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Amniotic fluid derived mesenchymal stromal cells augment fetal lung growth in a nitrofen explant model

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ABSTRACT

Purpose: Recent experimental work suggests the therapeutic role of mesenchymal stromal cells (MSCs) during lung morphogenesis. The purpose of this study was to investigate the potential paracrine effects of amniotic fluid-derived MSCs (AF-MSCs) on fetal lung growth in a nitrofen explant model.
Methods: Pregnant Sprague–Dawley dams were gavage fed nitrofen on gestational day 9.5 (E9.5). E14.5 lung explants were subsequently harvested and cultured ex vivo for three days on filter membranes in conditioned media from rat AF-MSCs isolated from control (*AF-Ctr*) or nitrofen-exposed (*AF-Nitro*) dams. The lungs were analyzed morphometrically and by quantitative gene expression.
Results: Although there were no significant differences in total lung surface area among hypoplastic lungs, there were significant increases in terminal budding among E14.5+3 nitrofen explants exposed to *AF-Ctr* compared to explants exposed to medium alone (58.8±8.4 vs. 39.0±10.0 terminal buds, respectively; $p<0.05$). In contrast, lungs cultured in *AF-Nitro* medium failed to augment terminal budding. Nitrofen explants exposed to *AF-Ctr* showed significant upregulation of surfactant protein C to levels observed in normal fetal lungs.
Conclusions: AF-MSCs can augment branching morphogenesis and lung epithelial maturation in a fetal explant model of pulmonary hypoplasia. Cell therapy using donor-derived AF-MSCs may represent a novel strategy for the treatment of fetal congenital diaphragmatic hernia.

Pulmonary hypoplasia and pulmonary hypertension continue to be major problems in neonates with congenital diaphragmatic hernia (CDH). Despite ongoing advances in state-of-the-art care, including fetal tracheal occlusion, extracorporeal membrane oxygenation, and permissive hypercapnia, mortality rates for infants with CDH remain in excess of 30% [1–4]. Among survivors, the pulmonary morbidity associated with this disease can be quite significant, as validated by the spawning of multidisciplinary clinics involving surgeons, pulmonologists, and other specialists at major children's hospitals nationwide [5]. Novel treatments, aimed at better facilitating fetal and/or postnatal lung growth, are needed if further inroads are to be made in the management of this debilitating disease.

Over the past decade, stem cell-based approaches have been explored as an innovative strategy towards understanding lung regeneration in a variety of pediatric lung disorders [6–8]. In particular, recent experimental work suggests that an underlying disruption of stem cells within the lung mesenchyme, as opposed to lung epithelia itself, may be a major initiator of lung hypoplasia in CDH and other diseases such as bronchopulmonary dysplasia (BPD) [9]. In parallel with these findings, several investigators have demonstrated the potential therapeutic role of exogenously delivered mesenchymal stromal cells (MSCs) derived from either bone marrow or umbilical cord on lung regeneration in pediatric disease models [10,11]. The mechanisms involved in the observed lung repair by MSCs have not been well elucidated but may involve paracrine modulation of inflammatory responses by growth factors, possibly with activation of adjacent lung epithelial progenitors and endothelial cells [12].

Our laboratory, among others, has focused on the potential role of cells normally present within the amniotic fluid during organogenesis [13–15]. The amniotic fluid is known to contain a heterogeneous population of MSCs originally derived from placenta, lung, skin, and other organs [16–20]. Although these amniotic fluid-derived MSCs (AF-MSCs) are largely CD117-negative and do not have three germ layer differentiation potential [21], they express selected markers of pluripotency, are easy to isolate across a wide range of gestational ages, and have been shown to exert paracrine effects including the acceleration of fetal wound healing [21–24]. Moreover, compared to postnatal bone marrow MSCs, fetal derived MSCs express higher levels of stimulatory pulmonary morphogens, including hepatocyte growth factor (HGF), a heterodimeric heparin-binding growth factor regarded as a major initiator of normal lung organogenesis [19,25]. To date, little is known about the possible salutary effects of AF-MSCs on lung development in both normal and pathological states. In this study, we explored the novel theoretical concept of fetal MSC-

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mediated lung growth by investigating the paracrine action of AF-MSCs on branching morphogenesis in a nitrofen lung explant model.

1. Methods

1.1. Nitrofen-induced lung explant model

This study was approved by the University of Michigan Unit for Laboratory Animal Medicine under protocol 10495-1 in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals. To induce fetal lung hypoplasia, timed-pregnant Sprague–Dawley rats (Harlan Laboratories) were gavage fed 100 mg of nitrofen (2,4-dichlorophenyl *p*-nitrophenyl-ether; Sigma-Aldrich) dissolved in 1 ml of olive oil on gestational day 9.5 (E9.5) as previously described elsewhere [26]. Control dams were gavage fed 1 ml of olive oil only. On day 14.5 (E14.5), dams were deeply anesthetized, and all fetuses were harvested by cesarean section. Fetal lungs were dissected out intact in Hank's buffered saline under a dissecting microscope.

1.2. Amniotic fluid mesenchymal stromal cells

Amniotic fluid samples were collected from pregnant control and nitrofen-exposed Sprague–Dawley dams (n=5 each) on E14.5. Rat AF-MSCs were isolated and cultured based on established protocols in the laboratory [13]. Plastic adherent, spindle-shaped cells between passage 3 and 5 were evaluated using criteria as described elsewhere [27,28]. For flow cytometry analyses, 5×10^5 AF-MSCs were incubated with 5% fetal bovine serum, and primary antibody at 4 °C for 30 min. Antibodies against CD11 (BD Biosciences), CD34 (Santa Cruz), CD45, CD73 (BD Biosciences), CD79 (Novus Biologicals), CD90 (BD Biosciences), and CD105 (Novus Biologicals) were used as well as same isotype antibodies as negative controls. Evaluation of staining was performed using the LSRII flow cytometer (BD Biosciences), and data were analyzed using FloJo software (Tree Star).

