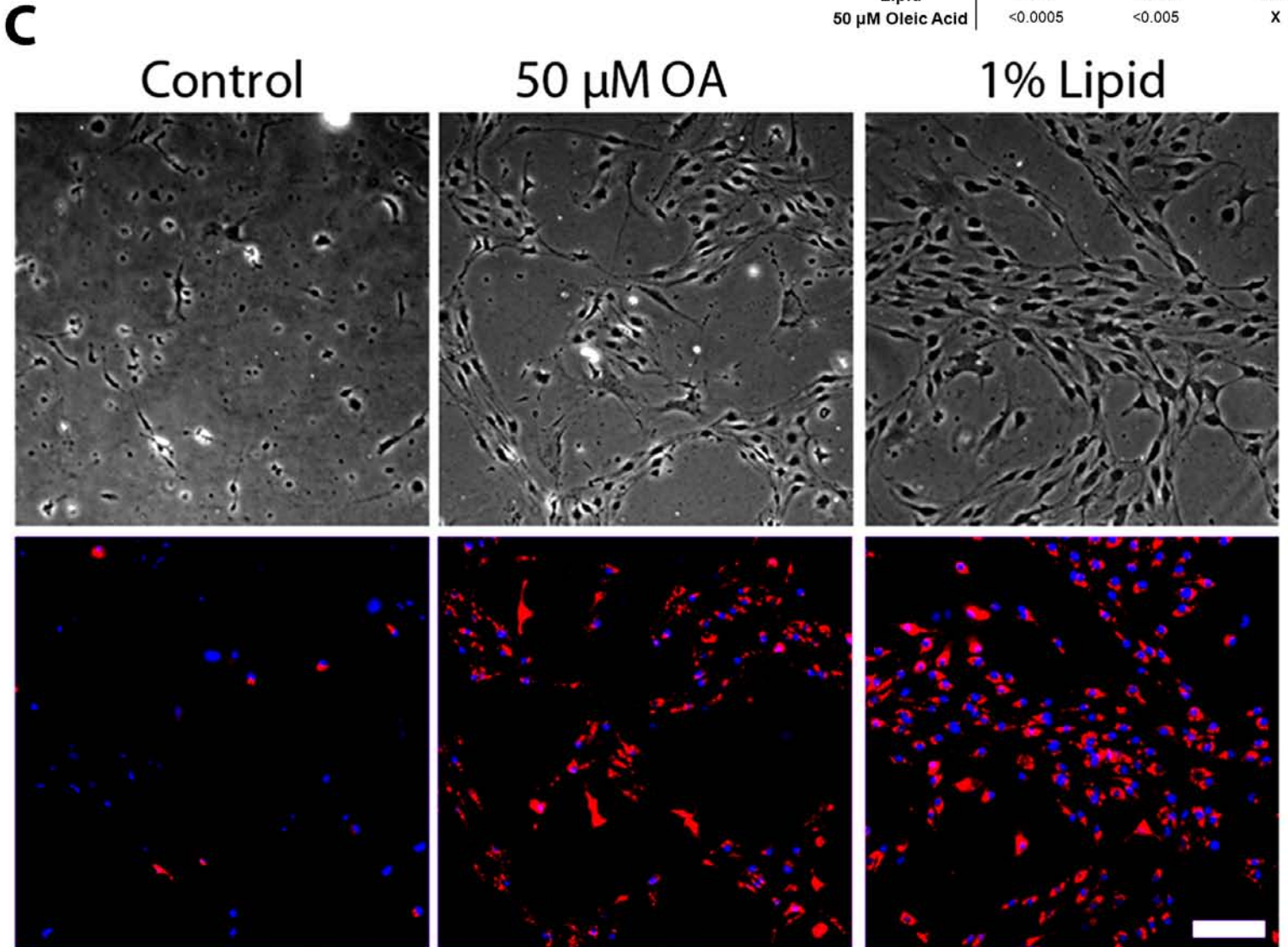


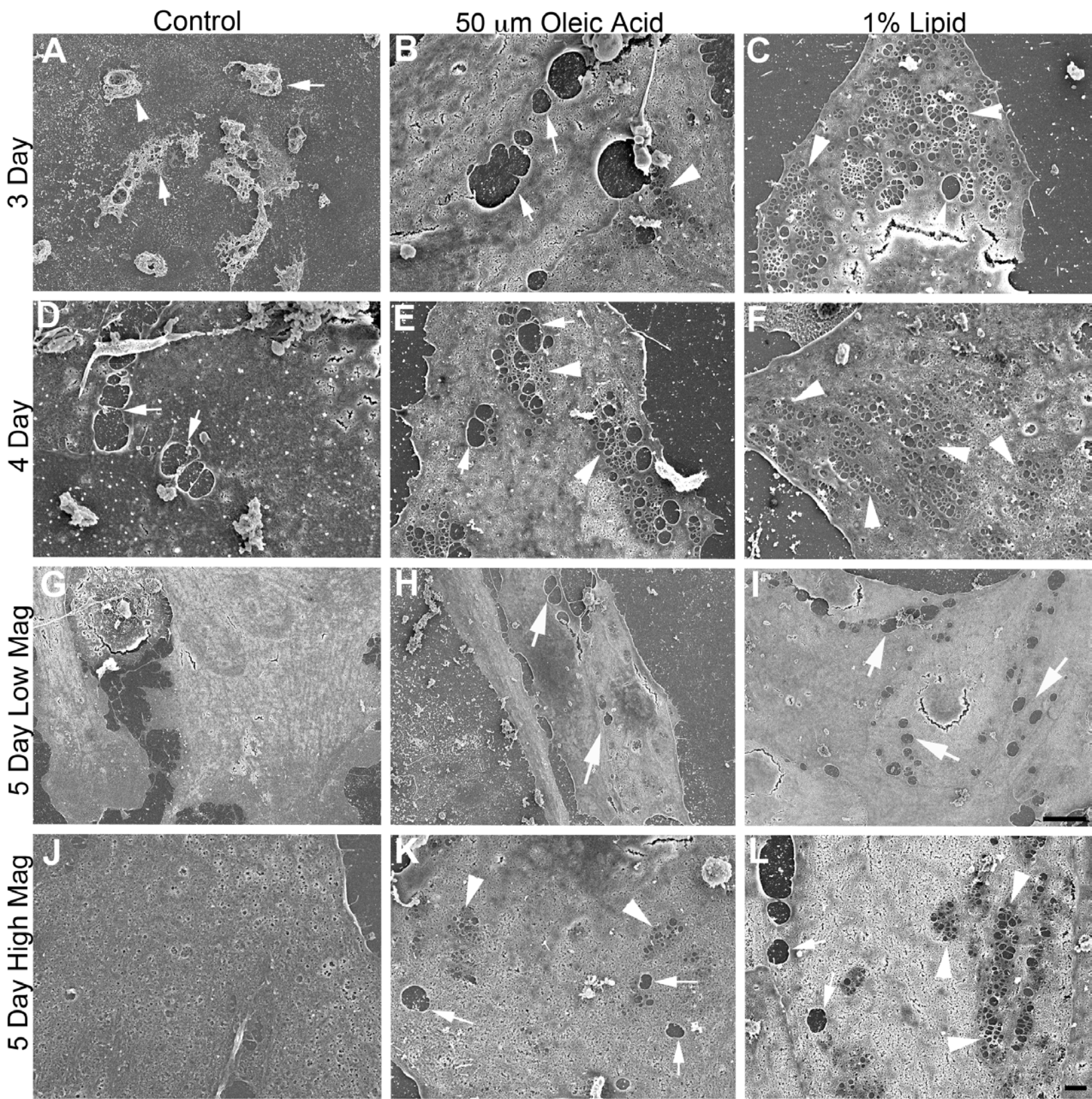
□ Control  
 □ 50  $\mu$ M PC  
 ▨ 50  $\mu$ M LPC  
