Characterization in mice of the resident mesenchymal niche maintaining AT2 stem cell proliferation in homeostasis and disease

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Abstract
Resident mesenchymal cells (rMCs defined as Cd31NegCd45NegEpcamNeg) control the proliferation and differentiation of alveolar epithelial type 2 (AT2) stem cells in vitro. The identity of these rMCs is still elusive. Among them, Axin2Pos mesenchymal alveolar niche cells (MANCs), which are expressing Fgf7, have been previously described. We propose that an additional population of rMCs, expressing Fgf10 (called rMC-Sca1PosFgf10Pos) are equally important to maintain AT2 stem cell proliferation. The alveolosphere model, based on the AT2-rMC co-culture in growth factor-reduced Matrigel, was used to test the efficiency of different rMC subpopulations isolated by FACS from adult murine lung to sustain the proliferation and differentiation of AT2 stem cells. We demonstrate that rMC-Sca1PosFgf10Pos cells are efficient to promote the proliferation and differentiation of AT2 stem cells. Co-staining of adult lung for Fgf10 mRNA and Sftpc protein respectively, indicate that 28% of Fgf10Pos cells are located close to AT2 cells. Co-ISH for Fgf7 and Fgf10 indicate that these two populations do not significantly overlap. Gene arrays comparing rMC-Sca1PosAxin2Pos and rMC-Sca1PosFgf10Pos support that these two cell subsets express differential markers. In addition, rMC function is decreased in obese ob/ob mutant compared to WT mice with a much stronger loss of function in males compared to females. In conclusion, rMC-Sca1PosFgf10Pos cells play important role in supporting AT2 stem cells proliferation and differentiation. This result sheds a new light on the subpopulations of rMCs contributing to the AT2 stem cell niche in homeostasis and in the context of pre-existing metabolic diseases.

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INTRODUCTION

Lung fibrosis is characterized by an accumulation in the respiratory airways of mesenchymal cells, called activated myofibroblasts (MYF) which over time, lead to impaired lung function.1 Through the use of single cell transcriptomic approach, the heterogeneity of the different resident mesenchymal (rMC) populations present in the lung is starting to emerge. A key stromal cell is represented by the lipofibroblasts (LIFs), which are rich in lipid-droplets and can be stained and isolated using the vital dye LipidTox (LT). They express Perilipin 2, Platelet derived growth factor receptor alpha (Pdgfra) and are negative for Acta2 and most importantly are located close to alveolar type 2 cells (AT2).2 They are proposed to supply AT2s with the triglycerides needed for the elaboration of surfactant. Using a co-culture assay of Pdgfrα+ cells, we previously reported the reversible trans-differentiation of the LIFs into activated MYFs, thereby leading to fibrosis. Pertinent to this background, obesity/diabetes and gender have been proposed to be predictive factors for the severity of the disease.12,13

In this study, we used the in vitro alveolosphere model based on the AT2-rMC co-culture in growth factor-reduced Matrigel to test the efficiency of different rMC subpopulations isolated by FACS from adult murine lungs. We used antibodies against Cd45, Cd31, Epcam and Stem cell antigen 1 (Sca1) to define the initial rMC population responsible for the proliferation of AT2 stem cells. Using a fluorescent substrate for β-galactosidase activity, rMC-Sca1+/Fgf10+/LIFs as well as rMC-Sca1+/LIPos cells were sorted. Additional sorting was achieved using LipidTOX (LT) staining for cells containing high level of neutral lipids. After 2 weeks of co-culture, organoid size and colony forming efficiency were quantified. RNAscope with Fgf7- and Fgf10-labeled riboprobes, to label rMC-Axin2+/pos and rMC-Fgf10+/pos populations, respectively, combined with Sftpc immunofluorescence on adult lungs was carried out. Gene array analyses were used to characterize sorted rMC-Sca1+/Fgf10+/pos vs rMC-Sca1+/Axin2+/pos cells. We used males and females C57BL6 and corresponding ob/ob mutant mice to determine the impact of obesity and gender on the functional capacity of the corresponding rMCs-Axin2+/pos to trigger AT2 stem cells proliferation.

