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ORIGINAL ARTICLE

Endoplasmic reticulum stress response is activated in pulmonary hypoplasia secondary to congenital diaphragmatic hernia, but is decreased by administration of amniotic fluid stem cells

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Abstract

Purpose Pulmonary hypoplasia secondary to congenital diaphragmatic hernia (CDH) is characterized by impaired epithelial homeostasis. Recently, amniotic fluid stem cells (AFSCs) have been shown to promote growth in hypoplastic lungs of rat fetuses with CDH. Herein, we investigated whether CDH hypoplastic lungs mount an endoplasmic reticulum (ER) stress response and whether AFSCs could re-establish pulmonary epithelial homeostasis.

Methods Primary epithelial cells were isolated from fetal rat lungs at E14.5 from control and nitrofen-exposed dams at E9.5. Nitrofen-exposed epithelial cells were grown in medium alone or co-cultured with AFSCs. Epithelial cell cultures were compared for apoptosis (TUNEL), cytotoxicity (LIVE/DEAD assay), proliferation (5′EdU), and ER stress (CHOP, Bcl-2) using one-way ANOVA (Dunn's post-test).

Results Compared to control, nitrofen-exposed epithelial cells had increased cytotoxicity and apoptosis, reduced proliferation, and activated ER stress. AFSCs restored apoptosis, proliferation, and ER stress back to control levels, and significantly reduced cytotoxicity.

Conclusions This study shows for the first time that ER stress-induced apoptosis is activated in the pulmonary epithelium of hypoplastic lungs from fetuses with CDH. AFSC treatment restores epithelial cellular homeostasis by attenuating the ER stress response and apoptosis, by increasing proliferation and migration ability, and by reducing cytotoxicity.

Keywords CDH · Nitrofen · ER stress · Cellular homeostasis · Regenerative medicine

Introduction

Congenital diaphragmatic hernia (CDH) is a birth defect that affects 1 in 3000 live births and that is still burdened by high mortality and morbidity rates, despite extensive research and critical care advancements [1, 2]. The primary determinant for the poor outcome of babies with CDH is considered to be pulmonary hypoplasia (PH) [3]. PH is characterized by abnormally small lungs with a reduced number of bronchiolar divisions to the level of the terminal bronchioles and a marked reduction and enlargement of the airspaces [4]. In the past, the mechanical compression of the abdominal organs herniated through the diaphragmatic defect was traditionally thought to be the cause of PH [5]. However, there is evidence that PH in fetuses with CDH originates before visceral herniation and that lung development in these fetuses is intrinsically delayed by undetermined factors [5]. One of the observations that support this concept is the altered cellular homeostasis in the pulmonary epithelium of CDH lungs, with a disrupted interplay between cell proliferation and apoptosis [6].

One of the conserved protective mechanisms that maintain cellular homeostasis is the endoplasmic reticulum (ER) stress response [7, 8]. The ER is an organelle that regulates protein folding and lipid synthesis. Under stressful conditions, the ER accumulates misfolded proteins and activates the ER stress response, which, if prolonged, induces

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apoptosis [7]. It is known that alveolar epithelial cells exhibit an ER stress response in the lung of infants with bronchopulmonary dysplasia [9, 10], a neonatal condition characterized by underdeveloped lungs similar to those of babies with PH. However, to the best of our knowledge, the ER stress response has not been investigated in hypoplastic lungs of CDH fetuses.

In search for novel strategies that would improve lung maturation and growth in babies with CDH, researchers have investigated the use of in utero stem cell therapy [11–13]. Interestingly, these studies showed that amniotic fluid stem cells (AFSCs) promoted lung regeneration in in vitro and in vivo nitrofen-based models of CDH via an undetermined paracrine mechanism. This is in line with previous studies reporting that AFSC have regenerative potential in the damaged lung [14], are able to differentiate into epithelial lung lineages, and exert a beneficial effect by reducing lung fibrosis [15] and repairing damaged type 2 pneumocytes [16]. However, the mechanism behind the AFSC beneficial effect on hypoplastic lungs of fetuses with CDH remains incompletely understood.

