A case report on filamin A gene mutation and progressive pulmonary disease in an infant

A lung tissue derived mesenchymal stem cell study

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Abstract

Rationale: Mesenchymal stem cells (MSC) play a crucial role in both the maintenance of pulmonary integrity and the pathogenesis of lung disease. Lung involvement has been reported in patients with the filamin A (FLNA) gene mutation. Considering FLNA’s role in the intrinsic mechanical properties of MSC, we characterized MSCs isolated from FLNA-defective lung tissue, in order to define their pathogenetic role in pulmonary damage.

Patient concerns: A male infant developed significant lung disease resulting in emphysematous lesions and perivascular and interstitial fibrosis. He also exhibited general muscular hypotonia, bilateral inguinal hernia, and deformities of the lower limbs (pes tortus congenitales and hip dysplasia). Following lobar resection, chronic respiratory failure occurred.

Diagnosis: Genetic testing was performed during the course of his clinical care and revealed a new pathogenic variant of the FLNA gene c.7391_7403del; (p.Val2464AlafsTer5). Brain magnetic resonance imaging revealed periventricular nodular heterotopia.

Interventions and outcomes: Surgical thoracoscopic lung biopsy was performed in order to obtain additional data on the pathological pulmonary features. A small portion of the pulmonary tissue was used for MSC expansion. Morphology, immunophenotype, differentiation capacity, and proliferative growth were evaluated. Bone marrow-derived mesenchymal stem cells (BM-MSC) were employed as a control. MSCs presented the typical MSC morphology and phenotype while exhibiting higher proliferative capacity (P < .001) and lower migration potential (P = .02) compared to control BM-MSC.

Lessons: The genetic profile and altered features of the MSCs isolated from FLNA-related pediatric lung tissue could be directly related to defects in cell migration during embryonic lung development and pulmonary damage described in FLNA-defective patients.

Abbreviations: BM-MSC = bone marrow-derived mesenchymal stem cells, cPD = cumulative population doubling, FLNA = filamin A, MSC = mesenchymal stem cells, SDF =stromal cell-derived factor.

Keywords: children, filamin A, lung, mesenchymal stem cells, tissue

1. Introduction

Filamins are cytoskeletal proteins that organize actin filaments into networks and link these networks to cell membranes. Filamin A (FLNA) is the most abundant and widely-expressed protein among the 3 filamin isoforms. As a mediator of cytoskeleton reorganization, it regulates actin filament disassembly at the leading edge of motile cells and directs these filaments to the filopodia, lamellipodia, stress fibers and focal contacts. It is ubiquitously distributed, and null mutations have strong consequences on embryonic development in humans, with organ defects which suggest deficiencies in cell migration.[11]

Recently lung involvement has been reported in patients with the FLNA gene mutation.[12–9] The specific role of FLNA in the development of lung disease is still not fully understood. Many lung diseases are driven by the maladaptive proliferation of mesenchymal, epithelial, and endothelial cells, which results in dysfunctional lung remodeling.[10]

Recent studies indicate that lung mesenchymal stem cells (MSCs) may contribute to lung injury and repair and to pulmonary disease progression.[11] Considering FLNA’s role in the intrinsic mechanical properties of MSC, including cell adhesion and migration, in this brief report, we characterized...
MSCs isolated from FLNA-defective pediatric lung tissue in terms of morphology, phenotype, differentiation capacity, proliferative growth, and gene signature. These data should be useful in defining the role of MSCs in pulmonary damage described in FLNA-defective patients.

2. Material and methods

2.1. Ethical approval

This study was approved by the ethics committee of The Children’s Hospital G. Di Cristina, ARNAS Civico-Di Cristina-Benfratelli, Palermo, Italy

2.2. Consent statement

After written informed consent was obtained from the parents, a small portion of the pulmonary tissue destined for histological analysis was used for MSC expansion.

Informed consent was obtained from the parents for the publication of this study.

3. Methods

3.1. Isolation and culture

Isolation and expansion of MSCs from pulmonary tissue were performed following standard bone marrow culture procedures, as previously described. The pulmonary sample, after cutting into small pieces, was incubated in the presence of 1 mg/mL collagenase type II (Sigma Aldrich, Milan Italy) in α-MEM (Gibco, Milan Italy) at 37°C, 5% CO2 for 30 minutes. Collagenase activity was blocked by adding α-MEM (Gibco) 10% FBS (Euroclone, Milan Italy) and the cell suspension was collected and plated in polystyrene culture flasks (Corning Costar, Corning, NY) at a density of 160,000/cm² in complete culture medium: D-MEM (Gibco) 10% FBS (Euroclone), gentamicin 30 mg/mL, and penicillin 100 U/mL.