Our results indicate that an rMC-Sca1+/pos/Fgf10+/pos/Lippos subpopulation is essential for the proliferation of the AT2 stem cells. This subpopulation is likely different from the previously described MANC. In addition, obesity and gender impact the capacity of rMC-Sca1+/pos cells to maintain the proliferation of AT2 stem cells indicating that future therapeutic approaches should be focussed on restoring the function of the mesenchymal niche.

MATERIALS AND METHODS

2.1 Mice

SftpcCreERT2/+ knock in (gift from Harold Chapman, UCSF), tdTomatofox reporter (Stock 007908, Jacksonlab), Fgf10fox2 reporter (Mailleux
et al., 2005), Axin2lacZ reporter (stock 009120, Jackson lab), Lepob/ob (aka ob/ob) mutant (stock 00632, Jackson lab) and wild type mice were maintained on the C57BL/6 background. All animal studies were performed according to protocols approved by the Animal Ethics Committee of the Regierungspräsidium Giessen (permit numbers: G7/2017-No. 844-GP and G11/2019-No. 931-GP).

2.2 | Lung dissociation and fluorescence-activated cell sorting

Lungs from C57BL6 (WT) mice were collected at 6-8 weeks of age and processed into a single-cell suspension using collagenase type IV (0.5% ml per lung; Gibco #9001-12-1) and DNase I (SIGMA#DN25). Cd45NegCd31NegEpcamNegSca1Pos cells were collected to establish organoid model combined with AT2 cells. The total AT2 population (SftpcPosTomPos cells) was isolated from lungs of 6-8 weeks old SftpcCreERT2/++; tdTomatofox/fox mice. For the single-cell preparation, lungs were inflated intratracheally with 3 mL dispase (5 U/mL; BD Biosciences #354235) and for further digestion, each lung was incubated in 3 mL dispase solution (5 U/mL) at room temperature for 30 minutes. For the isolation of epithelial cells from the SftpcTom lungs, we first sorted a population of Cd45NegCd31NegEpcamPos cells. To isolate enriched population of mature AT2 cells, we additionally used Lysotracker (100 μM; Green DND-26 fluorescent dye Invitrogen #L7526) and collected LysoposTompos cells. In general, we have considered the fluorescence values higher than 10^3 as a positive selection to gate cells of interest. The values around 0 were gated as a negative selection. In all experiments, we used the negative control to adjust gating to distinguish negative and positive populations.

2.3 | FluoReporter lacZ flow cytometry

Fluorescein di (b-D-galactopyranoside) (aka FDG) (Thermo Fischer Scientific #F1930) was used to isolate by FACS, cells expressing β-galactosidase from Fgf10lacZ and Axin2lacZ reporter lines. Lungs were collected from 6-8 weeks old Fgf10lacZ and Axin2lacZ reporter mice. According to manufacturer’s instruction, single-cell suspension and FDG working solution were prewarmed and the cells were resuspended with chloroquine followed by loading by FDG. After incubation for 20 minutes, FDG loading is stopped by adding ice cold staining medium containing propidium iodide and chloroquine. Cells are then placed on ice and incubated with antibodies against Cd45, Cd31, Sca1, and EpCam (for details see Lung dissociation and Fluorescence-Activated Cell Sorting) before sorting using the FACSAria III (BD Bioscience) cell sorter. Cells were sorted through a flow chamber with a 100-μm nozzle tip under 25 psi sheath fluid pressure. Cells were collected in sorting media (advanced DMEM:F12 [Gibco#12634-010] plus 10% FBS and 1% P/S).