In this study, we aimed to investigate whether the lack of homeostasis described in hypoplastic lungs of fetuses with experimental CDH was associated with an activation of an ER stress response. Moreover, we explored whether AFSCs could re-establish the pulmonary epithelial homeostasis and the potential mechanism through which this could occur.

Methods

Cell culture and treatment groups

Following ethical approval [AUP #39168], pregnant Sprague-Dawley rats at E9.5 were randomly assigned to be gavaged nitrofen (2,4-dichloro-4-nitrophenyl ether, Sigma Aldrich, Missouri, USA) (100 mg in 1 ml olive oil) to induce CDH and PH in the litter, or olive oil (control). Fetal rat lungs were dissected at E14.5 from nitrofen-exposed and non-exposed dams. Pulmonary epithelial cells were isolated and cultured in minimal essential medium (Thermo Fisher Scientific, Massachusetts, USA) supplemented with 10% FBS (Wisent Bioproducts, Quebec, Canada) in a humidified incubator at 37 °C as previously described [17]. Epithelial cells were checked daily for proper morphology and confirmed to be positive for EpCAM + and negative for vimentin via immunofluorescence staining assays. After 8–10 days of cell culture, nitrofen-exposed epithelial cells were divided into the AFSC co-culture treatment group (nitrofen + AFSC group) and the non-treatment group (nitrofen group). The nitrofen + AFSC group was co-culture with rat AFSCs by seeding 500 cells/well in the top compartment of the transwell for 24 h. Control and untreated nitrofen pulmonary epithelial cells were incubated with fresh media only (minimal essential medium supplemented with 10% FBS) for 24 h. Experiments followed immediately after the 24 h incubation for each treatment group.

Cell proliferation assay

Proliferation was measured using the Click-iT® EdU Imaging Kit (Thermo Fisher Scientific, Massachusetts, USA) following supplier-recommended protocol. Briefly, EdU (10 μM final concentration) was added directly into the medium of each well for 3 h at 37 °C. Cells were fixed in 3.7% paraformaldehyde (Sigma Aldrich, Missouri, USA) at room temperature protected from light for 15 min, then washed with 3% bovine albumin serum (Sigma Aldrich, Missouri, USA) in PBS. Cells were subsequently permeabilized with 0.25% Triton X-100 (Sigma Aldrich, Missouri, USA) in PBS for 15 min at room temperature. After cell permeabilization, each well was incubated with the azide-conjugated AlexaFluor®488 cocktail mixture (Thermo Fisher Scientific, Massachusetts, USA) for 30 min at room temperature protected from light. Cells were then washed with PBS and counterstained with Hoechst 33342 (Thermo Fisher Scientific, Massachusetts, USA) (1:2000 in PBS) for 10 min at room temperature protected from light. Immunofluorescence imaging was conducted using an inverted epifluorescence microscope (Leica DMI6000B, Leica Microsystems, Wetzlar, Germany) with the appropriate filters.

LIVE/DEAD® cytotoxicity assay

Cytotoxicity was measured using the LIVE/DEAD® assay (Thermo Fisher Scientific, Massachusetts, USA) which allowed simultaneous detection of live (calcein+) and dead (ethidium+) cells. After experimental treatment for each group, 20 μL of calcein/ethidium mixture (1 μM and 3 μM final concentration, respectively) was added directly into each well and incubated for 20 min at 37 °C. Following incubation, the cells were imaged using an inverted epifluorescence microscope (Leica DMI6000B, Leica Microsystems, Wetzlar, Germany) with appropriate filters.

Cell apoptosis assay

In order to label cells with fragmented DNA, a marker of late apoptosis, a TUNEL assay was performed (Click-iT™ Plus TUNEL Assay) using AlexaFluor®594 picolyl azide (Thermo Fisher Scientific, Massachusetts, USA). Cells were fixed in 3.7% paraformaldehyde and permeabilized with 0.25% Triton X-100 in PBS both at room temperature. Cells were incubated with the TUNEL TdT enzyme mixture at 37 °C on a rocker for 3 h to allow EdUTP incorporation into dsDNA breaks. Cells were incubated once more at

37 °C on a rocker for 1.5 h with a Click-iT AlexaFluor®594 cocktail mixture to detect incorporation of EdUTPs. After incubation, cells were washed with PBS, counterstained with Hoechst 33342 (1:2000 in PBS) (Thermo Fisher Scientific, Massachusetts, USA), and imaged using an inverted epifluorescence microscope (Leica DMI6000B, Leica Microsystems, Wetzlar, Germany) with the appropriate filters.