 ▩ 50  $\mu$ M OA  
 ■ 1% Lipid

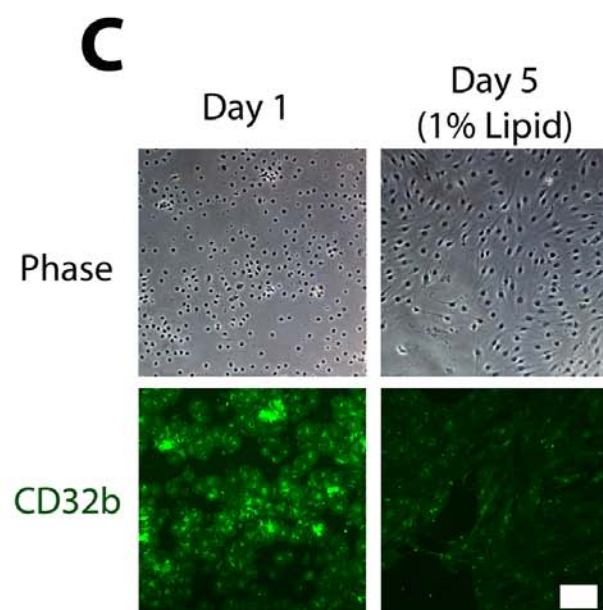
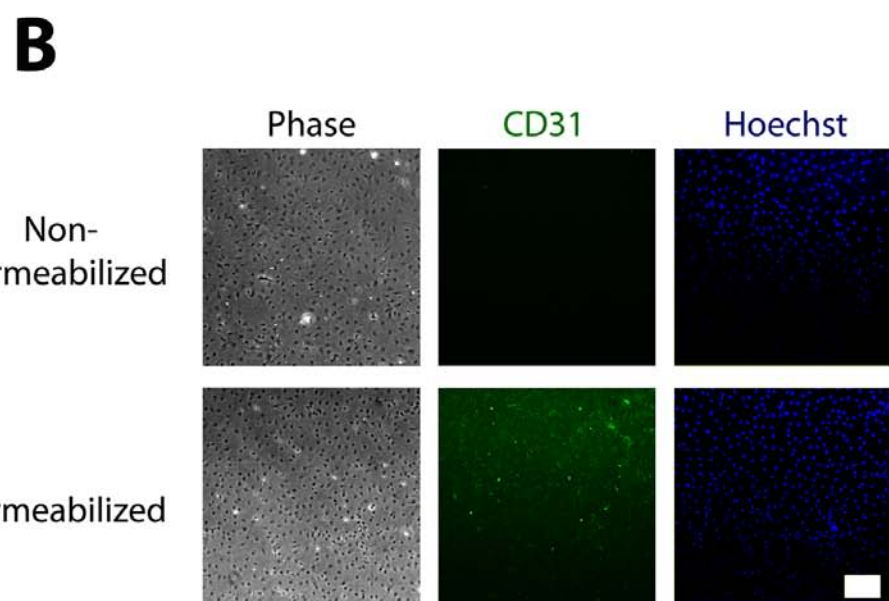
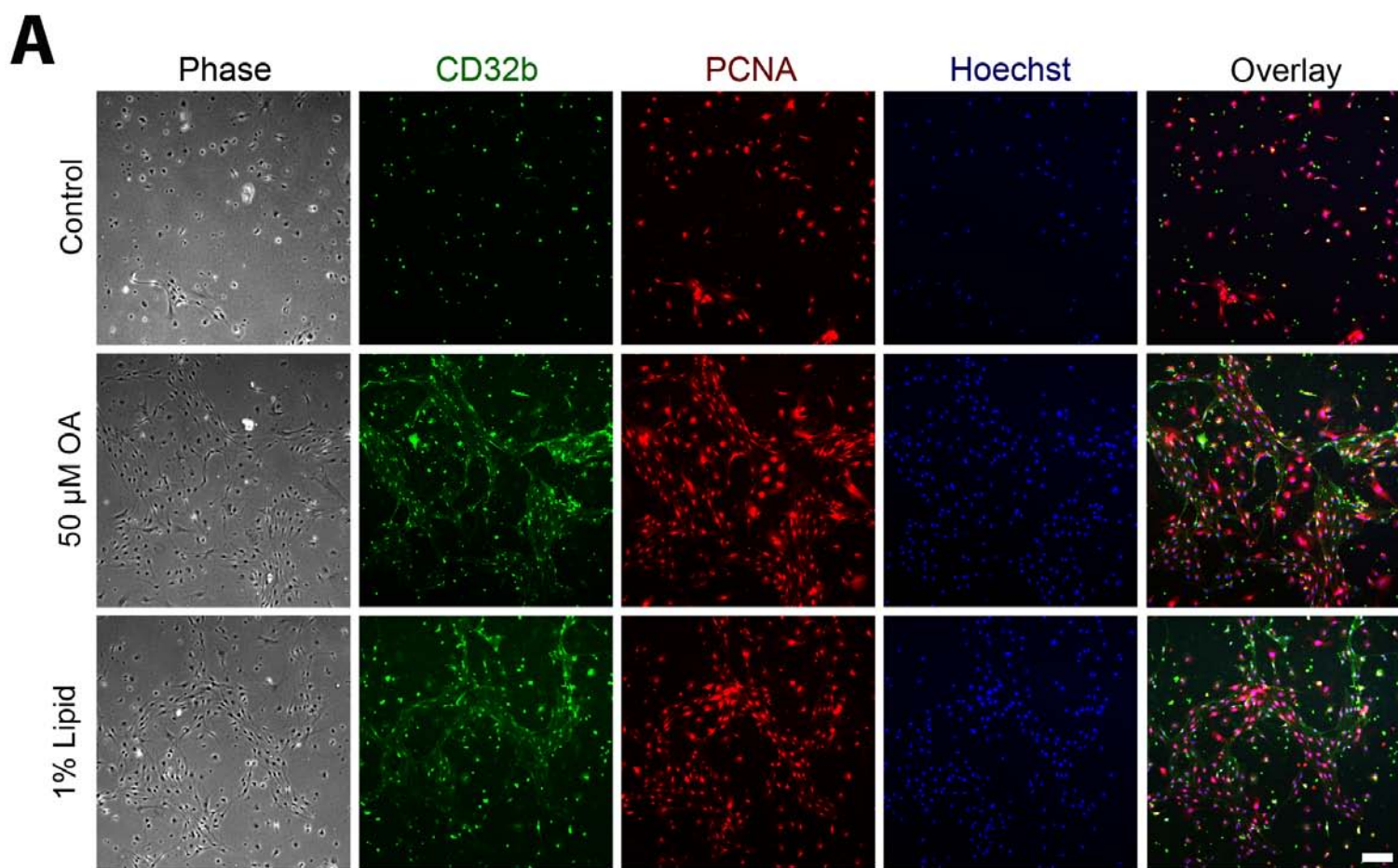