Cultures were maintained at 37°C, 5% CO2, and the medium was replaced twice a week. When MSCs reached ≥80% confluence, they were harvested with Trypsin EDTA (Lonza, Milan, Italy) and replated for expansion at a density of 4000 cells/cm².

4. Characterization of ex-vivo expanded MSCs

4.1. Proliferative capacity

Proliferative capacity was defined as cumulative population doubling (cPD) calculated following the formula PD=log (n. of harvested cells/n. of seeded cells)/log. Bone marrow-derived mesenchymal stem cells (BM-MSC) from healthy donors were employed as a control.

4.2. Immune phenotype

Cell immunophenotyping was performed by flow-cytometry. Fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies specific for CD73, CD34, CD90, CD14, CD45, CD31, CD105, HLA-I and HLA-DR and CXCR4 (Beckman Coulter, IL, Milan, Italy) were used. Appropriate, isotype-matched, non-reactive fluorochrome-conjugated antibodies were employed as controls. Cell populations were analyzed by direct immunofluorescence with a FACS Navios flow-cytometer (BC).

4.3. Differentiation capacity

The differentiation capacity of MSCs was assessed by incubating cells for at least 2 weeks at 37°C, 5% CO2 in differentiation medium: αMEM (Euroclone), 10% FBS, 10−7M dexamethasone, 50mg/ml L-ascorbic acid and 5 mM β-glycerol phosphate (all from Sigma-Aldrich). For adipogenic differentiation 100mg/mL insulin, 50mM isobutyl methylxanthine, 0.5mM indomethacin (Sigma-Aldrich), and 5 mM β-glycerol phosphate were also added. To detect osteogenic differentiation, cells were stained for alkaline phosphatase (AP) activity using Fast Blue (Sigma-Aldrich) and for calcium deposition with Alizarin Red (Sigma-Aldrich). Adipogenic differentiation was assessed based on the morphological appearance of fat droplets after staining with Oil Red O (Sigma-Aldrich).

4.4. Senescence assay

Cells were maintained in culture until they reached senescence. In order to reveal any change in morphology and/or proliferation rate, MSCs were closely monitored during senescence for up to 8 to 12 weeks before interrupting the cultures. Senescence of MSCs was assessed by staining with the β-galactosidase (SA-β-gal) staining Kit (Cell Signaling Technology, Danvers, MA), according to the manufacturer’s instructions and evaluated by direct-light microscopy.

4.5. Migration assay

The cell migration assay was performed with the transwell migration chamber system (Merck Millipore, Milan, Italy), utilizing polycarbonate inserts with an 8 μm pore size, as previously described. Briefly, 5 × 10⁴ cells/well were seeded in the upper chambers of the 96-well plate. For each condition, the lower chambers were filled with 150 μL of basal medium with or without stromal cell-derived factor (SDF). The cells were left to migrate for 16 hours at 37°C and 5% CO2. At the end of the incubation, the underside of the membranes was fixed with 4% paraformaldehyde for 20 minutes. Cell nuclei were then stained with Hoechst 33342 (Life Technologies, Monza, Italy). Three replicates were evaluated for each condition. Images were acquired using an Olympus BX51 microscope (Olympus, Deutschland GmbH, Hamburg, Germany). The number of migrated cells was blindly-counted analyzing 15 random fields of the membranes per condition. Data were expressed as the means of the number of migrated cells ± standard error (SE).

The number of migrated FLNA-MSCs was compared to the number of migrated BM-MSCs from healthy donors.

4.6. Genetic characterization

After obtaining informed consent for the genetic analyses, hybridization target enrichment and parallel sequencing were performed on genomic DNA extracted from pulmonary MSCs of the FLNA-defective patient. Sequences of the FLNA gene were enriched by using a custom NimbleGen SeqCap EZ Choice kit (Roche NimbleGen Inc., Madison, WI, USA) according to the manufacturer’s protocol, and sequenced on the Illumina NextSeq500 platform. The BaseSpace pipeline (Illumina, https://basespace.illumina.com/) and the TGexTM software (LifeMap Sciences) were used for the variant naming and
annotating variants, respectively. Sequencing data were aligned to the hg19 human reference genome.

4.7. Statistical analysis

Proliferative capacity of the patient’s MSCs was compared with controls using the z-statistic, while the number of migrated cells was compared between the patient and controls with the \( t \) test for independent samples. Statistical significance was defined as a \( P \) value < .05. Data analyses were performed with the STATA statistical package (release 14.2, 2012, Stata Corporation, College Station, TX).

