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Production of Reactive Oxygen Species by Multipotent Stromal Cells/Mesenchymal Stem Cells Upon Exposure to Fas Ligand

Melanie Rodrigues[*], **Omari Turner**[†], **Donna Stolz**[‡], **Linda G. Griffith**[§], and **Alan Wells**[*]

[*]Department of Pathology, University of Pittsburgh, Pittsburgh, PA, USA

[†]Meharry Medical College, Nashville, TN, USA

[‡]Department of Cell Biology, University of Pittsburgh, Pittsburgh, PA, USA

[§]Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA

Abstract

Multipotent stromal cells (MSCs) can be differentiated into osteoblasts and chondrocytes, making these cells candidates to regenerate cranio-facial injuries and lesions in long bones. A major problem with cell replacement therapy, however, is the loss of transplanted MSCs at the site of graft. Reactive oxygen species (ROS) and nonspecific inflammation generated at the ischemic site have been hypothesized to lead to MSCs loss; studies in vitro show MSCs dying both in the presence of ROS or cytokines like FasL. We questioned whether MSCs themselves may be the source of these death inducers, specifically whether MSCs produce ROS under cytokine challenge. On treating MSCs with FasL, we observed increased ROS production within 2 h, leading to apoptotic death after 6 h of exposure to the cytokine. *N*-acetyl cysteine, an antioxidant, is able to protect MSCs from FasL-induced ROS production and subsequent ROS-dependent apoptosis, though the MSCs eventually succumb to ROS-independent death signaling. Epidermal growth factor (EGF), a cell survival factor, is able to protect cells from FasL-induced ROS production initially; however, the protective effect wanes with continued FasL exposure. In parallel, FasL induces upregulation of the uncoupling protein UCP2, the main uncoupling protein in MSCs, which is not abrogated by EGF; however, the production of ROS is followed by a delayed apoptotic cell death despite moderation by UCP2. FasL-induced ROS activates the stress-induced MAPK pathways JNK and p38MAPK as well as ERK, along with the activation of Bad, a proapoptotic protein, and suppression of survivin, an antiapoptotic protein; the latter two key modulators of the mitochondrial death pathway. FasL by itself also activates its canonical extrinsic death pathway noted by a time-dependent degradation of c-FLIP and activation of caspase 8. These data suggest that MSCs participate in their own demise due to nonspecific inflammation, holding implications for replacement therapies.

Keywords

Reactive oxygen species (ROS); Multipotent stromal cells; Marrow stromal cells; Multipotent stem cells; Mesenchymal stem cells

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Address correspondence to Alan Wells, S 713 Scaife Hall, 3550 Terrace St., Pittsburgh, PA 15261, USA. wellsa@upmc.edu.

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INTRODUCTION

Multipotential stromal cells, also called mesenchymal stem cells (MSCs), have the ability to differentiate into a wide range of cell types in vitro including cells of the bone, cartilage, and fat (8,35,49,52). However, there lies a major void between studies done with these cells in vitro and with their use in a wound microenvironment in vivo since MSCs once implanted rarely survive. While there have been reparative effects seen after MSC implantation into sites of injury, the regeneration is attributed to the paracrine signaling by the MSCs or immunosuppressive aspects rather than MSC differentiation into the desired tissue (4,36,67). Tests conducted in vivo in small animals show that MSCs implanted both in the ischemic as well as the normal heart start dying within 7–10 days and completely disappear by the fourth week (38). Similar engraftment studies display less than 3% of the administered MSCs after 2 weeks, a number that is hardly sufficient to reform lost tissue (39,53). MSCs used for treatment of inflammatory bowel disease display clinical improvements in all mice injected with MSCs; however, only 40% of mice showed engraftment of cells, with as little as 0.13% of MSCs traceable in the intestine after 3 days (60). Similarly decreased numbers of MSCs are observed after delivery to the ischemic kidney (43). The reduced MSC numbers are consistent in studies across tissues.

Several factors have been hypothesized and reported for making the environment in which MSCs are injected poorly conducive to cell survival. Lack of trophic factors is one. But also, these cells must survive a surge of nonspecific inflammation involving the release of prodeath cytokines (21,57). Other nonexclusive causes may include reduced blood flow at the wound site causing hypoxia (77), reactive oxygen species (ROS) generation (70), and nutrient starvation (34,40), leading to cellular stress. All these factors would culminate in loss of the transplanted MSCs.

The microenvironment in which MSCs are implanted are generally wound regions with high prodeath cytokine activity. Based on the assumption that MSCs may be susceptible to prodeath cytokines, Fan et al. tested for survival of MSCs in the presence of several prodeath factors and found that FasL was the most efficient in triggering MSC death (21). This cell death is enhanced when MSCs are stressed with nutrient deprivation/ protein limitation; in vitro this is mimicked with the protein synthesis inhibitor cycloheximide (21). MSCs have been shown to be killed by $CD8^+$ T lymphocytes and activated natural killer (NK) cells (17,25), the two cell types that abundantly and perhaps exclusively produce FasL (63) with several groups showing interleukin (IL)-2 activated NK cells to be detrimental to MSCs (54,56,62). Le Blanc's group also notes that both fetal and adult MSCs are susceptible to death by activated NK cells, dependent on FasL, an innate immune response triggered upon implantation of foreign materials into the body (25). However, there is one contrary report where Mazar et al. show that activation of Fas, the receptor for FasL by anti-Fas antibody, does not lead to MSC death (41). These differences in outcomes may be explained by the mode of activation of Fas and culture surfaces, the study by Fan et al. being done using Fas ligand on bioengineered polymer surfaces and the studies by Mazar et al. using anti-Fas antibody on tissue culture plastic (21,41).

The target of MSC therapy is either a wound/injury bed and/or implantation; in both these cases, there is active inflammation, which involves the generation of ROS. ROS evoke many intracellular events in cells including cellular proliferation, migration, and cell cycle arrest, but mainly cell death (13,26,37). Increased ROS in cells brings about damage not only by oxidizing DNA, proteins, and lipids but also by activation of cellular stress-activating pathways. Recently, there have been studies indicating that paracrine signaling of ROS on MSCs can be detrimental to these cells. According to one report hydrogen peroxide, a major contributor to oxidative damage, when added to MSCs in culture, activated the caspase

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cascade and induced apoptosis in MSCs (37). In another study, ROS was seen to inhibit adhesion of MSCs to the ischemic myocardium (61). Additionally, senescent human umbilical cord MSCs have shown to be killed in the face of increased ROS (39). These reports suggest that MSCs will undergo cell death in an ischemic environment where there is excessive ROS production.

Based on the independent studies that reported FasL as a first phase immune response cytokine detrimental to MSCs (21,25), the primary goal of our study was to investigate whether cytokines like FasL induce ROS production in MSCs and contribute to death by causing oxidative damage. Our hypothesis is based on reports that FasL generates ROS in several cell types, which causes further death receptor clustering, activation of caspases and apoptosis (15,75,76). We also probed for uncoupling proteins (UCPs), protein transporters present in the inner mitochondrial membrane that are upregulated to counter increases in ROS in several cell types (1,6,10,38,51). As such, UCP-deficient animals show higher ROS production and greater oxidative damage (9). We found that FasL increased ROS within a few hours of treatment, causing high levels of intracellular ROS levels and subsequently caused activation of caspase 3 leading to MSC death. MSCs with UCP2 reduced by genetic means and showed faster onset of apoptosis upon treatment with FasL compared to MSCs without UCP2 knockdown, suggesting that UCP2 does negate intracellular ROS produced by FasL. To our knowledge this is the first time FasL has been linked to changes in uncoupling protein expression in terms of moderating oxidative stress in any cell type. Our data suggest that MSCs will not only be susceptible to paracrine signaling of ROS when implanted in ischemic wound regions but will also produce damaging ROS in response to implantation owing to nonspecific inflammation.