* (Day 3)				
p-value	Control	50 $\mu$ M PC	50 $\mu$ M LPC	50 $\mu$ M OA
Lipid	<1E-7	<0.005	X	X
50 $\mu$ M Oleic Acid	<1E-7	<0.005	<0.05	

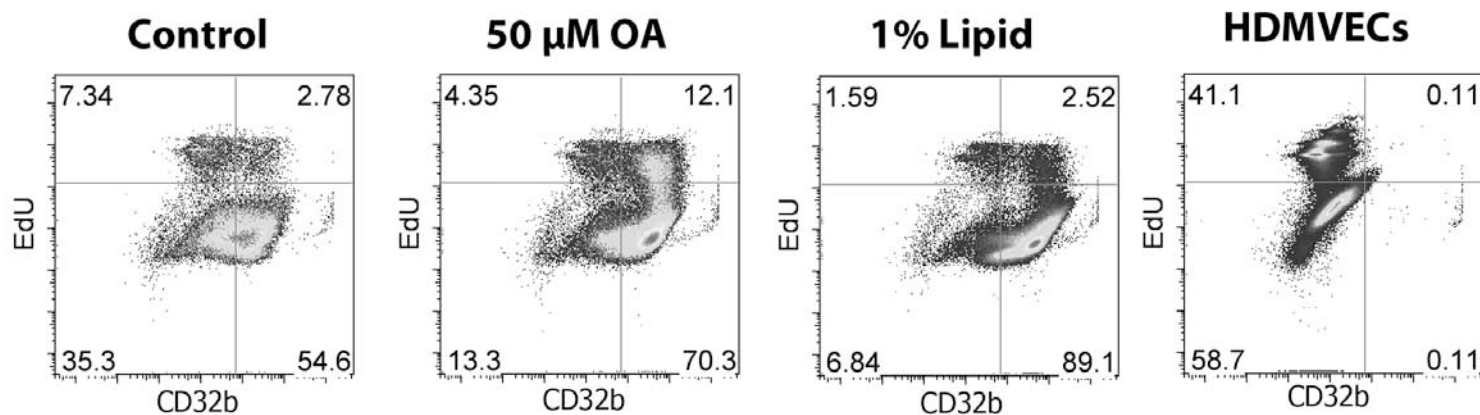
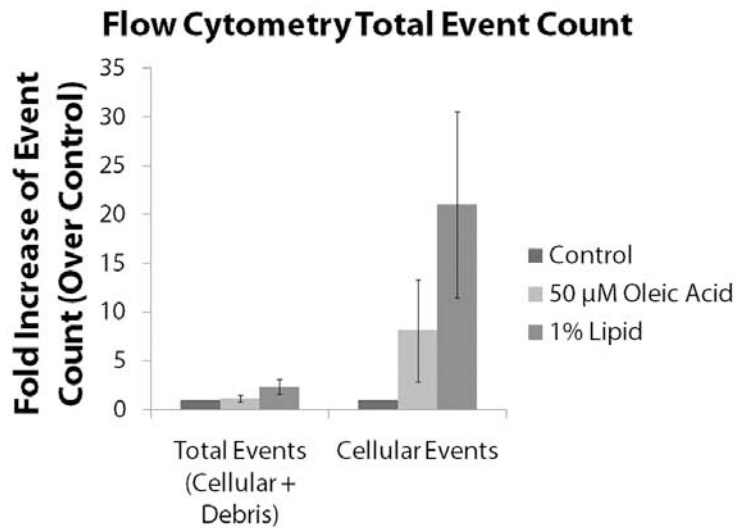
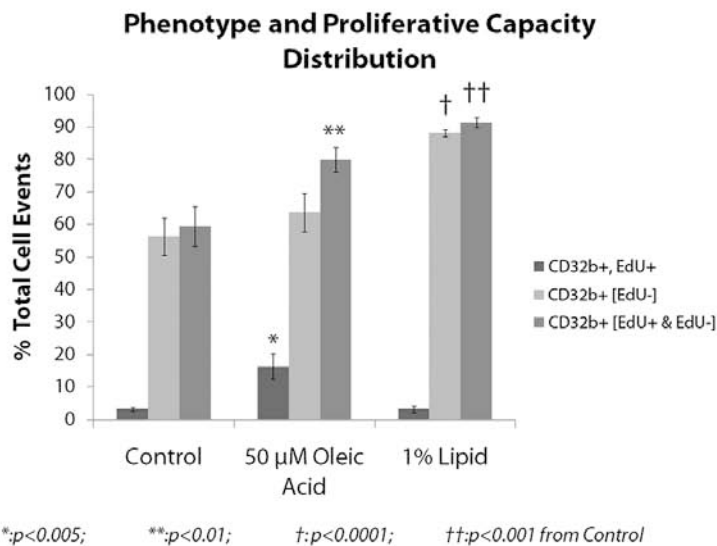
† (Day 5)				
