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Lysophosphatidic Acid Protects Human Mesenchymal Stromal Cells from Differentiation-Dependent Vulnerability to Apoptosis

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The survival of transplanted cells and their resulting efficacy in cell-based therapies is markedly impaired due to serum deprivation and hypoxia (SD/H) resulting from poor vascularization within tissue defects. Lysophosphatidic acid (LPA) is a platelet-derived growth factor with pleiotropic effects on many cell types. Mesenchymal stromal cells (MSC) exhibit unique secretory and stimulatory characteristics depending on their differentiation state. In light of the potential of MSC in cell-based therapies, we examined the ability of LPA to abrogate SD/H-induced apoptosis in human MSC at increasing stages of osteogenic differentiation *in vitro* and assessed MSC survival *in vivo*. Undifferentiated MSC were rescued from SD/H-induced apoptosis by treatment with both 25 and 100 μM LPA. However, MSC conditioned with osteogenic supplements responded to 25 μM LPA, and cells conditioned with dexamethasone-containing osteogenic media required 100 μM LPA. This rescue was mediated through LPA_1 in all cases. The addition of 25 μM LPA enhanced vascular endothelial growth factor (VEGF) secretion by MSC in all conditions, but VEGF availability was not responsible for protection against apoptosis. We also showed that codelivery of 25 μM LPA with MSC in alginate hydrogels significantly improved the persistence of undifferentiated MSC *in vivo* over 4 weeks as measured by bioluminescence imaging. Osteogenic differentiation alone was protective of SD/H-induced apoptosis *in vitro*, and the synergistic delivery of LPA did not enhance persistence of osteogenically induced MSC *in vivo*. These data demonstrate that the capacity of LPA to inhibit SD/H-induced apoptosis in MSC is dependent on both the differentiation state and dosage. This information will be valuable for optimizing osteogenic conditioning regimens for MSC before *in vivo* implementation.

Introduction

Mesenchymal stromal cells (MSC) are popular candidates for regenerative medicine due to their ready availability, nonimmunogenicity, and ability to differentiate into multiple tissue types.[1,2] In particular, MSC can be differentiated toward an osteoblastic lineage using chemical cues and supplements[3,4] and hold the potential for treating millions of critical-sized bone defects and nonunion fractures that occur annually. Current approaches under investigation seek to direct differentiation of MSC in conjunction with biocompatible scaffolds and other delivery systems,[5,6] but successful translation to *in vivo* environment is initially hampered by the harsh environment, including serum deprivation and reductions in local oxygen tension (hypoxia) (SD/H) at the defect or fracture site. Indeed, 99% of MSC do not survive culture under ischemia after 3 days[7] and 99% of MSC implanted into ischemic heart tissue die within 96 h,[8] severely limiting the therapeutic potential of such treatments. Without overcoming such poor conditions, extensive apoptosis can significantly impede or prevent tissue formation, regardless of the cell transplantation method.[9–11]

Although growth factors such as angiopoietin-1 have been shown to protect MSC against ischemia-induced apoptosis,[12] the high cost of producing and purifying recombinant proteins and the difficulty of accurate delivery render large-scale implementation impractical. Lysophosphatidic acid (LPA) is a glycerophospholipid signaling molecule that binds to cognate G-protein-coupled receptors and has a wide range of effects on many different cell types.[13–16] Naturally present in serum at low micromolar concentrations,[15] LPA plays regulatory roles in the adhesion, migration, and proliferation of endothelial cells as well as neurons.[14,17,18]

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Additionally, LPA affects actin polymerization in fibroblasts, osteoblasts, and other cell types to modulate cytoskeletal tension and contractile forces.[13,15]

Of particular interest for tissue engineering applications is the capacity for LPA to reduce apoptosis in MSC. Previous studies have demonstrated that LPA rescues rat MSC from SD/H-induced apoptosis *in vitro*[9] and *in vivo*[19] through ERK-dependent mediation of endoplasmic reticulum stress and signaling pathways.[10] The ability of LPA to attenuate apoptosis,[9,19] combined with its relative low cost (currently, ~$10/mg versus ~$12,000/mg for recombinant human angiopoietin[20]), makes LPA an attractive target for continued investigation as a means to enhance the effectiveness of MSC-based tissue engineering strategies by improving cell viability in ischemia.

Despite the promising, but limited, antiapoptotic effects of LPA in rat cells, further assessment of its potential for clinical application requires thorough characterization of its effects on human MSC. Undifferentiated MSC can be used to support angiogenesis in defect sites or modulate immune responses,[21] while treatment strategies that target direct bone repair may benefit from inducing MSC toward the osteoblastic lineage before implantation to promote rapid bone formation and calcium deposition.[22,23] Since MSC exhibit unique secretory and stimulatory characteristics depending on their differentiation state,[4,24] it is important to account for variations in cellular responses to LPA at different degrees of differentiation.

We hypothesized that LPA would rescue human MSC from SD/H-induced apoptosis and that the capacity for LPA to attenuate apoptosis is a function of the degree of osteogenic differentiation. We investigated this hypothesis by conditioning MSC with three media types of increasing osteogenic capacity and exposing them to SD/H in the presence of varying concentrations of LPA. We assessed apoptosis in cells maintained in each media type and also probed the expression of LPA receptors to elucidate the ones involved in promoting cellular survival. Additionally, we quantified the effect of LPA on the production of vascular endothelial growth factor (VEGF) and examined possible mechanisms for LPA-mediated apoptotic attenuation. Finally, we used an alginate hydrogel delivery vehicle and noninvasive bioluminescence imaging (BLI) to track cell survival *in vivo* over 4 weeks.