MATERIALS AND METHODS

Reagents

Human recombinant epidermal growth factor [hrEGF; 354052] was procured from BD BioSciences (Franklin Lakes, NJ) and human soluble recombinant SuperFasL [ALX-522-020-3005] was obtained from Enzo Lifesciences (Plymouth Meeting, PA). MitoSOX Red [M36008] and 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluo-rescein diacetate, acetyl ester [CM-H_2DCFDA;C6827] were from Molecular Probes, Invitrogen (Carlsbad, CA). RNeasy Minikit and QuantiTect reverse transcription kit were procured from Qiagen (Valencia, CA). Brilliant SYBR Green qPCR Master Mix was from Stratagene (Santa Clara, CA). Ac DEVD AFC (7-amino-4-trifluoromethyl coumarin) [556574] and Ac IETD AFC [556552] were from BD Pharmigen (San Diego, CA). UCP2 [SAB 2501087] and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies were from Sigma (St. Louis, MO); cellular caspase 8 (FLICE)-like inhibitory protein2 [c-FLIP] antibody [ab8421] was from AbCam (Cambridge, MA); phospho-c-Jun N-terminal kinase 1 (JNK) antibody [17–466] was from EMD Millipore (Billerica, MA); phospho-extracellular signal-regulated kinase 1/2 (ERK1/2) antibody [4377], phospho-p38 mitogen-activated protein kinase (p38MAPK), phospho-B-cell CLL/lymphoma 2 (bcl2) antagonist of cell death (Bad) antibody [9291], cytochrome oxidase IV (Cox IV antibody) [4844], poly (ADP-ribose) polymerase (PARP) [9532], and survivin antibody [2808] were from Cell Signaling Technology (Boston, MA). Caspase inhibitor Z-VAD-FMK [G7231] was from Promega (Madison, WI). UCP1 and UCP2 forward and reverse primers were from Integrated DNA Technologies (Coralville, IA). siRNA for UCP2 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

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Cell Culture

Immortalized human bone marrow stromal cells (imhMSCs) (47), a kind gift from Dr. Junya Toguchida's lab (Kyoto University, Japan), were cultured in Dulbecco's modified Eagle's medium (DMEM) low glucose from Cellgro (Mediatech, Washington, DC) with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 1 μM nonessential amino acids and 100 units/ml penicillin– streptomycin. The 10th passage of these cells was used for experiments. Primary human marrow stromal cells (prhMSCs) (59) provided by Dr. Darwin Prockop (Texas A&M) were cultured in α-MEM without ribonucleotides or deoxyribonucleotides from Gibco (Carlsbad, CA) with 16.5% FBS from Atlanta Biologicals (Norcross, GA) and 2 mM L-glutamine. We have previously characterized both these cell lines in terms of differentiation potential, proliferation in response to growth factors and death in the presence of cytokines (21,65). The use of the human cells has been approved the IRB committees of the University of Pittsburgh and Pittsburgh VAMC.

Concentration of Cytokines/Reagents

MSCs were treated with 100 nM EGF, 100 ng/ml FasL, 20 μM cycloheximide (CHX), 50 μg/ml *N*-acetyl cysteine (NAC), 0.03% H_2O_2, and 50 μg/ml zVAD-FMK.

MitoSOX Red Assay

MitoSOX Red is a live cell permeant probe that gets oxidized rapidly by superoxide in the mitochondria and fluoresces red on superoxide generation. imhMSCs and prhMSCs were grown in six-well plates until 60% confluent. Cells were washed in PBS, 20 μM MitoSOX Red reagent in PBS and Hoescht 33342 were added, and cells were incubated at 37°C for 10 min to allow for cells to intake the probe. Cells were rinsed in PBS to wash out all extracellular MitoSOX Red, and MSCs were treated with EGF/FasL/CHX/NAC. Images were taken at regular time periods up to 8 h and quantified by ImageJ analysis. Since cell numbers were less with onset of death, increases in ROS were represented as the ratio of MitoSOX intensity to total cell numbers.

CM-H_2DCFDA Assay

CM-H_2DCFDA is a cell permeable indicator that fluoresces only upon oxidation and after loss of its acetate group and is a marker for hydroxyl radical. imhMSCs and prhMSCs were grown in 96-well plates. On the day of assay, media were aspirated, and cells were washed with PBS and 10 μM CM-H_2DCFDA reagent in PBS added to cells. Cells were treated with EGF/FasL/CHX/ NAC alone and in combinations, incubated at 37°C and fluorescence at an excitation of 485 nm and emission of 535 nm measured at regular intervals up to 8 h.

Immunoblots

After treatment of cells with cytokines at defined time points, both floating cells in the media and cells left on the culture plate were lysed in sodium dodecyl sulfate (SDS) lysis buffer containing 0.1 M Tris–HCl, 4% SDS, 0.2% bromophenol blue, and 5% β-mercaptoethanol. Cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. Blots were probed for UCP2 PARP, phospho-JNK, phospho-p38MAPK, phospho-ERK1/2, phospho-Bad, survivin, CoxIV, and GAPDH primary antibodies, followed by horse radish peroxidase (HRP)-conjugated secondary antibodies and developed with an ECL kit.

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FLICA (Fluorochrome Inhibitor of Caspase Assay)

MSCs were grown on Lab-tek 8 chamber slides, and once the cells were 70% confluent, cells were changed into media with 0.5% dialyzed FBS. Cells were treated with cytokines like FasL alone, EGF + FasL, etc., for 6 h, following which FLICA reagent and Hoescht 33342 dye was added to cells in media and incubated for 30 min at 37°C. Media were aspirated, cells were washed, and live cells were imaged for fluorescence. FLICA intensity was measured by ImageJ analysis software. To account for cell loss, intensity of FLICA was normalized to the number of remaining cells.

Real-Time Quantitative RT-PCR Analysis

Total RNA from MSCs was isolated using RNeasy Minikit. RNA (1 μg) was reverse-transcribed into first-strand c-DNA using QuantiTect reverse transcription kit. Real-time PCR was performed on a MX3000P instrument from Stratagene using 1 μl of c-DNA and Brilliant SYBR Green qPCR Master Mix and the following primers: UCPI forward primer: 5′-CTGCCACTC CTCCAGTCGTT-3′, UCP1 reverse primer: 5′-CCG CCTCTCTCAGGATCGGCCT-3′, UCP2 forward primer: 5′-GACCTATGACCTCATCAAGG-3′, UCP2 reverse primer: 5′-ATAGGTGACGAACATCACCACG-3′, GAP DH forward primer: 5′-GAGTCAACGGATTTGGT CGT-3′, GAPDH reverse primer: 5′-TTCATTTTGGAG GGATCTCG-3′; the GAPDH primers were the normalization controls. All reactions were performed in triplicates, and the fold change in transcript levels was calculated based on ΔCt method with GAPDH as reference.

Caspase 3/Caspase 8 Activity Assay

To 15 μg of protein sample, obtained by lysis of imhMSCs with RIPA buffer and measurement of protein by BCA assay, 2 μl of 2 mM Ac DEVD AFC or 2 μl of 2 mM Ac IETD AFC was added to 200 μl of buffer to detect caspase 3 or caspase 8 activity, respectively. The buffer comprised of 20 mM PIPES, 100 mM NaCl, 1 mM EDTA, 0.1% CHAPS, 10% sucrose in water, with pH of 7.2. The samples were incubated with the reporter substrate for 8 h, and fluorescence was measured at an excitation of 400 nm and an emission of 505 nm.

UCP2 siRNA Knockdown

siRNA (0.5 nM) was transfected into imhMSCs using Lipofectamine 2000 for 6 h (in OptiMem), followed by a complete media change. Cells were left in media for 24 h and assayed for MitoSOX and FLICA after treatment with FasL and CHX and FasL. A fluorescein-conjugated scrambled RNA sequence also obtained from Santa Cruz Biotechnology (Santa Cruz, CA) was used as control siRNA.

Statistical Analysis

ROS levels and cell numbers were analyzed using paired *t* tests. Significance was set at $p < 0.05$ or a more stringent $p < 0.01$, as noted in the figure legends. The graphs of ROS that increases with different treatment conditions were compared to one another using two-way ANOVA and Tukey's tests on SPSS software. Significance for these tests was set as $p < 0.05$.

RESULTS

FasL Induces Superoxide Anion Production in MSCs

Prodeath cytokines such as FasL and tumor necrosis factor (TNF)-a cause increased ROS generation in several cell types causing intensive damage to cells and leading to cell death

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(12,27,31,58). In the present study, we used MitoSOX Red staining to determine increases in levels of the potent ROS superoxide anion after treatment of MSCs with FasL. MSCs are known to undergo cell death in the presence of FasL, and this death is further enhanced when protein synthesis is inhibited using cycloheximide (CHX) (21). CHX was used at suboptimal concentrations, enough to induce cell stress, but not strong enough to kill MSCs. As seen in Figure 1, imhMSCs treated with FasL showed MitoSOX fluorescence within 2 h and the fluorescence intensity kept increasing through 8 h after treatment. While addition of CHX to FasL increased MitoSOX Red staining to a much greater extent, addition of the antioxidant NAC to FasL-treated cells prevented the increase of superoxide anion generation. The positive control comprising MSCs treated with H_2O_2 showed pronounced increase in MitoSOX fluorescence. We were thus able to conclude that FasL increases superoxide anion synthesis in MSCs.