p-value	Control	50 $\mu$ M PC	50 $\mu$ M LPC	50 $\mu$ M OA
Lipid	<1E-7	<0.001	<0.01	X
50 $\mu$ M Oleic Acid	<0.0005	<0.005	X	

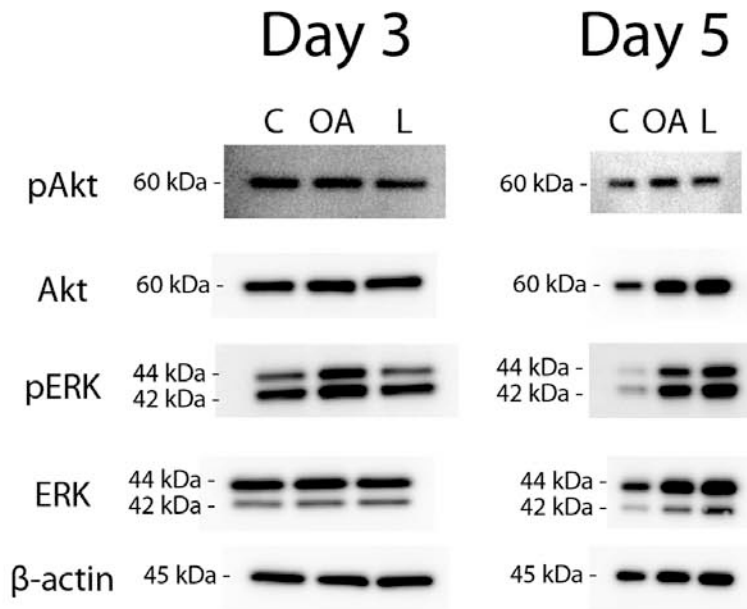