Materials and Methods

Cell culture

For *in vitro* studies, human bone marrow-derived MSC (Lonza, Walkersville, MD) were expanded without further characterization in a growth medium (GM) consisting of the minimum essential alpha medium (α-MEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; JR Scientific, Woodland, CA) and 1% penicillin–streptomycin (P/S; Mediatech, Manassas, VA). MSC were transduced to express firefly luciferase (MSC-Luc) for *in vivo* studies as previously described.[25,26] Cells were cultured under standard conditions in a humidified incubator and utilized at passages 5–6. To induce osteogenic differentiation, cells were cultured in either osteogenic media (OM: GM supplemented with 10 mM β-glycerophosphate and 50 μg/mL ascorbate-2-phosphate; Sigma-Aldrich, St. Louis, MO) or in OM supplemented with dexamethasone (OM+: OM with 10 nM dexamethasone, Sigma-Aldrich).[4] All media were replaced every 3 days.

For all experiments examining the effects of SD/H, MSC were preconditioned in GM, OM, or OM+ for 7 days in T-225 tissue culture flasks and subsequently seeded on six-well tissue culture plates at 30,000 cells/cm^2. After attaching overnight, cells were washed 3× with PBS to eliminate all traces of serum. To induce apoptosis, media were replaced with serum-free GM, OM, or OM+ supplemented with 0.1% (w/v) fatty acid-free BSA, and cells were incubated in hypoxia for 24 h ($n=6$). In studies examining caspase activity and VEGF secretion in SD/H, cells were incubated in airtight chambers (Billups-Rothenberg, Del Mar, CA) at an oxygen tension of ≤1%, as previously described.[27] In studies for LPA receptor expression, cells were incubated for in Heracell 150i tri-gas incubators (Thermo Scientific, Waltham, MA) at 1% oxygen. Negative controls for apoptosis were cultured for the same duration in 21% O_2 in GM, OM, or OM+ with full serum.

Biochemical characterization of osteogenic differentiation

MSC were plated at 30,000 cells/cm^2 on 12-well tissue culture plates in GM, OM, or OM+ and maintained for 7 days ($n=4$). The intracellular alkaline phosphatase (ALP) activity was quantified for cells in each medium at each time point and normalized to the DNA content as previously described.[28,29]

qPCR analysis of osteogenic markers and LPA receptor expression

Total RNA was collected from MSC exposed to SD/H after preconditioning in GM, OM, or OM+ ($n=4$) using an RNeasy Mini kit (Qiagen, Valencia, CA). About 270 ng of total RNA was reverse transcribed with the QuantiTect Reverse Transcription kit (Qiagen). qPCR was performed using a QuantiFast Probe PCR kit (Qiagen) on a Mastercycler realplex2 (Eppendorf, Westbury, NY). Primers and probes for *RPL13* (HS00204173_m1), *RUNX2* (Hs00231692_m1), *LPAR1* (Hs00173500_m1), *LPAR2* (Hs01113287_m1), *LPAR3* (Hs00173857_m1), *LPAR4* (Hs00271072_s1), and *LPAR5* (Hs00252675_s1) were purchased from Applied Biosystems (Foster City, CA). Amplification conditions were 95°C for 3 min, followed by 40 cycles at 95°C for 3 s and 60°C for 30 s. Quantitative PCR results were normalized to *RPL13* transcript levels to yield ΔCt, and fold change in expression relative to the housekeeping gene was calculated using $2^{-\Delta Ct}$.[30]

Visual and quantitative assessment of MSC exposed to SD/H

MSC conditioned in GM were exposed to SD/H as described above, and the morphological characteristics of MSC in each condition were observed and recorded at 100× magnification. DNA from MSC in each condition ($n=3$) was quantified using a Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen).

Induction and rescue of SD/H-induced apoptosis

MSC were preconditioned in GM, OM, or OM+ and exposed to SD/H for 24 h. To explore the antiapoptotic

potential of LPA, MSC were cultured in serum-free GM, OM, or OM+ containing 0.1% fatty acid-free BSA and supplemented with LPA (Enzo Life Sciences, Plymouth Meeting, PA) to a final concentration of 1, 10, 25, and 100 μM. A subset of cells received the $LPA_{1/3}$ inhibitor Ki16425 (10 μM; Cayman Chemical, Ann Arbor, MI) to abrogate LPA binding[10] in the presence of media containing 25 μM LPA. This LPA concentration was selected as an optimum dose due to its effectiveness in multiple media types. For experiments testing the effects of VEGF in SD/H, cells in GM were exposed to SD/H with 25 μM LPA and the saturating addition of 10 μg/mL $VEGF_{165/121}$ antibody (AB-293-NA; R&D Systems, Minneapolis, MN)[4] or 5 ng/mL recombinant VEGF (Lonza) with 25 μM LPA and 10 μM Ki16425 ($n=6$).