The intensity of MitoSOX fluorescence was normalized to cell numbers and is displayed in Figure 2A. Treatment of imhMSCs with FasL alone showed a two and a half fold steady increase in MitoSOX fluorescence over the 8-h time frame compared to untreated cells, while treatment with CHX and FasL showed a threefold increase of MitoSOX fluorescence after 8 h compared to untreated imhMSCs. Subjecting MSCs to H_2O_2 showed a trend similar to CHX + FasL treatment of cells with the fluorescence after 8 h being three times more than untreated control cells. NAC protected cells from FasL for 6 h, after which the effects of NAC appeared to wear out, as readdition of NAC after the first 4 h was able to protect imhMSCs from FasL induced ROS even at 8 h (data not shown). Addition of EGF to both FasL alone and to CHX and FasL was able to delay onset of superoxide generation by 2 h and alleviated the increased superoxide seen with both FasL alone and with CHX + FasL. EGF alone or CHX alone did not bring about any change in superoxide anion levels over the 8 h compared to untreated imhMSCs. The comparison of the various treatments over time was analyzed by ANOVA and Tukey's test and is represented in the first supplementary figure (see Fig. 1 of supplemental data; http://dl.dropbox.com/u/47761182/Cell%20Transplantation%20Supplemental/Rodrigues%20et%20al%20Supplemental%201.jpg).

To determine whether the increase in ROS was specific to immortalized MSCs, we also tested primary human MSCs with the death cytokine. prhMSCs showed a similar trend of MitoSOX intensity normalized to cell numbers with the various treatments as imhMSCs. The primary MSCs were more sensitive to both FasL and CHX + FasL treatments, with MitoSOX fluorescence increasing to three and a half times that of untreated cells after 8 h on addition of FasL alone and five times that of untreated prhMSCs with CHX + FasL treatment after 8 h (Fig. 2B). EGF added to both MSC treated with FasL alone and with CHX + FasL and offered protection against increase of ROS. EGF alone or CHX alone did not bring about any changes in ROS; however, H_2O_2 showed an increase in MitoSOX staining with time, as expected. The statistical comparison of the curves over time is represented in the second supplementary figure (see Fig. 2 of supplemental data; http://dl.dropbox.com/u/47761182/Cell%20Transplantation%20Supplemental/Rodrigues%20et%20al%20Supplemental%202.jpg).

FasL Induces Severe Oxidative Stress in MSCs

As high levels of superoxide anion were generated with FasL in MSCs, we hypothesized that there should also be high levels of other damaging ROS such as hydroxyl radicals. This would be the case, since superoxide anion gets dismutated spontaneously or by superoxide dismutases to generate hydrogen peroxide and molecular oxygen, and hydrogen peroxide further breaks down by the Fenton reaction to produce hydroxyl radical (22). To look for hydrogen peroxide and hydroxyl radicals, we used CM-H_2DCFDA. CM-H_2DCFDA fluorescence after treatment of imhMSCs with FasL showed a linear increase with time and ended six times higher than untreated cells after 8 h, while CHX + FasL rapidly increased

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CM-H_2DCFDA fluorescence to over 10 times that of untreated imhMSCs within the first 2 h and maintained this value to over five times that of untreated cells even after 8 h of treatment (Fig. 2C). Addition of NAC to FasL-treated cells entirely suppressed hydroxyl radical generation during the 8-h time frame, and addition of EGF to FasL alone and CHX + FasL reduced hydroxyl radical generation in both cases. CHX alone and EGF alone did not bring about any change in hydrogen peroxide or hydroxyl radical generation compared to untreated cells over the entire treatment time frame as seen with the MitoSOX results. H_2O_2 was used as the positive control and showed rapid increases in CM-H_2DCFDA fluorescence. The statistical comparison of the different treatments over time by ANOVA and Tukey's test is displayed in the third supplementary figure (see Fig. 3 of supplemental data; http://dl.dropbox.com/u/47761182/Cell%20Transplantation%20Supplemental/Rodrigues%20et%20al%20Supplemental%203.jpg).

Demonstrating that this hydroxyl generation is not unique to the immortalized MSCs, prhMSCs showed a 30-fold increase in generation of hydroxyl radicals with CHX + FasL compared to untreated cells after 8 h and a 10-fold increase with FasL compared to untreated cells in the same time frame (Fig. 2D). NAC again was able to completely block this increase of hydroxyl radicals, while EGF protected both prhMSCs treated with FasL alone and with CHX + FasL significantly, although these cells still display CM-H_2DCFDA fluorescence. CHX alone and EGF alone did not produce any hydrogen peroxide or hydroxyl radicals compared to control cells during the entire treatment. From the results obtained with both imhMSCs and prhMSCs, we were able to conclude that FasL produces severe oxidative stress in MSCs and this stress is amplified when protein synthesis is curtailed. The comparison of the effects on oxidative stress in prhMSCs with the different treatments is represented in the fourth supplementary figure (see Fig. 4 of supplemental data; http://dl.dropbox.com/u/47761182/Cell%20Transplantation%20Supplemental/Rodrigues%20et%20al%20Supplemental%204.jpg).

Heightened ROS Levels on FasL Treatment Leads to the Onset of Apoptosis and Death of MSCs

As FasL enhanced ROS and earlier reports show that FasL causes death in MSCs, we hypothesized that the increased oxidative stress on FasL treatment would cause activation of caspases and apoptosis. To investigate this, we examined caspase 3 activity in prhMSCs using FLICA. After 6 h of treatment with FasL or CHX + FasL, prhMSC showed active caspase 3 indicated by the red fluorescence in Figure 3A. EGF added to both these treatments was protective at 6 h (though longer challenges do lead to enhanced cell death (21)). Addition of NAC to FasL suppressed caspase 3 activation. H_2O_2 alone also induced caspase 3 activation. When zVAD-FMK, the pan-caspase inhibitor, was added to prhMSCs treated with CHX + FasL, caspase 3 activity was completely suppressed. EGF alone and CHX alone did not bring about any caspase 3 activation. The intensity of FLICA fluorescence was analyzed using ImageJ and is represented in Figure 3B. These results indicate that FasL induced ROS activates apoptosis in MSCs.

FasL Also Kills MSCs Via the Extrinsic Pathway Independent of ROS

MSCs were next subject to more prolonged treatment with FasL or CHX + FasL with or without the protective factors EGF or NAC. Eight hours after treatment, MSCs subjected to CHX + FasL displayed very few cells, as most cells had died by this time point and had detached from the surface. The few cells left behind on tissue culture plastic at the last stage of apoptosis showed diminished FLICA fluorescence. Addition of either EGF or NAC to CHX + FasL-treated cells was unable to protect from caspase 3 activation. Addition of zVAK-FMK however curbed caspase 3 activation (Fig. 4A). We were able to conclude from these results that the ROS produced in response to FasL generates enough oxidative stress to

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cause caspase 3 activation and death of MSCs; EGF is able to delay this death. NAC is able to suppress ROS generation until 6 h; however, MSC die after 8 h of exposure to FasL even in the presence of NAC, most likely through direct signaling of FasL via the caspase 8 pathway. To test further if EGF does not protect MSCs from death on longer exposures with the death factor FasL, we treated MSCs with the different factors for 8 h, and the lysates collected at this time point were immunoblotted for PARP, the DNA repair enzyme, which is a caspase 3 substrate. We observed FasL-induced degradation of PARP after 8 h, and this degradation increased with CHX + FasL treatment (Fig. 4B). Addition of EGF was unable to protect against CHX + FasL, observed by a distinct cleaved PARP band. Addition of the pan-caspase inhibitor zVAD-FMK to CHX + FasL-treated MSCs prevented PARP cleavage.

To confirm that FasL was activating the caspase 8 pathway, we examined expression of cellular caspase 8 FLICE like inhibitory protein (c-FLIP), which modulates the activity of caspase 8 by binding the Fas signaling complex and preventing the binding and activation of precaspase 8 to this complex. Upon treatment with FasL alone or CHX and FasL, FLIP levels were reduced in a time-dependent manner (Fig. 4C), consistent with reports in other cell types (32). Directly probing for caspase 8 by Ac IETD AFC demonstrated significant activation of caspase 8 after 8 h of treatment with FasL or CHX + FasL. EGF was not able to protect from caspase 8 activation (Fig. 4D). Similar increases with FasL alone/CHX + FasL/EGF + CHX + FasL was seen in caspase 3 activity. Although H_2O_2 displayed increased caspase 3 activity, the activated caspase 8 levels were not as high as either FasL alone or CHX + FasL, confirming that H_2O_2 and ROS has no role to play in activating the extrinsic death pathway.

Uncoupling Protein Expression Increases in Response to FasL-Induced Oxidative Stress

As cells upregulate mitochondrial UCPs as a feedback loop to attenuate increases in ROS (19,20,46,68), we first looked at basal expression levels of UCPs by qPCR and found that UCP2 was by and large the main form in these cells (Fig. 5A). This prompted us to examine the changes in UCP2 levels after treatment with FasL alone and CHX + FasL, the two conditions with which we saw distinct increase in ROS over the time course of 8 h. We found increases in UCP2 expression within 2 h of treatment in both these cases (Fig. 5B). In addition, we asked whether protective factors EGF or NAC when added to FasL or CHX + FasL changed UCP2 since these factors reduced ROS. Inclusion of NAC with FasL prevented the increased UCP2 expression; this correlated with the suppression of ROS. EGF also was able to suppress CHX + FasL-induced UCP2 expression for the first 4 h; again correlating with lessened ROS generation. As H_2O_2, the positive control, increased UCP2 expression as expected (Fig. 5B), we take this to suggest that the UCP2 upregulation is compensatory.