4.8. Case report

A male infant was referred to our surgical department, with a presumptive diagnosis of congenital lobar emphysema. He developed significant lung disease resulting in emphysematous lesions and perivascular and interstitial fibrosis. He also exhibited general muscular hypotonia, bilateral inguinal hernia, and deformities of the lower limbs (pes tortus congenitalis and hip dysplasia). Following lobar resection, chronic respiratory failure occurred, necessitating mechanical ventilation to assist the patient’s breathing. The decision to perform a surgical thoracoscopic lung biopsy was made in order to obtain additional data on the pathological pulmonary features for prognostic predictions and therapeutic decisions.

Genetic testing was performed during the course of his clinical care and revealed a new pathogenic variant of the FLNA gene c.7391_7403del; (p.Val2464AlafsTer5). Brain magnetic resonance imaging revealed periventricular nodular heterotopia. A small portion (1 cm \( \times \) 1 cm) of the pulmonary tissue obtained to lung biopsy was used for MSC expansion.

4.9. MSC expansion and characterization

Cells were isolated and expanded from pulmonary tissue of the FLNA-defective patient using the same standard culture conditions used for BM-MSC. The cells were plastic adherent, displayed the spindle shape morphology (Fig. 1A) and typical cell surface markers: positive for CD73, CD90, CD105, and HLA-I and negative for CD34, CD45, CD14, CD31 and HLA-DR. The percentage of cells expressing CXCR4 was \( > 2\% \) both in FLNA-MSCs and in BM-MSCs. While the FLNA MSC proliferative capacity, defined as cPD, was significantly higher \( (P < .00001) \) than BM-MSC, (Fig. 1B).

The in vitro osteogenic and adipogenic differentiation assays confirmed an osteogenic differentiation potential similar to BM-MSCs and lack of adipogenic differentiation capacity (Fig. 1C). Cells entered senescence phases at passage 14 (P14) (Fig. 1D). Considering these characteristics we defined these in vitro expanded cells from the patient’s pulmonary tissue as lung-MSCs.

4.10. Migration assay

Since the FLNA mutation may lead to a defect in cell motility, we evaluated lung-MSC in vitro migration in comparison with BM-MSC. The results of the transwell migration assay reported as the

![Figure 1](image_url)

**Figure 1.** Panel A: MSCs expanded from pulmonary tissue of the FLNA-defective pediatric patient exhibiting the typical spindle-shaped morphology. Magnification 4X; Panel B: cPD of FLNA-MSC and BM-MSC cultured to passage (P)5 (continuous line FLNA-MSC, broken line BM-MSC); Panel C: Adipogenic and osteogenic differentiation capacity of BM-MSC and FLNA-MSCs. Differentiation into adipocytes, revealed by the formation of lipid droplets (stained with oil red O staining) present in control BM-MSC resulted absent in FLNA-MSC. Differentiation into osteoblasts, demonstrated by the histological detection of AP activity (purple reaction) and calcium deposition stained with Alzarin red staining, was similar in FLNA-MSC and BM-MSC. Magnification 10X; Panel D: \( \beta \)-gal staining of senescent FLNA-MSC at P14 (stained in blue), BM-MSC = bone marrow-derived mesenchymal stem cells, cPD = cumulative population doubling, FLNA = filamin A, MSC = mesenchymal stem cells.
mean of the number of migrated cells per field, showed a significantly reduced number (P=.026) in lung-MSC compared with control BM-MSC (11.87 ± 2.54 and 37 ± 9.5, respectively), Figure 2. No increases in cell migration were observed in wells filled with SDF supplemented medium for either lung-MSCs or control BM-MSC (9.0 ± 1.5 and 21.5 ± 5.3, respectively).

4.11. Genetic characterization

DNA sequencing analysis of pulmonary MSCs confirmed the presence of the heterozygous frameshift variant, NM_001110556.1:c.7391_7403del; (p.Val2464AlafsTer5), in the FLNA gene, previously identified in the DNA from peripheral blood of the same patient (Fig. 3).

5. Discussion

To the best of our knowledge, this is the first report on a gene mutation in lung resident MSCs of an FLNA-defective patient. This mutation may be responsible for defective MSC migration and could have a causative role in the pulmonary damage described in these patients.