Total protein was collected in 50 μL of lysis buffer containing 0.1% Triton X-100 (Sigma-Aldrich), 1 mM Tris, pH 8.0, 1 mM EDTA (Sigma-Aldrich), and 1% Protease Inhibitor Cocktail Set I (EMD Chemicals, Darmstadt, Germany). Protein concentrations were determined using the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's instructions. Apoptosis was quantitatively assessed by analyzing 10 μg of protein per sample using a Caspase-Glo 3/7 assay (Promega, Madison, WI). Luminescence was detected on a microplate reader (BIO-TEK Synergy HTTR, Wisnooski, VT) and normalized to protein collected from control cells grown in 21% O_2 supplemented with 10% FBS.

Quantification of MSC VEGF production

VEGF secretion into media by MSC in response to SD/H, 25 μM LPA, and 10 μM Ki16425 was measured using a human VEGF ELISA kit according to the manufacturer's instructions (R&D Systems). Data were normalized to the quantity of total protein collected from the cells in each well ($n=4$).

Alginate gel synthesis

Alginate gels were fabricated largely as previously described.[31,32] Briefly, hydrogels were formed from a 2% (w/v) solution of irradiated alginate polymer coupled to oligopeptides containing the Arg-Gly-Asp (RGD) cell adhesion sequence. 6.66×10^6 undifferentiated or OM+ preconditioned MSC-Luc were suspended in each mL of the 2% solution, which was then mixed thoroughly with a supersaturated calcium sulfate slurry using two syringes and a three-way stopcock (40 μL $CaSO_4$ per mL alginate). When used, LPA was added to the 2% solution to yield a final concentration of 25 μM in the polymerized gels.

Murine subcutaneous injection model and noninvasive imaging of implanted cells

Treatment of experimental animals was in accordance with the UC Davis animal care guidelines and the all National Institutes of Health animal-handling procedures. Eight-week-old nonobese diabetic/severe combined immunodeficient gamma (NSG, NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ) mice (Jackson Laboratories–West, Sacramento, CA) were anesthetized and maintained under a 2% isoflurane/O_2 mixture delivered through a mask. Every animal received four subcutaneous injections of 150 μL each, containing MSC-Luc conditioned in GM or OM + and with or without 25 μM LPA ($n=12$). One injection was made over each shoulder and hip.

Cell persistence was measured using *in vivo* BLI at 1, 3, 7, 14, 21, and 28 days on an IVIS Spectrum (Perkin Elmer, Waltham, MA) as previously described.[25,26] Briefly, mice were injected with D-Luciferin, Firefly (Caliper, Hopkinton, MA; 10 μL/g body weight), and luminescence was measured using Living Image software (Perkin Elmer). Total photons per second per centimeter were recorded from each bioluminescent region of interest. Data are normalized to luminescence from gels containing undifferentiated cells within each animal at each time point.

Animals were euthanized 7 and 28 days postsurgery ($n=6$) and each gel was excised and fixed in 10% formalin for 72 h. Fixed gels were processed for histology using standard techniques. The presence of surviving MSC was assessed by immunohistochemistry for human CD90 (ab133350, 1:100; Abcam, Cambridge, MA) and a rabbit-specific HRP/DAB detection kit (ab64261; Abcam).

Statistical analysis

Data are presented as mean ± standard error unless otherwise stated. Statistical analysis was performed using paired Student's *t*-tests and one-way ANOVA with the Tukey's multiple comparison post-test, where applicable. *p*-values less than 0.05 were considered statistically significant.

Results

Media composition differentially drives osteogenic differentiation

MSC cultured in OM+ exhibited significantly higher levels of ALP activity at 7 days compared with cells maintained in GM and OM (Fig. 1A). MSC in OM exhibited modestly a greater ALP activity compared with GM, although we did not observe statistically significant increases. Similarly, cells conditioned in OM+ expressed significantly higher levels of the osteogenic transcription factor *RUNX2* compared with cells cultured in GM or OM (Fig. 1B). Based on these and previous data,[6] we selected 7 days as the consistent duration for osteogenic preconditioning for the remainder of these studies.

LPA receptor expression is dependent upon differentiation state

MSC exposed to SD/H had expressed greater transcript for *LPAR1*, *LPAR2*, *LPAR4*, and *LPAR5*, regardless of osteogenic preconditioning, compared with samples cultured at 21% O_2 in full serum. *LPAR1* expression was significantly increased by SD/H in MSC cultured in OM and OM+ (Fig. 2A), while *LPAR2* expression was higher in SD/H in all media (Fig. 2B). *LPAR4* and *LPAR5* similarly showed trends for increased expression in SD/H compared with 21% O_2, but did not achieve statistical significance (Fig. 2C, D). Notably, MSC preconditioned in OM expressed significantly lower levels of *LPAR1* and *LPAR2* compared with cells cultured in both GM and OM+. *LPAR3* was not found at detectable levels under the tested culture