2.4 | Alveolar organoid assay

Five thousand LysoposTompos cells (AT2s from adult SftpcCreERT2/++; tdTomatofox/fox) lungs and 50,000 nMCs cells were resuspended in 100 μL culture medium (sorting media plus 1% ITS [Gibco #14400-045]) and mixed 1:1 with 100 μL growth factor-reduced phenol Red-free Matrigel (Corning #356231). Cells were seeded in individual 24-well 0.4 μm Transwell inserts (Falcon, SARSTEDT). After incubation at 37°C for 15 minutes, 500 μL of culture was placed in the lower chamber and the plate was placed at 37°C in 5% CO2/air. The culture medium was changed every other day. ROCK inhibitor (10 μM, Y27632 STEMCELL#72304) was included in the culture medium for the first 2 days of culture. Organoids were counted and measured at day 14. Colony-forming efficiency (CFE) is calculated as the ratio between the numbers of spheres observed over the initial number (5000) of AT2 cells. At day 14, organoids were processed for whole-mount immunofluorescence staining.

2.5 | Whole-mount immunofluorescence staining of organoids

Organoids were fixed in 4% paraformaldehyde for 30 minutes followed by 3× washing steps with PBS and incubation in 0.1% Triton X-100 for 30 min. After washing 3× with PBST, organoids were blocked with 1× TBS, 3% BSA. 0.4% Triton X-100 for 1 hour at room temperature. Organoids were washed and then incubated at 4°C overnight with 1× TBS, 1.5% BSA, 0.2% Triton X-100, and primary antibody against Hopx (1:250 SIGMA #HPA030180). The next day, after 3× washing with TBST for 10 minutes, organoids were incubated with secondary antibodies (AlexaFlour 488 goat anti-Rabbit IgG Green [1:500] Cat. #11034 Invitrogen) at RT and washed three times with TBST before being mounted with Prolong Diamond Anti-fade Mountant with DAPI (Invitrogen 4′,6-diamidino2-phenylindole). Photomicrographs of immunofluorescence staining were taken using a Leica DMRA fluorescence microscope with a Leica DFC360 FX camera (Leica, Wetzlar, Germany). Figures were assembled using Adobe Illustrator.

2.6 | Quantitative RT-PCR

Total RNA was extracted from FACS-sorted Sca1PosLT^N^ and Sca1PosLT^N^ cells using RNeasy plus Micro kit (Cat. # 74034 QIAGEN), cDNA was synthesized using QuantiTect Reverse Transcription kit (Cat. #205314 QIAGEN). Quantitative real-time PCR (qRT-PCR) analysis was performed using LightCycler 480 II machine (Roche AppliedScience). Data were presented as expression relative to hypoxanthine-guanine phosphoribosyltransferase (Hprt) for mouse genes.

Primers: mFgf10: (For: ATGACTGTGTGACATCAG, Rev: CACT GTTCAGCCTTTTGA), mPdgfra: (For: GTCGTTGACCTGACTTGA Rev: CCAGCATGGTATACCTTG).
2.7 Co-staining: RNA in situ hybridization assay and IF

Murine lungs were perfused with PBS and fixed in 4% paraformaldehyde according to standard procedures. Next, they were embedded in paraffin and sectioned at 5 μm thickness. RNAscope Multiplex Fluorescent Reagent Kit v2 assay (document Nr:323100-USM, Advanced Cell Diagnostic, Newark, California) was performed according to the manufacturer's instructions, however, with slight changes in applying standard pretreatment conditions and target retrieval antigen. The pretreatment time was reduced to 7 minutes and the amplification steps were increased to 1 hour. Specific probes were used for the detection of Fgf10 and Fgf7 gene expression (Mm-Fgf10 Cat. #446371, Mm-Fgf7 Cat. #443521-C3). 3-plex Positive control (Cat. #320881) and 3-plex negative control (Cat. #320871) were carried out using probes specific to murine housekeeping-genes.