FLNA is a protein that crosslinks actin filaments into orthogonal networks in the cortical cytoplasm and participates in the anchoring of membrane proteins to the actin cytoskeleton establishing mechanical stability in cells.[1] Remodeling of the cytoskeleton is central to the modulation of cell shape and migration. The role of FLNA in cell migration is essential for embryonic development. More precisely, FLNA localizes to podosomes, which are cell structures involved in mesenchymal embryonic development. More precisely, FLNA localizes to podosomes, which are cell structures involved in mesenchymal embryonic development. FLNA is a protein that crosslinks actin filaments into orthogonal networks in the cortical cytoplasm and participates in the anchoring of membrane proteins to the actin cytoskeleton establishing mechanical stability in cells.[1] Remodeling of the cytoskeleton is central to the modulation of cell shape and migration. The role of FLNA in cell migration is essential for embryonic development. More precisely, FLNA localizes to podosomes, which are cell structures involved in mesenchymal migration via adhesion and matrix degradation properties.[15] Adhesion and migration of cells into tissues, including tissue-resident MSCs, are critical processes for organ formation, homeostasis, and repair. The organism’s fitness is highly dependent on the appropriate function of tissue stem cells.[16]

We demonstrated that MSCs isolated from FLNA-defective pediatric lung tissue, according to the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy Standards,[17], are plastic adherent when cultured under standard conditions, express the characteristic mesenchymal cell surface antigens and have the capacity to differentiate into osteoblasts; however, adipocyte differentiation capacity was not detected. The differentiation tendencies of stem cells are closely linked to several factors including adhesive context, mechanical signals, and the physical responses of the cells.[18] Cytoskeletal components composed of actin, microtubules, and intermediate filaments in the cytoplasm play an important role in the elastic properties of cells. These components vary in amount and arrangement during stem cell differentiation, resulting in changes in elasticity.[19–21] Therefore, due to the influence of FLNA on the intrinsic mechanical properties of these cells, the role of an FLNA mutation in defective MSC differentiation cannot be excluded.

MSCs from FLNA-defective pediatric lung tissue exhibited a lower migration capacity compared to control BM-MSC. MSCs express a number of chemokine receptors that are likely involved in their migration capabilities,[22] possibly in combination with growth factors and chemokines necessary for maximal effect.[23] The contribution of the CXCL12 (SDF-1a)/CXCR4 axis to the recruitment of bone marrow-derived stem cells in lung pathologies has been demonstrated in a number of studies.[24–26] The ability of MSCs to home and migrate appears to decrease during in vitro expansion also in relation to their loss of surface expression of chemokine receptors.[27–28] Moreover, the similar CXCR4 expression in MSCs isolated from FLNA-defective tissue compared with control BM-MSC suggests that the reduced migration capability is probably not related to a chemokine/receptor mechanism, but may be linked to an FLNA deficiency. This alteration may be directly related to defects in cell migration during embryonic lung development and in pulmonary damage described in FLNA-defective patients.

The lung is a complex organ deriving from the endodermal and mesodermal germ line. Normal lung development depends on the well-orchestrated interaction between mesenchymal, epithelial, and endothelial cells. Even though genetic and epigenetic regulation, as well as the specialized intra-, inter-, and extracellular mechanisms responsible for proper development of the respiratory system continue to be elucidated, each step in lung development is reliant upon inductive cues and reciprocal interactions between the pulmonary epithelium and the surrounding mesenchyme. Loss of or abnormalities in cells and in their critical interactions can lead to
severe anatomical and functional defects in the airway and alveoli. MSCs are key cells in the connective tissue hierarchy of many organs, including the lung, and given the intimate relationship between the epithelium and underlying mesenchyme in the lung, both during branching morphogenesis and in the adult it is likely that MSC play a crucial role in both the maintenance of epithelial integrity and pathogenesis of lung disease.

Even if a single case may represent a limit to the interpretation of the results, in our FLNA-defective patient, dysfunctional lung MSCs could have played a role in pathological lung development causing neonatal emphysematous lesions. Subsequently, they may limit repair, inducing dysfunctional matrix remodeling that results in the development of progressive fibrotic lung disease.

Due to their tissue-regenerative and immunomodulatory properties, MSCs have been proposed as cell therapy in lung disease associated with dysregulated immune responses (e.g., chronic inflammation in chronic pulmonary disease) and aberrant repair processes of lung tissue (e.g., idiopathic pulmonary fibrosis (IPF)). Results from preclinical studies using MSCs to treat the genetic syndrome, cystic fibrosis, have been reported. The potential therapeutic role of healthy BM-MSC cannot be excluded also in these defective patients. Further studies are mandatory to improve our understanding of the role of MSCs in lung development and disease in genetic disorders, in order to propose prospective cell therapies.

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