Wound healing assay

The wound healing assay was used to measure cell migration by monitoring the closure rate over a 24-h period. Primary epithelial cells were grown for 8–10 days to reach 90% confluency density. Cells were incubated with Hoechst 33342 (1:2000 in PBS) for 10 min at room temperature. An artificial wound was made by scratching the well once with a sterile pipette tip. Immunofluorescence imaging was conducted at 0, 12 and 24 h using an inverted epifluorescence microscope (Leica DMI6000B, Leica Microsystems, Wetzlar, Germany) with the appropriate filters. Hoechst 33342 was added before imaging at each time point to ensure that new unstained cells are tracked throughout the 24-h period.

RNA isolation and cDNA synthesis for qRT-PCR experiments

RNA was extracted from primary epithelial cells using Trizol reagent (Thermo Fisher Scientific, Massachusetts, USA) following supplier recommended protocols. RNA was quantified using NanoDrop spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). SuperScript® VILO™ (Thermo Fisher Scientific, Massachusetts, USA) kit was used to synthesized cDNA for each sample group. The resulting cDNA was used for qRT-PCR using SYBR green qPCR Master Mix (Wisent Bioproducts, Quebec, Canada) on a Viia7 real-time PCR machine (Thermo Fisher Scientific, Massachusetts, USA). The primer sequences for the targets include: GAPDH (forward 5′-GGGTGTGAACCACGAGAAAT-3′, reverse 5′-ACTGTGGTCATGAGCCCTTC-3′), CHOP (forward 5′-CTCCCAGAGCCCTCACTCTC-3′, reverse 5′-TGCTTGAGCCGTTCATTCTC-3′) and Bcl-2 (forward 5′-GCCGTCGAGCCGATGAAATA-’3, reverse 5′-GGTTGGCAAGGCGTGATCTA-3′). The relative expression levels of CHOP and Bcl-2 mRNA was determined by comparing the ΔCt to the mean ΔCt of the control group.

Statistical analysis

Data are reported as mean ± standard deviation. One-way ANOVA with Tukey post-test was applied for comparing three or more treated groups. Differences among groups was considered significant when $p < 0.05$. All statistical analyses were produced using GraphPad PRISM software version 6.

Results

The pulmonary epithelium of CDH lungs has impaired cellular homeostasis

Compared to control, the primary epithelial cells from nitrofen-exposed lungs had a significant lower proliferation rate (17 ± 21% vs. 3 ± 4%; $p < 0.01$; Fig. 1a), higher cytotoxicity rate (4 ± 6% vs. 17 ± 10%; $p < 0.0001$; Fig. 1b), and higher apoptosis rate (6 ± 4% vs. 10 ± 4%; $p < 0.01$; Fig. 1c). Moreover, pulmonary epithelial cell migration was significantly slower in nitrofen-exposed cells over the 0–12 h ($p < 0.0001$) and 0–24 h ($p < 0.0001$) period compared to control (Fig. 1d–f).

ER stress is activated in the pulmonary epithelium of CDH hypoplastic lungs

The expression levels of CHOP, a regulator and marker of ER stress [7], were significantly increased in the nitrofen-exposed pulmonary epithelium compared to control ($p < 0.0001$; Fig. 2a). Moreover, the mRNA expression levels of Bcl-2, a mediator of ER stress-induced apoptosis [7], were significantly decreased in epithelial cells exposed to nitrofen compared to control ($p < 0.01$; Fig. 2b).