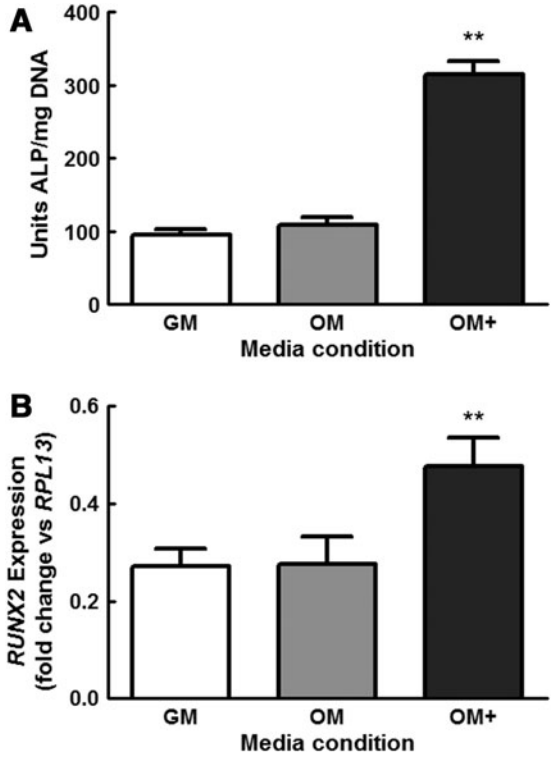

FIG. 1. Osteogenic differentiation of mesenchymal stromal cells (MSC) grown in growth medium (GM), osteogenic media (OM), and OM+ for 7 days as determined by **(A)** intracellular alkaline phosphatase (ALP) activity and **(B)** *RUNX2* expression. $^{**}p < 0.001$ versus GM and OM, $(n = 4)$.

conditions (data not shown), consistent with other reports indicating that MSC do not express LPAR3.

SD/H causes loss of MSC in culture

Compared with MSC maintained in 21% O_2 and full serum (Fig. 3A), we observed a significant loss of MSC exposed to SD/H (Fig. 3B). The number of MSC in culture was visually reduced, and the addition of 25 μM LPA mitigated this loss (Fig. 3C, D). These observations were quantitatively confirmed by measuring the DNA content in the culture (Fig. 3D).

LPA-mediated rescue of SD/H-induced apoptosis is dependent upon differentiation state

MSC in SD/H conditions exhibited increased caspase 3/7 activity compared with control cells maintained in 21% O_2 and 10% FBS, regardless of the differentiation state (Fig. 4A–C). The addition of LPA to the culture medium resulted in a dose-dependent effect on the caspase activity that was also related to the differentiation state. For MSC in GM and SD/H, the addition of 25 and 100 μM LPA significantly reduced caspase 3/7 activity, a widely accepted quantitative measure of apoptosis,[9,33] compared with no LPA (Fig. 4A). MSC preconditioned in OM exhibited significant reduction in caspase activity to control levels when stimulated with 25 μM LPA, and the addition of the $LPA_{1/3}$ receptor inhibitor Ki16425 negated this effect (Fig. 4B).

MSC preconditioned in OM+ displayed significantly greater cell death after 24 h in SD/H compared with OM+ conditioned control cells in 21% O_2 with full serum. Significant attenuation of caspase activity occurred in the presence of 100 μM LPA and was abrogated by Ki16425. Unlike MSC conditioned in OM or GM, 25 μM LPA was insufficient to protect cells against apoptosis, as measured by caspase 3/7 activity (Fig. 4C).

LPA increases VEGF production in MSC

VEGF production by MSC decreased as a function of increased osteogenic differentiation, as directed by the addition of dexamethasone to the cocktail of osteogenic supplements (Fig. 5A). MSC exposed to SD/H in GM increased VEGF secretion compared with control cells exposed to 21% oxygen with 10% FBS, but we did not observe increased VEGF secretion under SD/H for MSC cultured in other media. Compared with MSC in GM or OM, VEGF

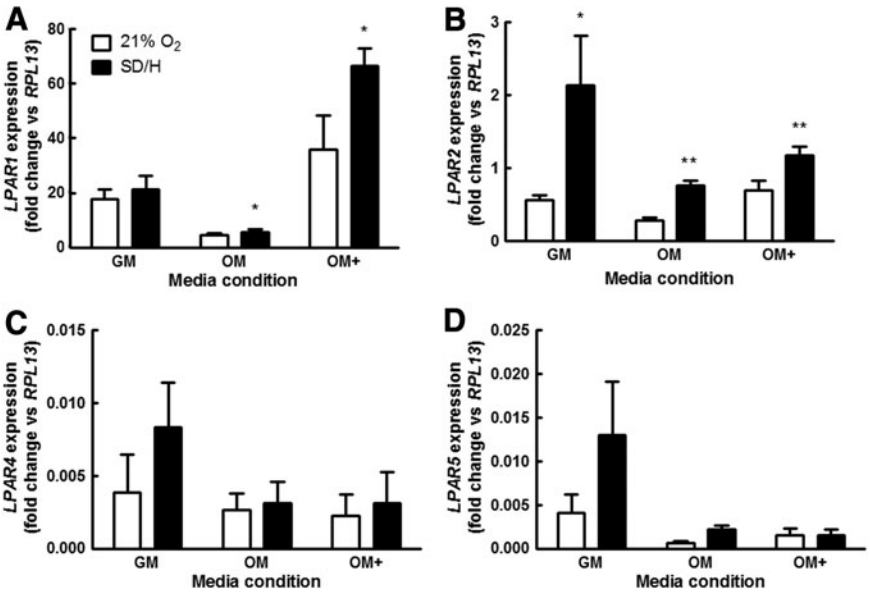

FIG. 2. Lysophosphatidic acid (LPA) receptor expression is significantly affected by the MSC differentiation state. **(A)** *LPAR1* expression is significantly increased by serum deprivation and hypoxia (SD/H) in OM and OM+, while **(B)** *LPAR2* expression is higher in SD/H for all media conditions. SD/H also increases expression of **(C)** *LPAR4* and **(D)** *LPAR5*, but not to statistically significant levels. *LPAR1* and *LPAR2* expression was significantly higher in GM and OM+ than OM. *LPAR3* was not detected MSC in any media type. $^{*}p < 0.05$ versus control, $^{**}p < 0.01$ versus control.