Since we earlier observed that high levels of ROS were established on FasL treatment after 4 h, we examined the differences in UCP2 expression across treatments at this time point. UCP2 expression was increased upon exposure to FasL alone and CHX + FasL. Addition of NAC to CHX + FasL-treated cells resulted in levels similar to untreated cells and much lower than cells treated with FasL alone as expected. The changes in protein were mirrored by changes in UCP2 transcript levels (Fig. 5D).

The trend of UCP2 increase was similar to the trend of ROS increase after 4 h (Fig. 5E). EGF added to FasL or to CHX + FasL at 4 h showed no significant decrease in UCP2 production compared to FasL alone or CHX + FasL. These results suggest that as ROS levels increase in MSCs, levels of UCP2 also increase as a compensatory mechanism to bring about attenuation of ROS and limit oxidative stress.

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Abrogation of UCP2 Leads to Higher Levels of Apoptosis in the Presence of FasL

Since FasL increased UCP2 expression and UCP2 is a negative regulator of ROS, we wanted to see if UCP2 either prevents ROS or delays ROS increase in MSCs. UCP2 levels were downregulated in imhMSCs using siRNA, and reduced levels were confirmed by an immunoblot for UCP2 (Fig. 6A); cells transfected with a control scrambled siRNA showed no downregulation of UCP2. Nontransfected imhMSCs, imhMSCs with downregulated UCP2, and imhMSCs transfected with scrambled RNA were treated with media alone, FasL, and CHX with FasL, and ROS generation was evaluated. Cells in which UCP2 was downregulated showed higher amounts of ROS compared to both nontransfected cells and cells with scrambled RNA, even in the absence of challenge. Cells with downregulated UCP2 showed greater amounts of ROS when further confronted with FasL alone or CHX + FasL (Fig. 6B). This suggested that UCP2 upregulation brought about by FasL reduces ROS levels in MSCs.

When we looked at the onset of apoptosis after 4 h of treatment with FasL, we saw imhMSCs downregulating UCP2 showing much higher levels of apoptosis and on treatment with CHX and FasL, MSCs with UCP2 siRNA with fragmented nuclei due to enhanced cell death (Fig. 6C). This led us to conclude that increases in UCP2 levels in the presence of FasL likely reduce ROS levels in MSCs and may delay the onset of apoptosis in MSCs, but that these compensatory changes are insufficient to alter ultimate outcome.

FasL-Induced ROS Activates Mitogen-Activated Protein Kinases and Alters Apoptotic Pathway Signaling

Studies suggest that oxidative stress and ROS activate the MAPK pathways including ERK, JNK, and p38 kinase (33,42). Moreover, studies in MSCs suggest that H_2O_2 induces phosphorylation of both JNK and p38 MAPK pathways leading to caspase 3 activation and cell death (38). We investigated if FasL-induced ROS was responsible for the activation of these MAPK pathways (Fig. 7). MSCs were treated with H_2O_2 alone as positive control or FasL alone or CHX and FasL over a time course of 8 h. In the presence of oxidative stressors (H_2O_2 or FasL), the MAPK pathways were activated, though the time courses varied, with some peaking prior to the full 8 h of observation. The addition of NAC to CHX and FasL-treated cells prevented the increases in phosphorylation of these pathways, suggesting that it is the oxidative stress aspects that trigger these signalings.

We next probed for specific pro- and antiapoptotic proteins of the mitochondrial death pathway, which might display changes in expression on FasL treatment and production of mitochondrial ROS. Survivin belongs to the inhibitor of apoptosis protein (IAP) family and plays a role in inhibiting mitochondrial apoptosis, inhibiting caspases by interacting with other IAPs and also modulates stress pathways by interacting with heat shock proteins (3). We observed a rapid reduction in expression of survivin on treatment with FasL alone or with CHX and FasL (Fig. 7D) displaying an onset of mitochondrial stress related apoptosis in MSCs, which can be linked to generation of mitochondrial ROS by FasL.

FasL also caused the activation of proapoptotic pathways in MSCs. Upon activation of stress pathways such as JNK and p38 MAPK, Bad becomes dephosphorylated and binds the antiapoptotic proteins Bcl-2 and Bcl-xl, displacing Bax from these proteins, causing the translocation of Bax to the mitochondrial membrane where it forms pores in the membrane, causes decrease in membrane potential and activates the intrinsic apoptotic pathway (74). With treatments of FasL alone or CHX and FasL, we saw decreased phosphorylation of Bad (Fig. 7E). Together these findings suggest a tip in the balance of signaling towards apoptosis.

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DISCUSSION

Considerable attention, including numerous clinical trials, has been devoted to MSCs due to the potential of using these cells to regenerate tissue. In addition, MSCs have been promoted as being immunoprivileged (23,72), making them ideal to be used not just by themselves to regenerate tissue but also with other implanted cells to suppress inflammation. However, tests have increasingly demonstrated that grafting of MSCs in animal models leads to inflammation and subsequent death of MSCs (2). MSC regenerative therapy has been curtailed by the failure of the cells to permanently engraft to form tissue. Threats to MSCs in the wound milieu are multiple: in addition to cytokines and reactive oxygen species in the microenvironment, hypoxia and serum starvation commonly seen during ischemia generate increased ROS. Hypoxia and serum starvation cause an increased expression of nicotinamide adenine dinucleotide (phosphate) [NAD(P)H] oxidase and reduced catalase, lowering cellular levels of the prosurvival protein survivin, causing increased Bcl-2/ Bax ratios, leading to MSC death (50). It is generally considered that nonspecific inflammation induced upon introduction of any new cell or material may contribute to the death. FasL is one such cytokine. FasL is also highly expressed on cytotoxic T lymphocytes and natural killer cells, both of which recently have been implicated to promote MSC death after an allogeneic transfer, while NK cells have been shown to promote MSC death on autologous transfer (17). Thus, we sought to understand the response of MSCs to FasL and ROS.

Extracellular ROS when applied to MSCs is known to reduce focal adhesions, induce caspase activation, and promote death by apoptosis. In addition, advanced glycation products seen in diabetic patients are shown to increase ROS in MSCs leading to reduced proliferation and migration of these cells (73). These studies suggest that MSCs may be susceptible to oxidative stress and damage. Our results, which suggest that MSCs promote their own death by increasing levels of ROS in the presence of inflammation, are in line with this hypothesis. We used FasL as a representative inflammatory cytokine since previous work has demonstrated MSC death in the presence of FasL (21,25). We were able to demonstrate that addition of an antioxidant, NAC (16), reduces FasL-induced ROS production in MSCs and is able to suppress MSC apoptosis. However, prolonged exposure to FasL causes MSCs to undergo apoptosis despite presence of NAC. This later death was due to activation of the extrinsic death pathway by FasL.

The general prosurvival growth factor EGF prevented FasL-induced ROS in MSCs and protected from apoptosis for a short time frame, after which the prodeath signals breakthrough. EGF has previously been shown to impair oxidative stress and protect cells from oxidative cell death in other cell types, and our results concur with these reports (11). EGF has also been shown to specifically protect from FasL-induced apoptosis in other cell types (24). However, the loss of protection offered by EGF is likely due to receptor internalization and degradation. We have reported that EGF causes a transient activation of downstream pathways due to EGFR internalization and degradation, which prevents it from protecting MSCs from FasL-induced cell death. However, restraining EGF to the cell surface, thus preventing the EGF-EGFR complex internalization, causes a more prolonged activation of downstream EGFR pathways involved in survival and offers a protective advantage to MSCs in the presence of FasL (21).

Uncoupling proteins, present on the inner mitochondrial membrane, contribute to the “uncoupling to survive” hypothesis wherein mitochondrial ROS is attenuated by partial uncoupling of the proton gradient while still maintaining ATP production. UCP1 (45), the first identified UCP, is mainly found in fat tissue and maintains thermogenesis. UCP2 is ubiquitously expressed, with the mRNA being expressed in all cells, but the protein restricted to only a few tissues. However, our MSCs by and large expressed only UCP2,

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which prompted us to look at changing expression of UCP2 with increases of ROS. UCP2 expression increased in a time-dependent manner with the addition of FasL or CHX + FasL, the pattern of increase following the increasing trend of ROS. Knocking down UCP2 in the presence of FasL or CHX + FasL increased ROS production and quickened the onset of apoptosis to just 4 h after treatment with FasL proving that UCP2 delayed ROS generation in MSCs. UCP2 has a very high turnover time, with a half-life of 1 h, which might explain rapid increases in protein expression on exposure of MSCs to FasL. Stem cells are thought to have low baseline ROS levels compared to other cell types due to relatively higher amounts of antioxidants glutathione and superoxide dismutase (69). Uncoupling proteins may be another such means by which MSCs under normal physiological conditions maintain their ROS levels.