AFSC treatment restores epithelial cellular homeostasis and reduces ER stress

Compared to untreated nitrofen-exposed pulmonary epithelial cells, pulmonary epithelial cells co-cultured with AFSC had a significantly higher proliferation rate (3 ± 4% vs. 11 ± 12%; $p < 0.05$; Fig. 1a), lower cytotoxicity (17 ± 10% vs. 9 ± 6%; $p < 0.01$; Fig. 1b), and a reduced apoptosis rate (10 ± 4% vs. 5 ± 3%; $p < 0.001$; Fig. 1c). AFSC treatment rescued pulmonary epithelial cell proliferation, apoptosis, and migration levels back to those of control epithelium (p = ns; Fig. 1). Moreover, AFSC treatment significantly decreased the expression levels of CHOP ($p < 0.0001$) and increased the expression of Bcl-2 ($p < 0.05$) compared to untreated nitrofen-exposed primary epithelial cells (Fig. 2a, b) and rescued them back to control levels.

Discussion

In the present study, we first confirmed that the pulmonary epithelium of rat fetuses with PH and CDH experiences an alteration in its cellular homeostasis, which we have shown

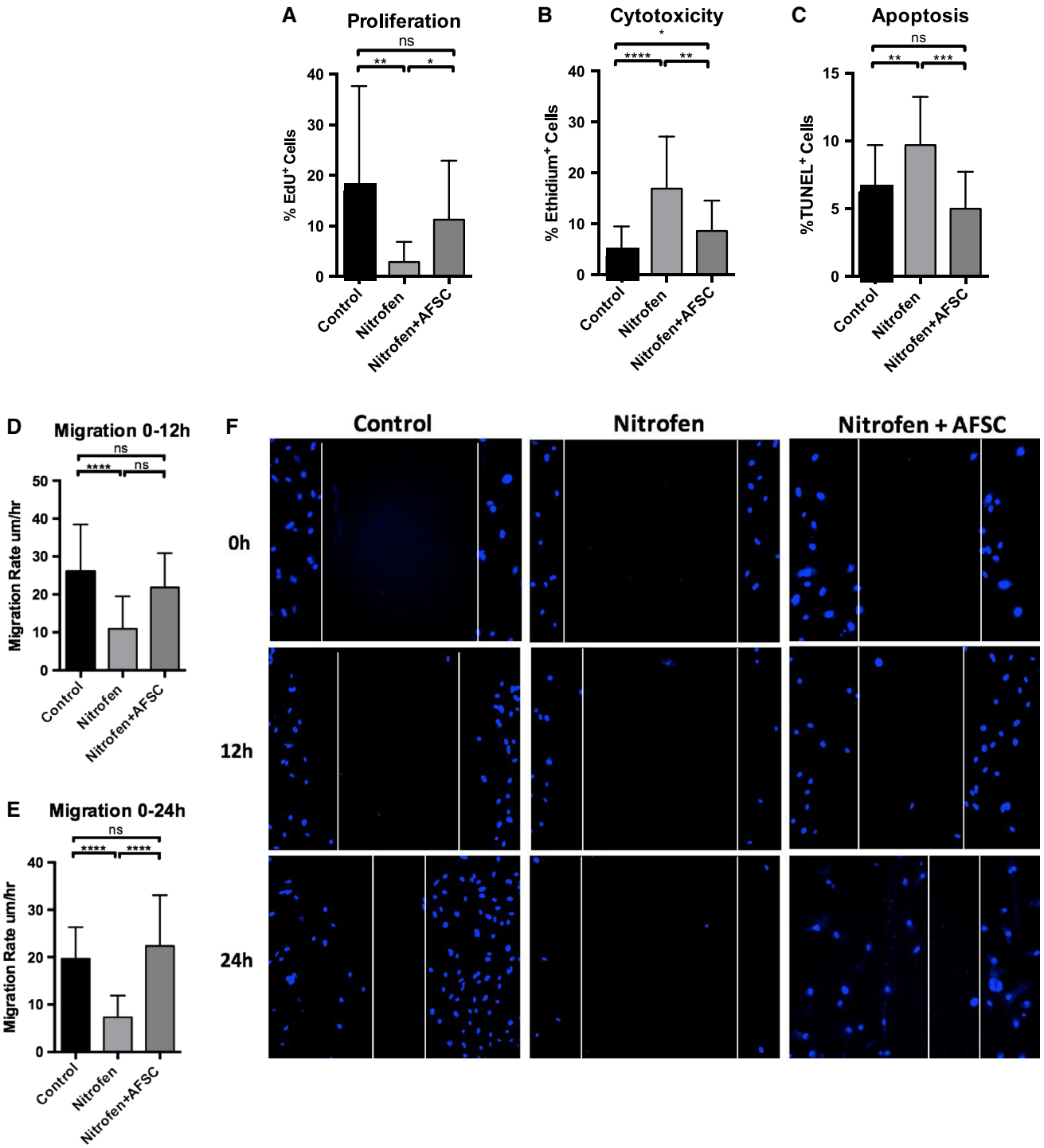

Fig. 1 Cellular homeostasis is impaired in primary lung epithelial cells in the nitrofen model of CDH. Three groups were compared: control, nitrofen-exposed epithelial cells, and nitrofen-exposed epithelial cells co-cultured with AFSCs. Cellular homeostasis was assessed by measuring: **a** proliferation rate (% EdU^+ cells), **b** cytotoxicity (% ethidium stained cells), **c** apoptosis rate using the TUNEL assay, and **d**–**f** migration over the total 24-h period. Data are reported as mean ± standard deviation. p values are represented as asterisks, where ns: $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$

for the first time to be associated with an ER stress response. Moreover, we have shown that administration of AFSCs exerts a beneficial effect on the pulmonary epithelium of hypoplastic lungs in part via an attenuation of the ER stress response, which AFSCs revert back to normal control levels.

Several studies using the nitrofen-based model of CDH have shown that hypoplastic lungs have impaired epithelial

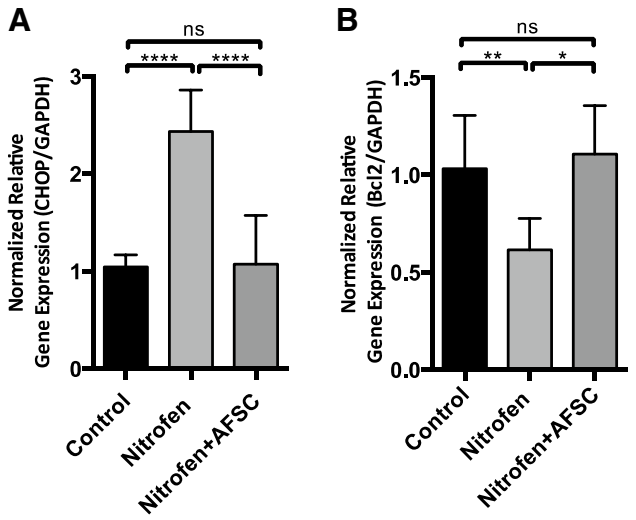