These samples were followed by IF for Sftpc. Samples were washed in TBST buffer then incubated at 4°C overnight with 1× TBS, 1.5% BSA, 0.2% Triton X-100, and primary antibody against Sftpc (Anti-Pro-Surfactant Protein C, polyclonal Ab Cat. # AB3786). The next day, after 3× washing with TBST for 10 minutes, samples were incubated with secondary antibodies (AlexaFlour 488 goat anti-Rabbit IgG Green [1:250] Cat. #11034 Invitrogen) at RT and washed three times with TBST before being mounted with Prolong Diamond Anti-fade Mountant [1:250] Cat. #11034 Invitrogen) at RT and washed three times with TBST before being mounted with Prolong Diamond Anti-fade Mountant. Photomicrographs of immunofluorescence staining were taken using a Leica DMRA fluorescence microscope with a Leica DFC360 FX camera (Leica, Wetzlar, Germany). Figures were assembled using Adobe Illustrator.

2.8 Microarray analysis

Gene expression profiles of two sub-lineages of the resident mesenchymal niche cells were carried out. Purified total RNA was amplified using the Ovation PicoSL WTA System V2 kit (NuGEN Technologies, Bemmel, Netherlands). Per sample, 2 μg amplified cDNA was Cy5-labeled using the SureTag DNA labeling kit (Agilent, Waldbronn, Germany). Hybridization to 8x60K 60mer oligonucleotide spotted microarray slides (Agilent-074809: Catalog gene expression microarray for Mouse, v2 8x60K) and subsequent washing and drying of the slides was performed following the Agilent hybridization protocol in Agilent hybridization chambers, with following modifications: 2 μg of the labeled cDNA were hybridized for 22 hours at 65°C. The cDNA was not fragmented before hybridization. Each sample was processed and hybridized twice (technical replicates).

The dried slides were scanned at 2 μm/pixel resolution using the InnoScan is 900 (Innovys, Carbone, France). Image analysis was performed with Mapix 6.5.0 software, and calculated values for all spots were saved as GenePix results files. Stored data were evaluated using the R software and the limma package from BioConductor. Log2 mean spot signals were averaged, and from several different probes addressing the same gene only the probe with the highest average signal was used. Data from technical replicates was averaged. The data were deposited in a public database (GEO accession: GSE162859). Genes were ranked for differential expression using a moderated t-statistic. Pathway analyses were done using gene set tests on the ranks of the t-values. Pathways were taken from the KEGG database (http://www.genome.jp/kegg/pathway.html).

Heatmaps are generated from the normalized log2 spot intensities (l) and show the gene-wise z-values (where $z_j = (l_j - \text{mean}(l))/\text{SD}(l)$ for $j = 1,...,n$).

2.9 Statistics

All results are mean ± SEM. All error bars on graphs represent SEM. Statistical tests are 2-tailed t tests. $P \leq .05$ was considered statistically significant.

3 RESULTS

3.1 The capacity of Cd45negCd31negEpcamneg resident mesenchymal cells (rMCs) to functionally support the proliferation and differentiation of alveolar epithelial type 2 (AT2) stem cells is associated with Stem cell antigen 1 (Sca1) expression

The aim of this study is to refine the adult lung resident mesenchymal cell population, called thereafter rMC and defined by flow cytometry as Cd45negCd31negEpcamneg cells. It was already shown that the Cd45negCd31negEpcamnegpdgfrpos cell population was capable of sustaining AT2 stem cell renewal using the so-called alveolosphere in vitro assay. In this approach, the use of antibodies against Cd45 and Cd31 allowed removing, from the target subpopulation, the hematopoietic and endothelial cells, respectively.

In the context of a previous milestone study generating organoids in Matrigel arising from a co-culture of EpcamHigh Cd24Low epithelial cells (previously called epithelial stem/progenitor cells or epiSPC) with rMC-Sca1pos (Cd45negCd31negEpcamnegpdgfrpos) cell population was capable of sustaining AT2 stem cell renewal using the so-called alveolosphere in vitro assay. It was found, in this approach, the use of antibodies against Cd45 and Cd31 allowed removing, from the target subpopulation, the hematopoietic and endothelial cells, respectively.