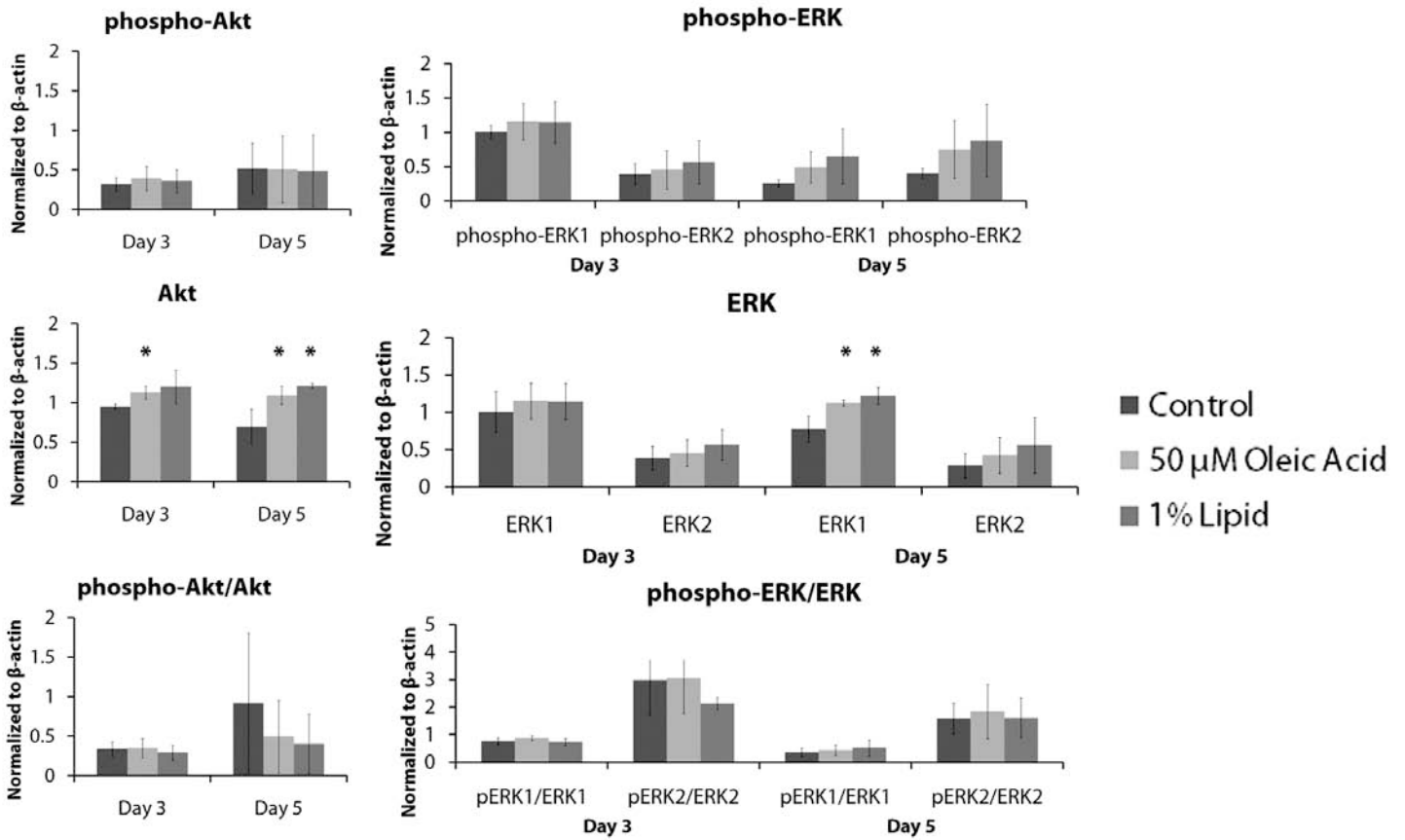








**A****B****C**

**A****B**

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