Muscle-derived stem cells (MDSCs) display lower levels of oxidative stress-related death compared to their more differentiated phenotype, myoblasts (48). However, the reduced baseline ROS does not prevent MDSCs from undergoing cell death in the presence of oxidative stress. This has been shown in studies where pretreatment of MDSCs with NAC prior to implantation of cells in the heart increases their survival, while on the contrary addition of a pro-oxidant like diethyl maleate decreases survival (18). Our results concur with these reports and suggest that addition of NAC to MSCs at sites of inflammation will moderate ROS and increase the chance of MSC survival.

In an initial step of linking the Fas receptor to the death pathways, we examined the main MAPK species in MSCs, based on reports in other cell types of FasL activating ERK and JNK leading to oxidative damage (33). This prompted us to look at FasL-induced ROS activating the MAPK pathways. ROS generated in response to FasL in MSCs activated both the MAPK ERK and MAPK stress pathways: p38MAP kinase and JNK. While ERK has traditionally been considered as an anabolic MAPK pathway, supporting mitogenesis, motility, and survival, there have been several studies, which highlight ERK signaling in driving cell death (14,55). ROS activation of ERK is also implicated in leading to DNA damage (44). JNK and p38MAPK are canonical stress-activated pathways and have been implicated in activating apoptosis. JNK primarily activates mitochondrial apoptosis by modulating the Bcl2 family of proteins and activating Bcl2-associated X (Bax) and Bad (71). Our results show activation of Bad and a link of FasL-induced ROS stimulation of JNK to the mitochondrial death pathway. Activation of p38 MAPK by ROS on the other hand has been shown to kill and limit the population of hematopoietic stem cell in vivo (28).

FasL has previously been described to stimulate ROS in two waves: the first by activation of NADPH oxidase and nitric oxide (NO) synthase (5,7,64) and the second via induction of mitochondrial permeability transition (58). The first wave does not generally lead to caspase 3-mediated apoptosis; the second wave, however, helps in formation of the apoptosome, mitochondrial permeability transition, release of cytochrome *c* and apoptosis. Although we did not check in particular for these ROS waves produced in MSCs, our results tilt in the direction of the second wave being responsible for apoptosis in these stromal cells, since we describe the ROS generated by FasL to be mitochondrially derived. We also see activation of the mitochondrial death pathway in MSCs, which is the pathway activated by the second ROS wave.

In conclusion, we have demonstrated that MSCs are capable of enhancing their own demise in the presence of inflammation, which is a major hurdle in terms of MSC therapy. In order for translational therapy to be effective, we need to better understand ways in which MSC survival can be promoted. Use of antioxidants like NAC offers one such approach to increased survival. Our earlier reports and studies (65,57) herein suggest that prosurvival factors such as EGF may hold promise in protecting MSCs. However, while EGF appears

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protective early in the challenge but not over extended time periods, as noted herein, the abbreviated protection may be due to rapid internalization and downregulation of the few EGFR present on these cells (66). Thus, extended signaling ligands, including tethering (21,66) or low-affinity (29,30) EGFR ligands, may be useful in protecting implanted MSCs from para- and autocrine signaled apoptosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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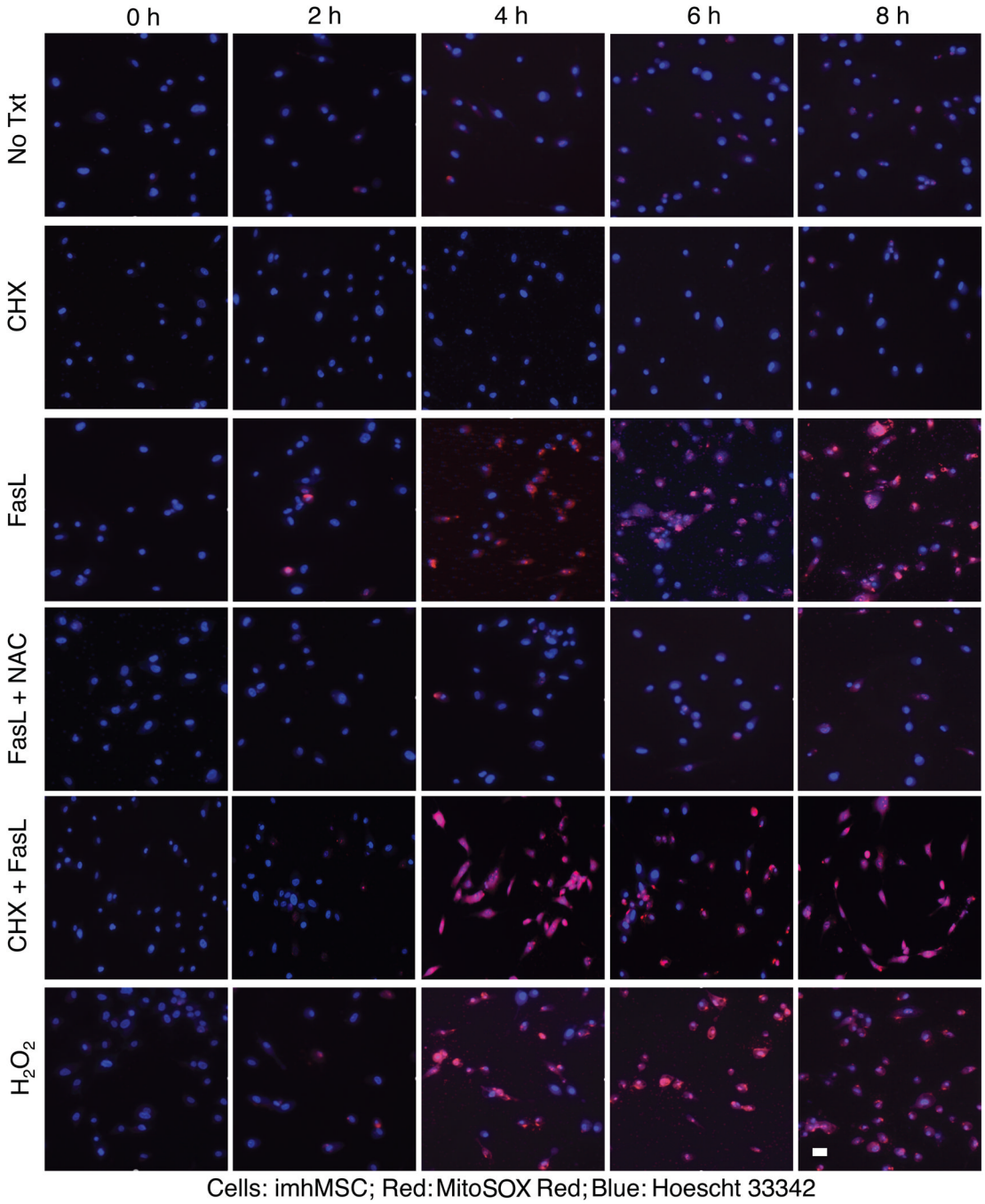

Figure 1.

FasL stimulates production of mitochondrial superoxide anion in MSCs. Immortalized human bone marrow stromal cells (imhMSCs) were treated with various factors and then tested for MitoSOX Red fluorescence at the indicated time points. MitoSOX Red fluorescence (red) is an indicator for mitochondrial superoxide generation. Shown are representative photomicrographs of cells, of three independent experiments. Original magnification, 20 ×. Scale bar: 10 μm. Not shown are similar experiments with primary human MSCs. Txt, transplant; CHX, cycloheximide; NAC, *N*-acetyl cysteine.