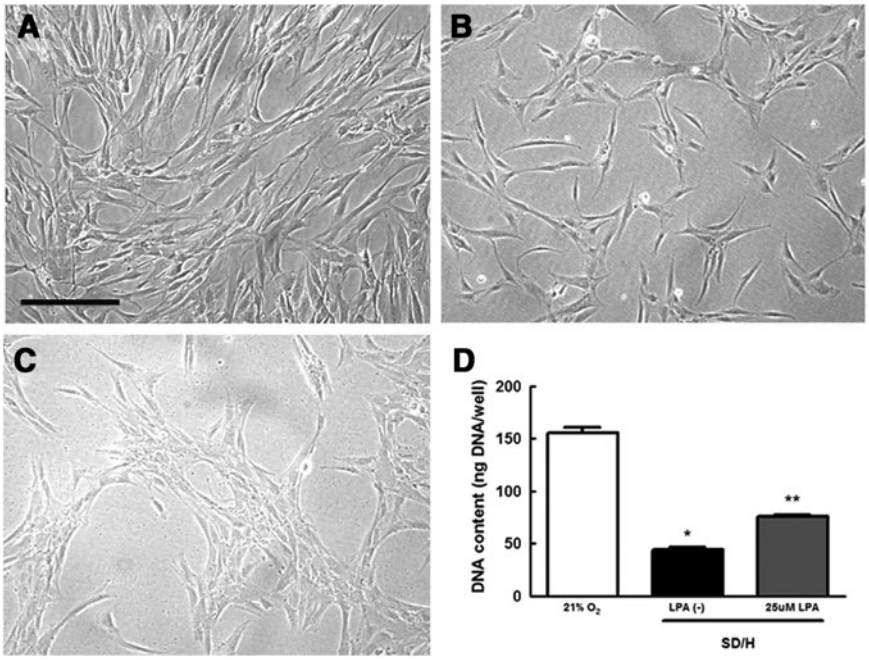

FIG. 3. SD/H induces apoptosis in MSC in GM after 24 h. **(A)** Representative images of MSC in 21% O_2 with 10% fetal bovine serum (FBS). **(B)** MSC exposure to SD/H results in significant loss of cells from the culture dish that is partially rescued by the addition of **(C)** 25 μM LPA. Images taken at 100×; scale bar represents 100 μm. **(D)** Quantification of cell number by DNA content confirms visual assessment. $*p < 0.001$ versus control, $**p < 0.001$ versus SD/H ($n = 3$).

secretion was impaired in MSC preconditioned in OM+ and significantly reduced further in SD/H. For MSC in GM, the addition of 25 μM LPA in SD/H increased VEGF secretion beyond concentrations seen for cells in 21% O_2 or SD/H, while Ki16425 abrogated this increase in VEGF in response to exogenous LPA. Compared with cells without LPA, we observed a trend for increased VEGF production in MSC cultured in OM upon the addition of 25 μM LPA in SD/H, although this did not achieve statistical significance. Similar to MSC in GM, the addition of LPA to MSC in OM+ in SD/H significantly increased VEGF production and Ki16425 negated this response.

To determine if LPA-mediated VEGF protects MSC against apoptosis, cells in GM were exposed to SD/H with 25 μM LPA and saturating addition of 10 μg/mL $VEGF_{165/121}$ antibody; no further decrease in the caspase activity was detected (Fig. 5B). Similarly, 5 ng/mL VEGF did not improve cell survival when added to GM in conjunction with 25 μM LPA and 10 μM Ki16425.

LPA and osteogenic differentiation promote MSC survival in vivo

To test whether the protective effects of LPA translate to *in vivo* applications, we entrapped MSC-Luc in alginate gels containing 25 μM LPA and injected them subcutaneously in NSG mice. We compared undifferentiated cells versus MSC preconditioned in OM+ because these groups had the