Fig. 2 qRT-PCR shows an activated ER stress response in nitrofen-exposed lung epithelial cells, but is attenuated upon administration of AFSCs. Relative normalized gene expression of **a** CHOP and **b** Bcl-2 is reported as mean ± standard deviation. *p* values are represented as asterisks, where ns: $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$

cell proliferation, especially at the branching terminal lung buds [6, 18–20]. Nitrofen has been reported to also induce cytotoxicity and apoptosis on different cell populations [6, 21–24]. While it has been clearly reported that mesenchymal fibroblasts from hypoplastic lungs of rat fetuses with CDH suffer from impaired cell proliferation and apoptosis [6], the activation of an uncontrolled apoptosis process in the pulmonary epithelium has remained controversial [5, 6, 18, 24]. In the present study, we intentionally focused on the pulmonary epithelium of hypoplastic lungs, by deriving epithelial primary cells from fetal lungs and growing them in cell culture. Prior to the present study, only one other group had focused on the pulmonary epithelium in vitro and on its outcome following nitrofen administration [24]. However, that study used a human cell line of alveolar type II cells (A549 cells), and not rat cells derived from the CDH model [24]. Nonetheless, the results of our study are in line with those of the study from Tong et al. and have clearly demonstrated for the first time higher levels of apoptosis in the nitrofen-exposed pulmonary epithelium. Moreover, by investigating the ER stress response in the pulmonary epithelium, we were able to demonstrate that hypoplastic lungs undergo mitochondria-mediated apoptosis. Similar to the study from Tong et al. we also demonstrated that nitrofen induces caspase-independent apoptosis in the pulmonary epithelium. In fact, in our study, we found that nitrofen-exposed pulmonary epithelium has lower expression levels of Bcl-2, an anti-apoptotic protein that is responsible for maintaining the integrity of the mitochondrial outer membrane and cytochrome *c* release [25, 26].