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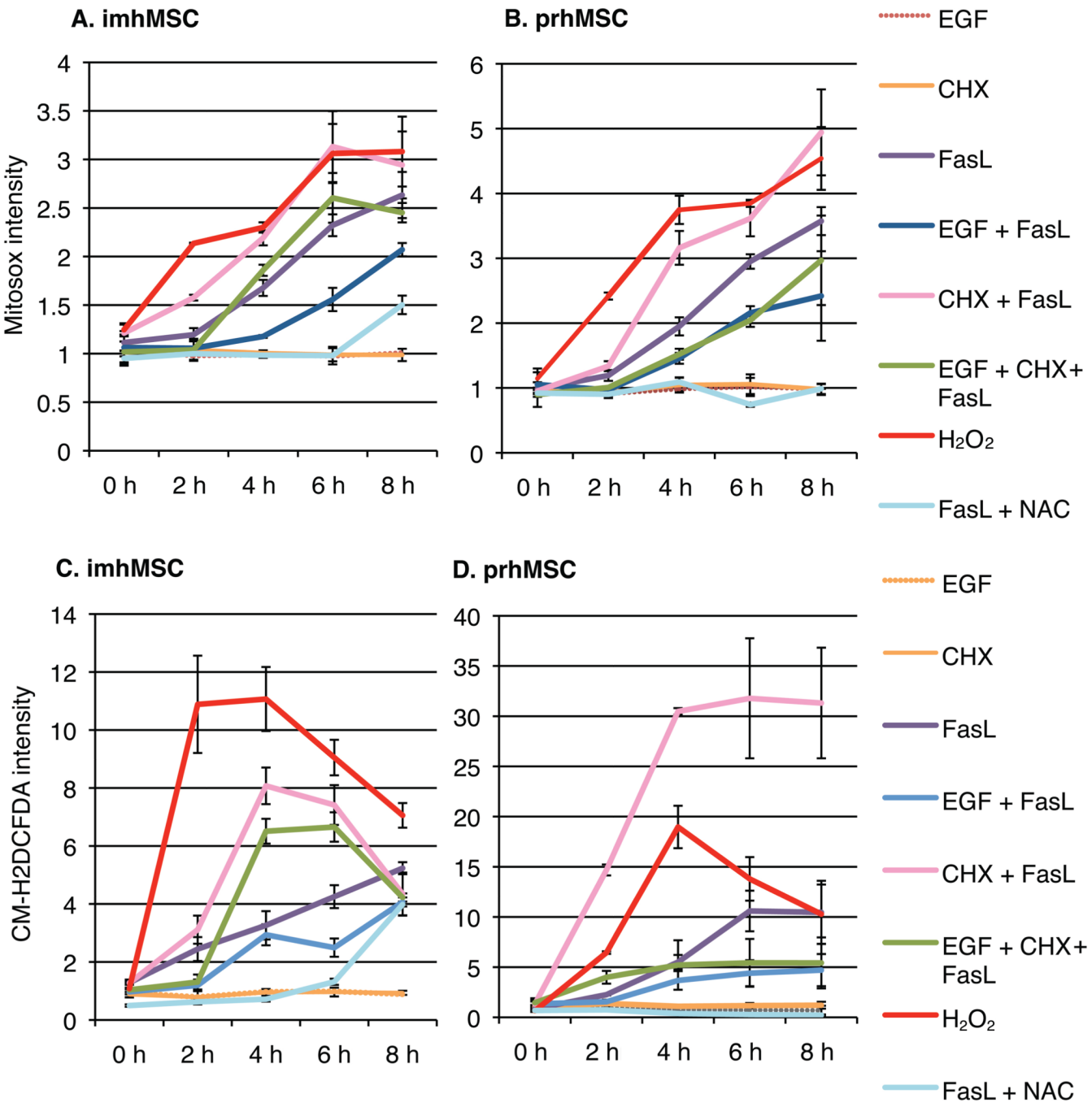

Figure 2.

FasL causes severe oxidative stress in MSCs. Both imhMSCs (A, C) and primary human marrow stromal cells (prhMSCs) (B, D) were treated and analyzed for superoxide generation as in Figure 1 (A, B) or hydroxyl radical generation (C, D). Superoxide anion generation was detected by MitoSOX Red fluorescence (A, B) and hydrogen peroxide and hydroxyl radical synthesis by CM-H_2DCFDA fluorescence (C, D). Shown are graphs of mean ± SEM of three independent experiments, each normalized to cell number and no treatment. EGF, epidermal growth factor.

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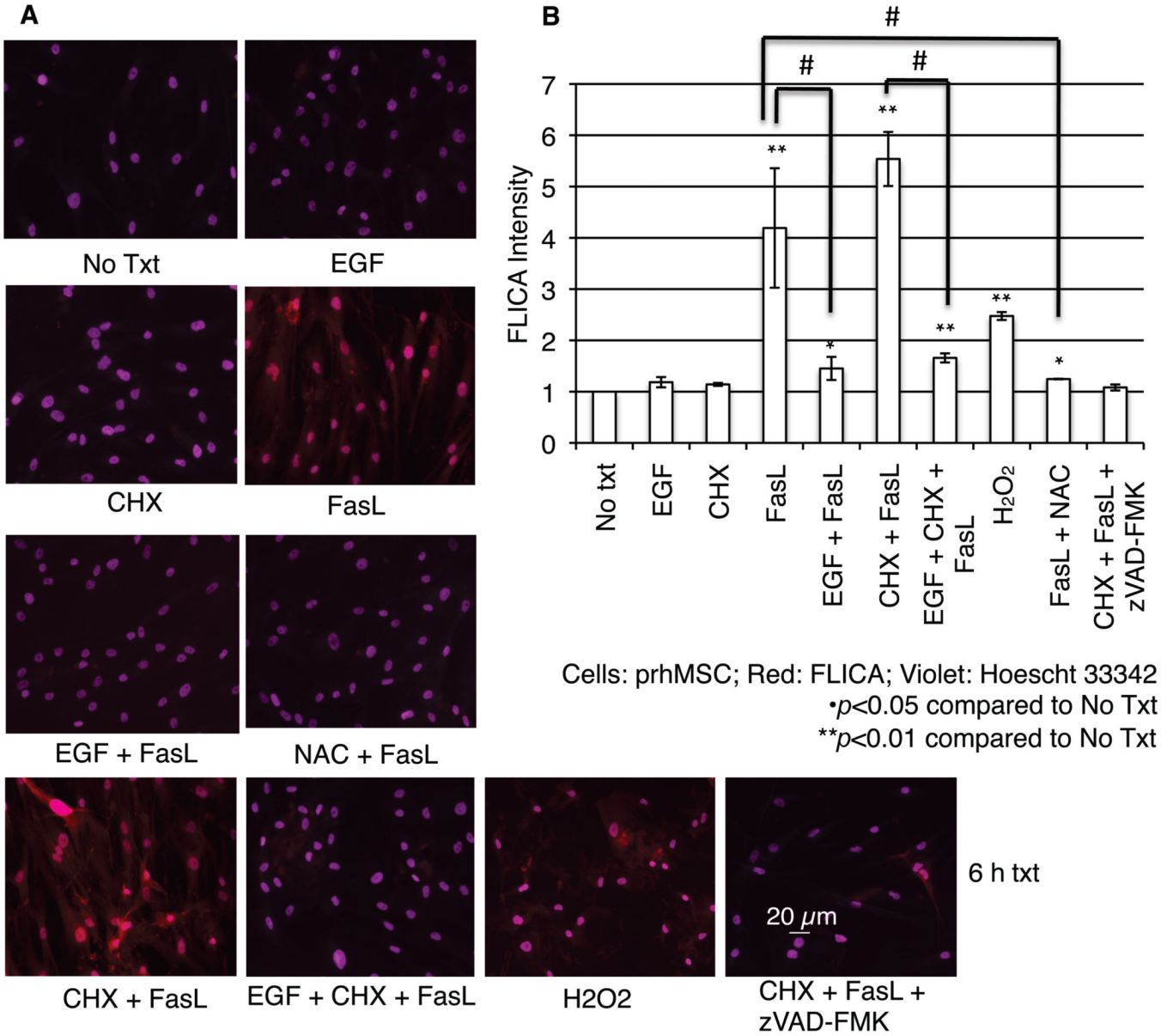

Figure 3.

FasL-induced reactive oxygen species (ROS) leads to apoptosis in MSCs. (A) prhMSCs were treated with the indicated agents for 6 h and tested for caspase 3 activation by fluorochrome inhibitor of caspase assay (FLICA). The red fluorescence represents FLICA, the violet fluorescence is Hoescht 33342 stain for nuclei. Shown are representative fields. The fluorescence is quantified in (B) with $*p < 0.05$ and represents mean ± SEM of three independent assays each viewing multiple fields.