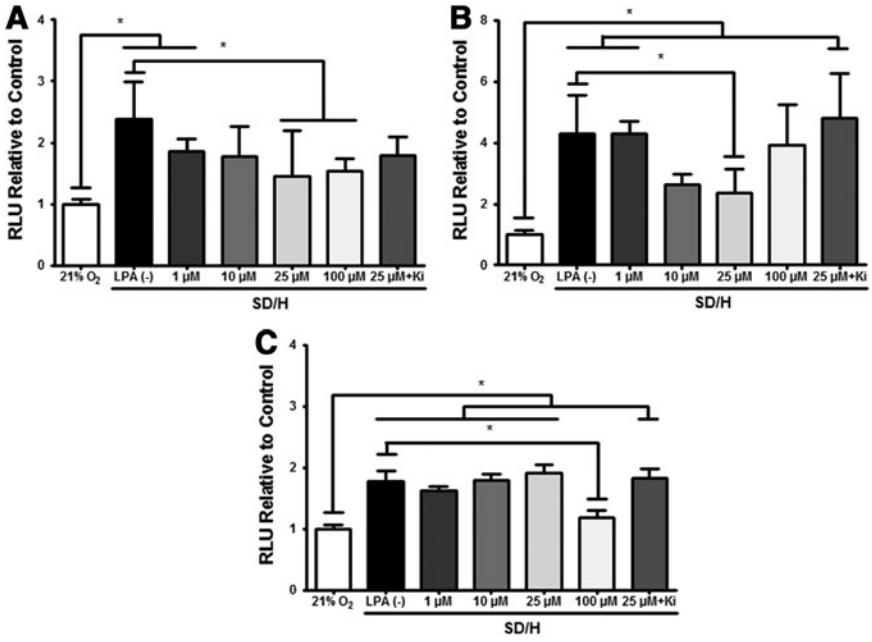

FIG. 4. LPA rescues MSC from SD/H-induced apoptosis in a dose-dependent manner that varies by differentiation state as induced by the following: **(A)** GM, **(B)** OM, and **(C)** OM+. Data are normalized to RLU from cells in 21% O_2 and media containing 10% FBS. $*p < 0.01$ ($n = 6$).

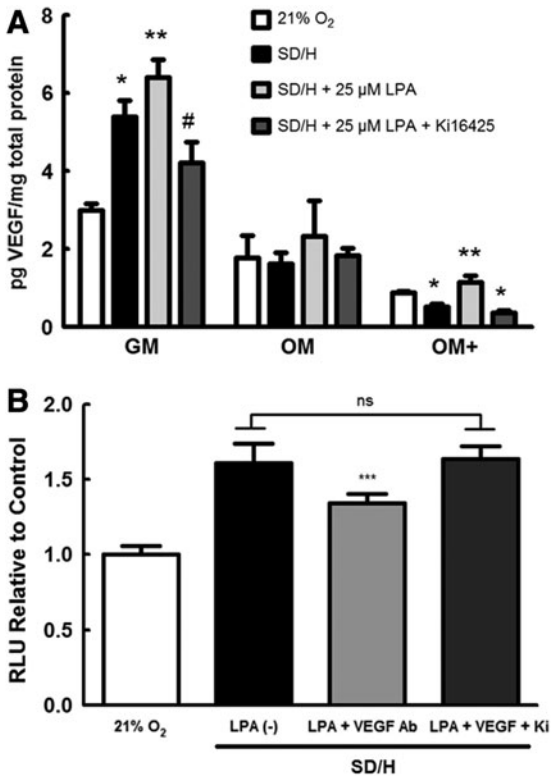

FIG. 5. **(A)** LPA significantly increases production of vascular endothelial growth factor (VEGF) by MSC in GM and OM+, which is mitigated by the addition of Ki16425 ($n=4$). However, **(B)** the addition of VEGF does not affect MSC survival under SD/H ($n=6$). $^{*}p<0.0001$ versus control, $^{**}p<0.01$ versus SD/H, $^{\#}p<0.001$ versus SD/H +25 μM LPA, and $^{***}p<0.01$ versus LPA(−).

widest range of LPA receptor expression in SD/H compared with 21% O_2 and exhibited less sensitivity to SD/H than cells in OM. Noninvasive BLI revealed that alginate-entrapped, GM conditioned MSC-Luc undergo significant cell death over 4 weeks *in vivo* (Fig. 5A, B). By the final time point, virtually no luminescence was detected from this group of gels. Codelivery of 25 μM LPA significantly improved survival of undifferentiated cells at 28 days and maintained sustained, readily observable luminescence.

In agreement with our *in vitro* caspase 3/7 data, MSC-Luc preconditioned in OM+ were dramatically more resistant to apoptosis. Similarly, the addition of 25 μM LPA did not further inhibit cell loss. CD90 staining of MSC at 28 days (Fig. 5C–F) confirms an almost total absence of undifferentiated cells that was improved with LPA treatment, as well as the continued presence of OM+conditioned cells.

Discussion

Widespread cell death following implantation into sites of ischemic injury remains a major hurdle to successful implementation of MSC-based solutions for tissue repair. Although recombinant proteins can promote cell survival, they are costly to produce in large quantities and carry distinct challenges for long-term stabilization and delivery. In this study, we examined the ability of LPA to inhibit SD/H-induced apoptosis in human MSC. Previous groups have shown that LPA, an inexpensive glycerophospholipid, inhibits apoptosis in undifferentiated rat MSC *in vitro*,[9] but these results lack translational relevance because of the inherent differences in behavior between human and rat MSC. Human MSC signaling and phenotype are affected by their differentiation state,[4] and many strategies for tissue repair utilize different preconditioning regimens[1,2,6] for increased efficacy. For example, osteogenically inducing MSC before implantation enhances *in vivo* bone regeneration,[6] whereas undifferentiated MSC secrete higher levels of growth factors.[34] Therefore, we specifically assessed the protective effects of LPA on cells exposed to three increasing degrees of osteogenic conditioning.