In the present study, we have shown for the first time that an ER stress response is activated in the epithelium from the hypoplastic lungs of rat fetuses exposed to nitrofen. To study ER stress, we investigated the levels of CHOP, a key transcriptional marker of ER stress activation [7]. Since the promoter of the CHOP gene can be stimulated by all three major ER stress response pathways, CHOP is one of the first transcription factors to be transcribed when there is an accumulation of misfolded proteins in the ER [7]. However, the influence of CHOP on pro-apoptotic proteins can be overcome by the activation of the pro-survival Bcl-2 protein [7, 27]. In our study, after 24 h treatment, the primary epithelial cells from nitrofen-exposed lungs experienced an increase in apoptosis, where CHOP gene expression was increased and the pro-survival Bcl-2 expression was decreased. Moreover, along with an activated ER stress response, primary epithelial cells from nitrofen-exposed fetal lungs experienced a reduction in proliferation, migration, and an increase in cytotoxicity. This associated imbalance in pulmonary epithelial cellular homeostasis with an activated ER stress response indicates that epithelial cell dysfunction is intrinsically present in PH.

In addition, we have also shown that AFSCs can rescue the damaged pulmonary epithelium from hypoplastic lungs by restoring cellular homeostasis. In particular, we have observed that AFSCs restore epithelial cell proliferation, apoptosis, and migration rates back to normal levels. Moreover, AFSC-treated epithelial cells had levels of apoptosis and of ER stress similar to those of epithelial cells from normal lungs. The attenuation of the ER stress response is similar to that obtained with the administration of mesenchymal stem cells (MSCs) in a bleomycin-induced model of idiopathic pulmonary fibrosis [28]. In this study, MSCs were able to ameliorate lung fibrosis by re-establishing the homeostasis in a similar fashion to that of AFSCs in our study.

It is still unclear how AFSCs exert their beneficial effects, but they act in a paracrine manner, as evidenced by co-culturing experiments in transwells. For a long time, the regenerative paradigm of AFSCs, as well as of other stem cell populations, was based on the assumption that stem cells play a critical role in tissue repair by means of their plasticity and differentiation potential. However, several studies have reported a relatively low degree of AFSC integration in the lung or in other organs, such as the intestine and the kidney, despite their potent regenerative effect [14, 29, 30]. Therefore, the AFSC beneficial effect has been ascribed to their paracrine modulatory effect, whereby biomolecules synthesized by AFSCs and present in their conditioned medium are more important than the differentiation of AFSCs in eliciting functional tissue repair. Previous studies on models of wound healing, limb ischemia–reperfusion, and acute hepatic failure have documented that AFSC-conditioned medium is a source of factors that promote tissue repair,

modulate local and systemic inflammation, and have pro-angiogenic regenerative effects [31–33]. There are ongoing studies aimed at identifying the factors secreted by AFSCs that are responsible for lung tissue regeneration.

In conclusion, the present study demonstrates for the first time that ER stress-induced apoptosis is activated in the pulmonary epithelium of hypoplastic lungs from fetuses with CDH. This phenomenon is responsible for the impairment in cellular homeostasis during PH. Moreover, we have demonstrated that AFSC treatment restores epithelial cellular homeostasis by attenuating the ER stress response and apoptosis, by increasing proliferation and migration ability, and by reducing cytotoxicity. The present study confirms that AFSCs exert a beneficial effect via a paracrine mechanism, whose effectors remain unknown. Nonetheless, this novel regenerative approach with a stem cell-based therapy could be a promising avenue for the treatment of hypoplastic lungs in fetuses with CDH.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Ethical approval for experiments conducted AUP #39168.

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