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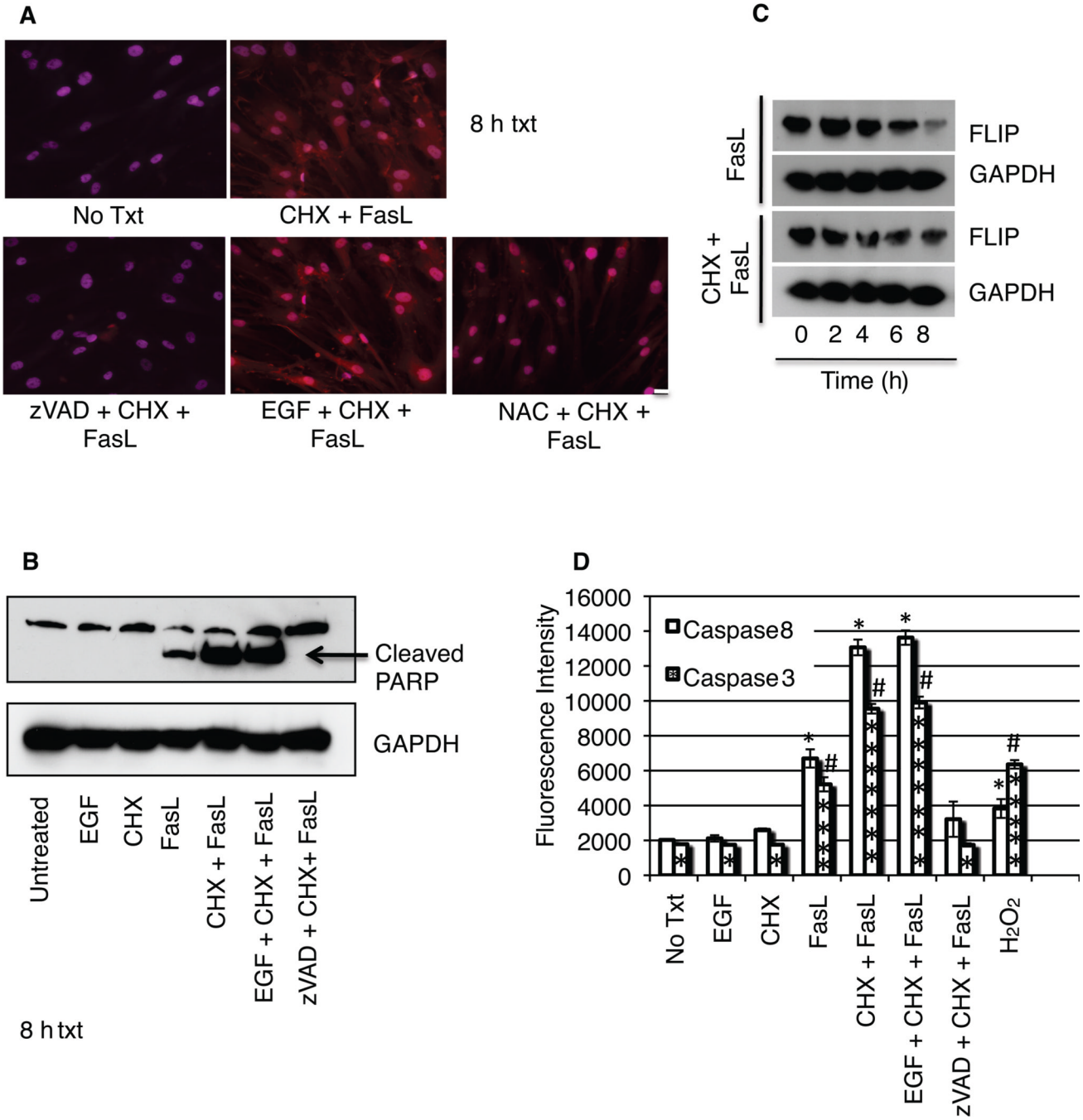

Figure 4.

FasL kills MSCs via the extrinsic pathway independent of ROS. (A) prhMSCs were treated with the indicated agents for 8 h and tested for caspase 3 activation by FLICA. Original magnification, 20 ×. Scale bar: 20 μm. Shown are representative fields. (B) imhMSCs were treated with the indicated agents for 8 h and tested for apoptosis by full length and cleaved poly (ADP-ribose) polymerase (PARP) immunoblot. The immunoblots are representative of at least three experiments. (C) Levels of cellular caspase 8 (FLICE)-like inhibitory protein2 (c-FLIP) in imhMSCs assessed by immunoblot after treatment with indicated agents. The immunoblots are representative of at least two experiments. (D) Caspase 8 and caspase 3 activity in imhMSCs by Ac IETD AFC and Ac DEVD AFC detection, after 8 h of treatment

with indicated agents. The *y*-axis represents emission at 505 nm and is mean ± SEM of two independent assays. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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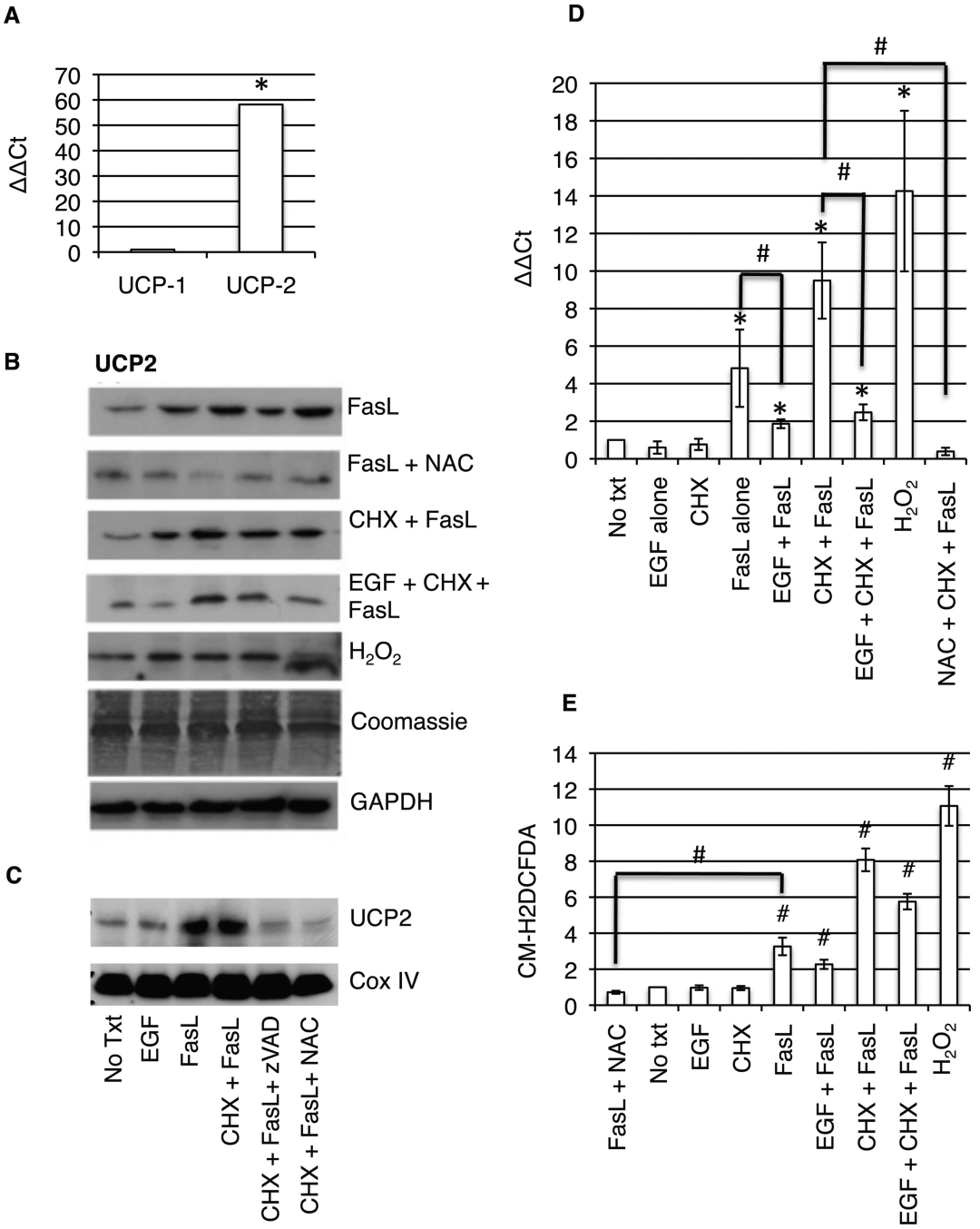

Figure 5.

FasL exposure leads to an increase in UCP2 expression. (A) Levels of uncoupling protein (UCP) mRNA in imhMSCs were assessed by quantitative RT-PCR. (B) Cells were treated with the indicated agents and then UCP2 protein level assessed at indicated times. (C) Immunoblotting after 4 h of treatment to show direct comparison of UCP2 levels. (D) qPCR analysis of UCP2 transcripts after 4 h of treatment. Cycle times are compared to GAPDH and further normalized to no treatment condition. *$p < 0.05$ compared to no treatment. #$p < 0.05$. (E) Changes in CM-H_2DCFDA fluorescence in imhMSC after 4 h of treatment. *$p < 0.05$ compared to no treatment. #$p < 0.05$. The immunoblots are representative of at least

two experiments, and the graphs are mean ± SD (A) or SEM (D, E) of three independent experiments. CoxIV, cytochrome oxidase IV.