For adipocyte differentiation of AF-MSCs, confluent cells were placed in adipogenic differentiation medium [0.5 mM isobutylmethylxanthine (EMD Millipore), 200 μM indomethacin (MP Biomedicals), 10^{-6} M dexamethasone (MP Biomedicals), and 10 μg/ml of insulin (Sigma-Aldrich) in αMEM (Gibco)] supplemented with 10% fetal bovine serum. For osteoblast differentiation, confluent cells were cultured in osteogenic differentiation medium [10^{-7}M dexamethasone (MP Biomedicals), 10 mM β-glycerophosphate (Alfa Aesar) in DMEM (Gibco)] supplemented with 10% fetal bovine serum.

Rat dermal fibroblasts were isolated from adult rat skin as described elsewhere [29]. One day prior to fetal lung dissection, cells were plated at a density of 20,000 cells/cm^2 in 12-well plates and grown for 24 h in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine at 37 °C in a 5% CO_2 atmosphere.

1.3. Explant culture system

E14.5 lung explants were placed into polyethylene terephthalate hanging insert filters (0.4 μm, EMD Millipore) and divided into four groups based on cell type: medium alone (*medium*, n=6), AF-MSCs from control rats (*AF-Ctr*, n=6), AF-MSCs from nitrofen-exposed rats (*AF-Nitro*, n=6), and rat dermal fibroblasts (*dermis*, n=3). Normal lungs harvested from E14.5 control dams were grown in medium alone. Explants were cultured under serum-free hypoxic conditions (3% O_2) for three days to mimic the low-oxygen tension of the fetal environment.

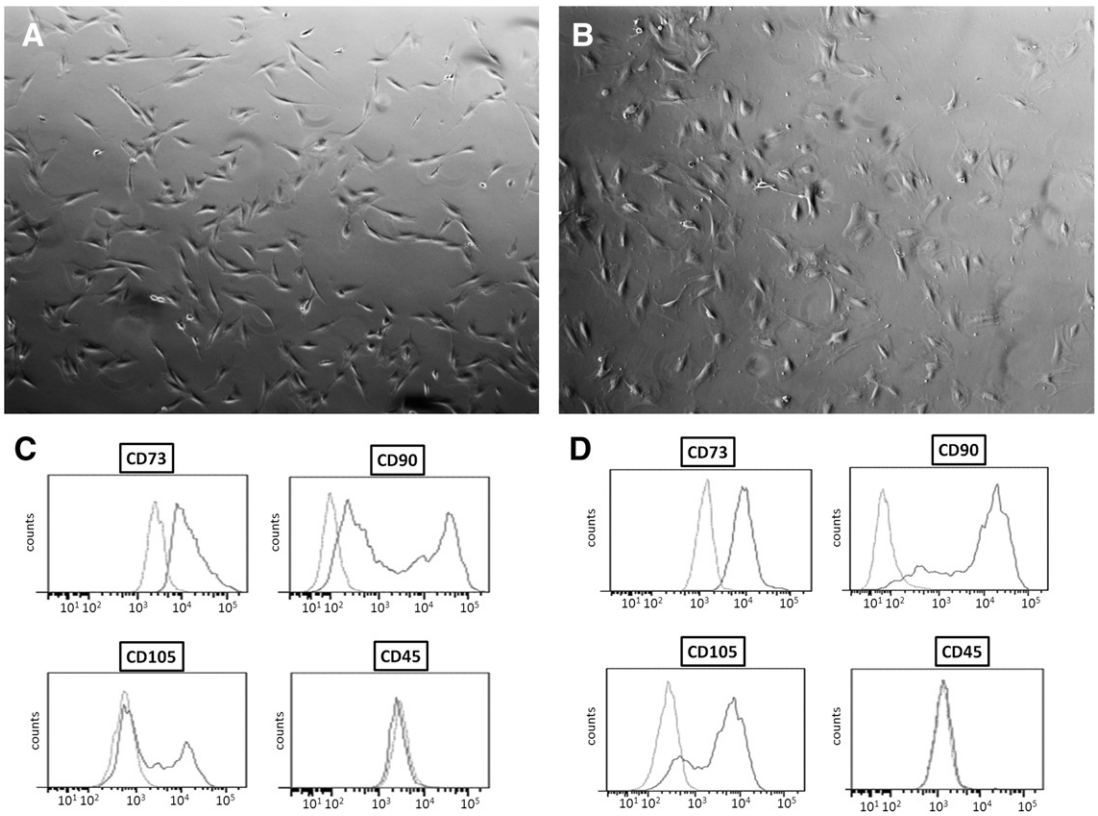

Fig. 1. Characteristics of rat amniotic fluid-derived mesenchymal stromal cells (AF-MSCs) harvested on gestation day 14.5. Phase contrast photomicrographs of AF-MSCs from control (A) and nitrofen-exposed (B) fetuses (20× magnification). Flow cytometry analyses of AF-MSCs from control (C) and nitrofen-exposed (D) fetuses, demonstrating a predominant population of CD45$^-$, CD73$^+$, CD90$^+$, and CD105$^+$ cells.