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For this purpose, we have sorted an enriched population of mature AT2 cells from the SftpcCreERT2/+;Tomatofox/+ lungs. We started first by isolating Cd31negCd45negEpcamneg cells and then applied two successive and stringent sorting approaches: first using LysoTracker, a fluorescent dye that labels acidic compartments found abundantly in the lamellar bodies of AT2 cells and second, using the tomato reporter expressed specifically in AT2 cells (Figure 1A). In addition, we sorted also rMCs-Sca1pos (Cd45negCd31negEpcamnegpdgfrpos) as well as rMCs-Sca1neg (Cd45negCd31negEpcamnegpdgfrpos). In order to have
sufficient cells to carry out our assay, we pooled the lungs of four adult (6-8 weeks old) C57BL/6 mice. This allowed us to carry out 6 independent cocultures. Sca1<sup>Pos</sup> cells represent around 25% of the total rMC population (Figure 1A). When co-cultured with sorted mature AT2s, rMC-Sca1<sup>Pos</sup> led to the formation of organoids (Figure 1B) with a colony forming efficiency of 5% (Figure 1C) which is in line with previously published alveolosphere experiments. Interestingly, rMCs-Sca1<sup>Neg</sup> fail to sustain alveolosphere formation (Figure 1B). The difference in organoid size and CFE is highly significant between the two rMC subpopulations (Figure 1C, P < .01).

### 3.2 | rMCs-Sca1<sup>Pos</sup> can be further functionally subdivided for the proliferation and differentiation of AT2 stem cells on the basis of LipidTOX staining

To better characterize rMCs-Sca1<sup>Pos</sup> lung cells and in particular whether this population may include the lipofibroblasts (LIFs), we used LipidTOX (LT), an efficient fluorescent stain for neutral lipid. As LIFs are abundant in neutral lipid droplets, LT has been previously used to quantify LIF population during lung development.<sup>2</sup>

Sorted rMCs-Sca1<sup>Neg</sup>LTHigh and rMCs-Sca1<sup>Neg</sup>LTLow were tested functionally in the alveolosphere model for their capacity to induce the proliferation and differentiation of AT2 stem cells (Figure 2A). For this experiment, we pooled the cells from three mice and carried four independent co-cultures. When co-cultured with sorted mature AT2s, rMCs-Sca1<sup>Pos</sup>LTHigh led to the formation of organoids (Figure 2B) with a colony forming efficiency of 2% (Figure 2C) while rMCs-Sca1<sup>Neg</sup>LTLow failed to sustain organoid formation. We also carried out at day 14, Immunofluorescence (IF) for LT (LIF marker), Hopx (AT1 cell marker), and DAPI in conjunction with the detection of the endogenous tomato reporter (Figure 2B). Our results indicate that LT<sup>Pos</sup> cells are located in the periphery of each organoid. In addition, we observed abundant expression of Hopx within the organoid indicating proper AT2 to AT1 differentiation.

RT-qPCR was also carried out to quantify the expression of Fgf10 and Pdgfra, two well-known markers enriched in LIFs (Figure 2D). Our results indicate a trend toward an increase in the expression of Fgf10 and Pdgfra in rMCs-Sca1<sup>Pos</sup>LTHigh compared to rMCs-Sca1<sup>Neg</sup>LTLow (n = 3 independent mice) (Figure 2D). These results are in line with the previous observation that Fgf10 and Pdgfra are also expressed in cells other than LIFs.<sup>2,16</sup> Overall, our data therefore support the previous conclusion that it is the LIF subpopulation of the rMCs that can preferentially support AT2 stem cell survival and differentiation.