After preconditioning MSC in varying media (GM, OM, and OM+) for 1 week, the cellular response to LPA in SD/H was significantly different at both the transcriptional and functional levels. qPCR analysis revealed that cells in 21% O_2 with 10% FBS expressed *LPAR1* and *LPAR2* at the highest levels, while *LPAR4* and *LPAR5* expression was much lower (Fig. 2). *LPAR3* was undetected in this population. This is in agreement with previous studies characterizing LPA receptor expression in MSC expressing recombinant human telomerase.[35] Following exposure to SD/H, LPA receptor expression increased in cells in all media types, although to varying degrees of statistical significance. Notably, the expression levels of *LPAR1* and *LPAR2* were statistically different in each media condition, and MSC preconditioned in OM showed lower levels of expression for all receptors compared with cells in GM and OM+.

We selected a luciferase-based caspase 3/7 activity assay to rapidly quantify protein markers directly involved in late-stage apoptosis. A number of techniques are available for apoptotic assessment, including flow cytometry and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL). However, these techniques do not reliably discriminate between apoptotic and necrotic cells, and TUNEL may yield false positives from cells undergoing DNA repair and transcription.[36] Caspase 3/7 activation has long been established as a late-stage marker of apoptosis that is conserved across many species.[37] Increases in the caspase activity among a cell population are widely accepted as being reflective of increases in apoptosis.[9,33] The caspase 3/7 activity revealed that LPA rescues SD/H-induced apoptosis in human MSC at all three differentiation states, but cells conditioned in each media type require different effective concentrations (Fig. 4). 25 and 100 μM of LPA inhibited SD/H-mediated increases in caspase activity for cells conditioned in GM, while only 25 μM LPA had significant effects in OM. 100 μM of LPA was required to detect reductions in apoptosis for cells in OM+. Furthermore, we found that caspase 3/7 activity in MSC conditioned in OM and exposed to SD/H were nearly twice as high as those in cells cultured in GM and OM+. Additionally, SD/H induced the lowest apoptotic response in MSC preconditioned in OM+. Because dexamethasone protects MSC from confluence-induced apoptosis,[38] we examined the potentially protective role of dexamethasone (10 nM) when added to cells in GM and exposed to SD/H for 24 h. No anti-apoptotic effects were found (data not shown), indicating that differences in caspase activity between OM and OM+ were not dexamethasone dependent.

The addition of the $LPA_{1/3}$ inhibitor Ki16425 abrogated LPA-mediated rescue of apoptosis in all media conditions, although significance was achieved only in OM and OM+. Combined with the absence of detectable *LPAR3*, this indicates that apoptotic inhibition is mediated through LPA_1, which is further supported by greater levels of transcript expression. Additional comparisons between apoptotic responses and LPA receptor expression levels show a correlation between relatively low levels of LPA_1 in OM-conditioned MSC and higher caspase levels in SD/H, suggesting that cells with fewer receptors are more sensitive to ischemic conditions. Similarly, MSC under hypoxia exhibit increased expression of receptors for erythropoietin and hepatocyte growth factor,[8] as well as receptors *CX3CR1* (interleukin 8 alpha) and *CXCR4*.[39] Ongoing work is examining the role of cAMP/PKA and Ca^{2+} in the ability of LPA to rescue cells from SD/H.

In addition to possessing osteogenic potential, MSC can play a critical role in tissue repair by serving as support cells that secrete trophic factors to accelerate angiogenesis and wound healing.[2,26,40] One such potent angiogenic factor, VEGF, is antiapoptotic for a number of cells, including endothelial cells,[41] cardiomyocytes,[42] and neurons.[43] Although MSC do not express VEGF receptors,[44] they produce increased amounts of VEGF in response to ischemia.[45–47] Indeed, MSC conditioned in GM secreted significantly higher levels of VEGF in SD/H, which was further augmented by the addition of 25 μM LPA (Fig. 5A). Ki16425 decreased VEGF production when added with LPA, again implicating LPA_1-mediated signaling. The addition of VEGF-neutralizing antibody or exogenous VEGF had no effect on caspase activity, confirming that LPA rescues apoptosis independently of VEGF (Fig. 5B).

As human MSC undergo osteogenic differentiation, they lose their proangiogenic potential,[4] which is in accordance with our observed decreases in VEGF production by OM- and OM+ treated cells. However, the addition of LPA to OM+ still resulted in significantly increased VEGF secretion, which was abrogated by Ki16425. The secretion of VEGF by MSC in OM in response to LPA exhibited a trend that mirrored OM+, but lacked statistical significance, possibly as a result of the lower receptor expression levels described previously.