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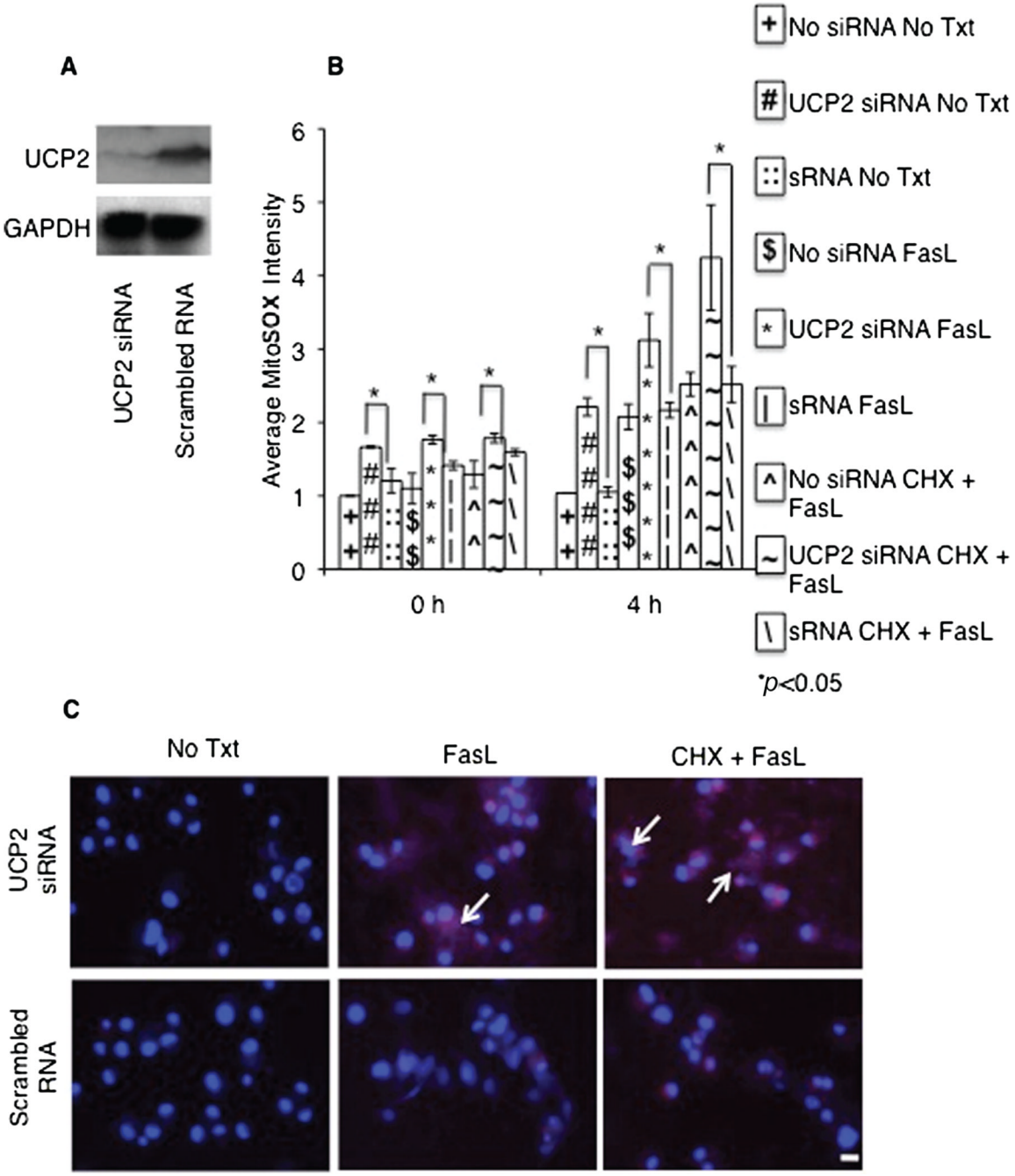

Figure 6.

UCP2 moderates ROS increase in MSCs upon exposure to FasL. (A) UCP2 levels were downregulated upon transfection of UCP2 siRNA compared with scrambled RNA. (B) Levels of ROS as assessed by average MitoSOX intensity in untreated MSCs, MSCs with downregulated UCP2, or MSCs with scrambled RNA (sRNA) with and without FasL or CHX and FasL. (C) Onset of apoptosis in MSCs with downregulated UCP2 4 h after treatment with FasL. The immunoblot (A), graph (B), and micrographs (C) are one of two replicates (*$p < 0.05$). Original magnification, 20×. Scale bar: 10 μm. White arrows indicate dying cells.

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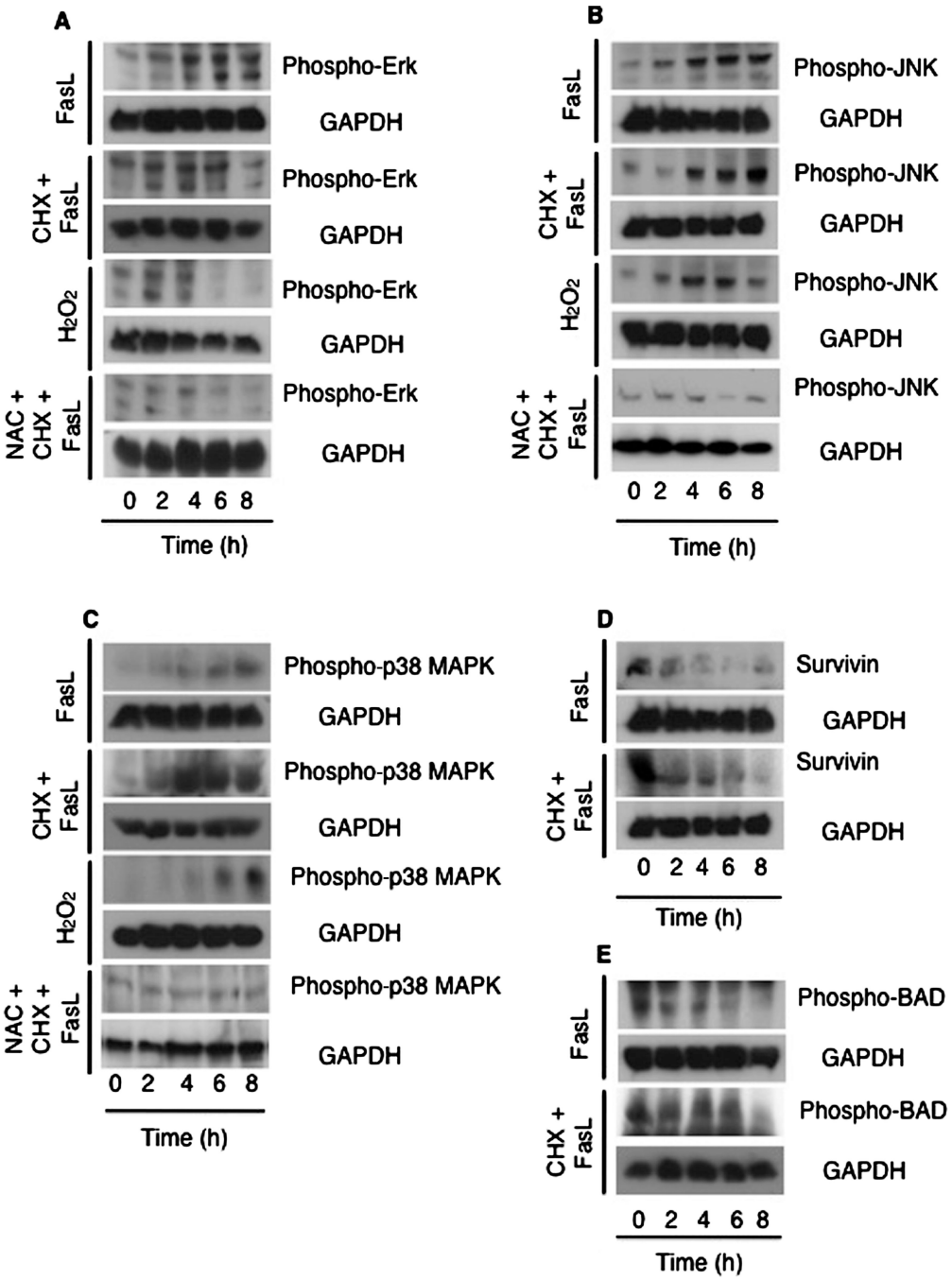

Figure 7.

FasL-induced ROS activates the mitogen activated protein kinase (MAPK) pathways in MSCs and proteins of the mitochondrial death pathway (A) expression of phospho-extracellular signal-regulated kinase (ERK), (B) phospho-c-Jun N-terminal kinase (JNK), (C) phospho-p38 MAPK, (D) survivin, and (E) phospho-B-cell CLL/lymphoma 2 (bcl2) antagonist of cell death (Bad) by immunoblot after treatment with indicated agents and times. The immunoblots are representative of at least two experiments.