### 3.3 | Fgf10<sup>Pos</sup> cells represent a niche for AT2 cells

We have previously reported that during development, a subset of Fgf10<sup>Pos</sup> cells are progenitors for lipofibroblasts (LIFs) in the late stage of development and postnatally. We have also reported that during the early postnatal stage of lung development, only 28% of the LIFs express Fgf10 indicating that the LIFs, like Fgf10<sup>Pos</sup> cells,<sup>5</sup> are a
heterogenous population. To functionally evaluate the difference between rMCs-Sca1^Pos^Fgf10^Pos^ and rMCs-Sca1^Pos^Fgf10^Neg^ cells, we used the Fgf10^2a2^ reporter line in combination with the β-galactosidase fluorescent substrate FDG to sort, from rMCs, enriched populations of FDG^Pos^ Fgf10^Pos^ and FDG^Neg^ Fgf10^Neg^ cells. We then isolated the Sca1^Pos^ fraction for each subpopulation. Our results indicate that organoid only form with rMCs-Sca1^Pos^Fgf10^Pos^ with a CFE of 4% (n = 4 independent experiments) (Figure 3B.D). rMCs-Sca1^Pos^Fgf10^Neg^ are not capable of eliciting organoid formation. IF staining of the organoids for Hopx show that the AT2 cells properly differentiate into AT1 cells (Figure 3C).

3.4 | Comparison of rMCs-Sca1^Pos^Fgf10^Pos^ vs rMCs-Sca1^Pos^Axin2^Pos^

Fgf10^Pos^ cells are progenitor for lipofibroblast. LIF in alveolar region are functionally important to support AT2 cells during lung...
development and postnatal stages. In addition, Axin2, a marker for Wnt signaling activation, is expressed in a subset of mesenchymal cells in the adult lung. It has been reported that 74% of Axin2Pos cells in the alveolar region are also expressing Pdgfra. Axin2Pos PdgfraPos mesenchymal cells are called mesenchymal alveolar niche cells (MANC) and are located close to AT2 cells. Using the alveolosphere model, they have been reported to sustain AT2 stem cell proliferation and differentiation. As these cells have been described to express Fgf7 and not Fgf10, we propose that rMCs-Sca1PosFgf10Pos and rMCs-Sca1PosAxin2Pos represent two independent pools of niche cells for AT2 stem cells.

To better characterize these two rMC subpopulations, we used specific reporter lines; Fgf10LacZ and Axin2LacZ to monitor the distribution of LipidTOX staining in these two sub-lineages. By using FACS Aria III cell sorter, we analyzed 100 000 events, each sample contained harvested lung from one mouse -with the same age range (6-8 weeks old). Cd45NegCd31NegEpcamNegSca1Pos sorted cells were processed for further analysis (Figure 4A). For Fgf10LacZ lungs, we found 25% FDGPos (rMCs-Sca1PosFgf10Pos) cells out of total rMC-Sca1Pos. For Axin2LacZ lungs, our results indicate around 10% FDGPos (rMC-Sca1PosAxin2Pos) cells out of total rMC-Sca1Pos. Further analysis based on LipidTox staining indicate that 85% of Fgf10Pos and 98% of rMC-Sca1PosAxin2Pos cells were also LTHigh cells (Figure 4B, C, respectively). Based on LT staining, we also report that most of the rMCs-Sca1PosFgf10Neg as well as rMCs-Sca1PosAxin2Neg subpopulations contain a high percentile of LTLow/LTHigh cells suggesting again a functional heterogeneity at the level of the LIFs (in regards to the maintenance of AT2 stem cell proliferation) based on whether they express or not Fgf10 or Axin2.