We applied our findings *in vivo* by injecting alginate-entrapped MSC-Luc with LPA subcutaneously in NSG mice. Using BLI, we noninvasively observed persistence over 4 weeks and found that undifferentiated cells indeed suffered from widespread cell death upon implantation (Fig. 6). The addition of 25 μM of LPA significantly improved survival, confirming that LPA can be effectively implemented in a hydrogel delivery system. Of particular note, GM MSC entrapped in LPA-containing gels experienced sustained increases in survival from localized delivery of LPA at every time point over the course of the study. This prolonged response contradicts the burst release profile that frequently hampers the effectiveness of recombinant proteins.[48] Additionally, our ability to incorporate LPA and cells directly into gels during synthesis allows for straightforward clinical applications, especially compared with previously established methods that require rat MSC to be preconditioned with LPA in culture before injection *in vivo*.[19]

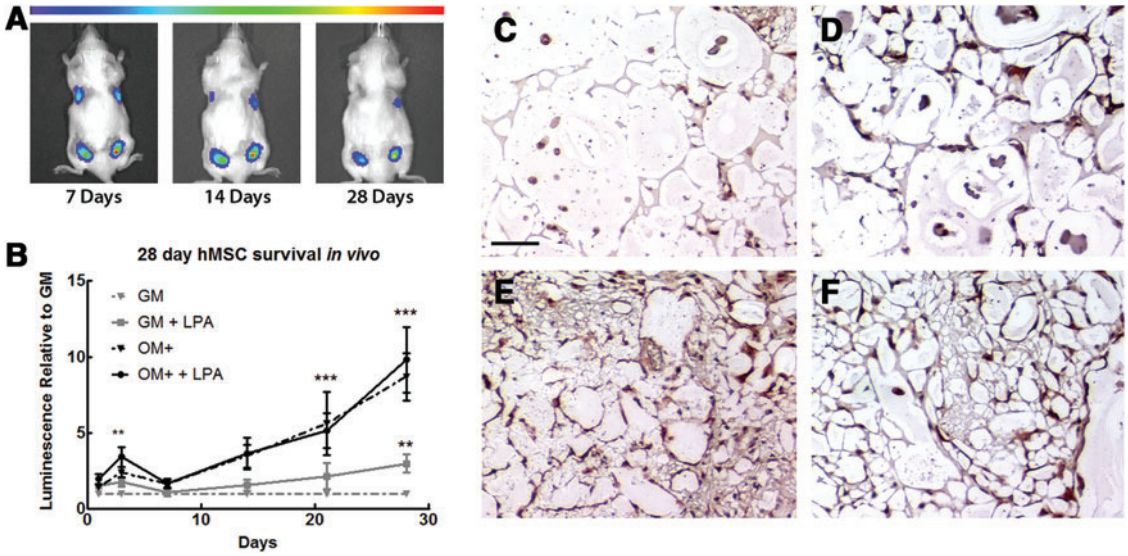

FIG. 6. LPA promotes cell survival of MSC over 4 weeks *in vivo*. **(A)** BLI reveals that more GM-preconditioned MSC-Luc survive over 28 days when delivered with 25 μM LPA (top right) compared with untreated cells (top left). OM+ preconditioned MSC-Luc (bottom left) have higher persistence over time, but also exhibit improved survival in LPA-containing gels (bottom right). **(B)** Quantification of luminescence over time shows that LPA significantly improves survival of GM MSC-Luc. OM+ conditioning alone had a protective effect over 28 days, regardless of LPA treatment. Data are normalized to luminescence from gels containing undifferentiated cells within each animal at each time point. **(C)** Representative CD90 staining from gels containing GM-conditioned MSC confirms poor cell survival at 28 days that is **(D)** improved with the addition of LPA. **(E, F)** MSC conditioned in OM+ persisted at 28 days both in the absence **(E)** or presence **(F)** of LPA. Magnification is 100×; scale bar represents 100 μm. $^{**}p < 0.01$ versus GM, $^{***}p < 0.001$ versus GM ($n = 6$). Color images available online at www.liebertpub.com/tea

Consistent with our *in vitro* data, osteogenically induced MSC-Luc were inherently more resistant to apoptosis, with LPA providing no significant additional protection at 25 μM. Temporal quantification of luminescence revealed a universal decrease in intensity over time in all conditions, indicating that differences between groups are not driven by proliferation (data not shown). These data have significant implications for cell-based therapies for tissue repair, with potential applications for two unique treatment strategies. In one case, LPA, which has mitogenic effects on endothelial cells,[49] could be used to promote survival and VEGF secretion in undifferentiated MSC to further enhance angiogenesis for a natural wound healing response. This approach may markedly enhance the efficacy of MSC when implanted to drive neovascularization for use in advanced vascular disease, slow healing wounds, or promoting collateralization during bone repair. Conversely, osteogenically induced MSC treated with LPA would exhibit increased resistance to SD/H-induced apoptosis in a defect site, potentially enabling these cells to more effectively and directly contribute to forming new bone. Either of these approaches will significantly improve the efficacy of MSC-based tissue engineering treatments in a cost-effective, readily applicable manner

Our results demonstrate that LPA-mediated rescue of human MSC viability in SD/H is indeed dependent on osteogenic differentiation, with cells conditioned in GM requiring the smallest effective dose and cells in OM exhibiting the most adverse reaction to ischemia. Additionally, LPA treatment significantly increased VEGF production in GM and OM+. All of these responses appear to be dependent on LPA_1, as determined by treatment with an $LPA_{1/3}$ inhibitor, coupled with the lack of *LPAR3* expression. We also successfully codelivered LPA with MSC *in vivo* to promote survival of undifferentiated cells and showed that differentiated MSC are more resistant to ischemic cell death. This study provides valuable insight into the considerations necessary to optimize *in vivo* cell survival in differentiation-mediated tissue engineering applications.

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Disclosure Statement

No competing financial interests exist.

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