In order to better define at the transcriptomic level, the difference between rMCs-Sca1PosFgf10Pos and rMCs-Sca1PosAxin2Pos, we performed gene array analysis using the Agilent platform. Figure 4D shows top 100 genes which differentially expressed between rMCs-Sca1PosFgf10Pos and rMCs-Sca1PosAxin2Pos subsets (see Figure S1 for higher magnification of the heatmap). The genes differentially regulated between these two subsets were then evaluated in rMCs-Sca1PosAxin2Pos cells. Our results indicate that several markers such as Ackr4, Jchain, Cd46, Alox5, Nfam1 and Col10a1 are differentially expressed between rMCs-Sca1PosFgf10Pos and rMCs-Sca1PosAxin2Pos and therefore could be used in the future to label these mesenchymal subpopulations. KEGG analysis of rMCs-Sca1PosAxin2Pos vs rMCs-Sca1PosFgf10Pos indicates an upregulation of metabolic pathways, RNA transport, DNA replication as well as cell cycle indicating that the Axin2Pos cells are metabolically active and proliferative (Table S1). Altogether, our data notably suggest that rMCs-Sca1PosFgf10Pos cells are likely different from rMCs-Sca1PosAxin2Pos cells.
3.5 Fgf10 expressing cells are located close to Sftpc<sup>Pos</sup> cells

To investigate the relative interaction between Fgf10 expressing cells and AT2 cells, we combined the in situ hybridization technique for detecting Fgf10 mRNA expression with immunofluorescence staining for pro-SPC in adult wild type lungs. We found that 28% ± 0.5% (n = 3) of total Fgf10 expressing cells are located close to pro-SPC expressing cells (Figure 5A). This number is very similar to the one we reported before in the newborn lung using the Fgf10<sup>LacZ</sup> reporter. This observation suggests that a subset only of the Fgf10 expressing cells constitutes a critical component of the alveolar niche that may robustly communicate with AT2 cells. To better define the heterogeneity of AT2 stromal niche, we have also performed co-staining of Fgf10 mRNA and Sftpc. Fgf10 expression has been proposed to be a hallmark of the MANC population. To validate the Fgf7 mRNA probe, we used Fgf7 KO adult lungs. While a clear signal was obtained with a positive control probe provided by the manufacturer, the Fgf7 mRNA probe failed to generate a signal in the Fgf7 KO lungs (Figure S2) indicating that the signal observed in wild type lung is specific (Figure 5B). We found that around only 2.0% ± 0.2% (n = 3) of Fgf7 expressing cells are in vicinity of Sftpc<sup>Pos</sup> cells (Figure 5B). Finally, we performed a co-staining for Fgf7 and Fgf10 mRNA using different fluorescent labeled probes. Our results indicate that 15.7% ± 1.3% (n = 3) of total cells are Fgf7 expressing cells vs 25.4% ± 0.9% (n = 3) for Fgf10 expressing cells. Finally, 4.0% ± 0.3% (n = 3) of total cells are double positive Fgf10/Fgf7 cells (Figure 5C). Taken together, these data suggest that the rMCs-Sca1<sup>Pos</sup>Fgf10<sup>Pos</sup> are distinct from Fgf7<sup>Pos</sup> MANC, while both subpopulations are located close to AT2 cells.
3.6 | rMCs-Sca1\textsuperscript{Pos} are impacted by obesity and gender

Massive damages to the AT2s occur following various injuries such as viral infection. As a consequence, the transdifferentiation of the LIFs into activated MYFs has been proposed, thereby leading to fibrosis formation.\textsuperscript{7} Obesity/diabetes and gender are thought to be aggravating pre-existing conditions predicting the severity of the disease.\textsuperscript{12} To explore the impact of obesity/diabetes and gender on the functionality of the rMCs-Sca1\textsuperscript{Pos} cells, we used 6-8 weeks C57BL6 males and females as well as Leptin-deficient ob/ob (aka ob/ob) mutant male and female mice (n = 3 for each gender and for WT and mutant). Figure 6A shows the analysis by flow cytometry of rMCs-Sca1\textsuperscript{Pos} in C57BL/6 mice vs C57BL/6 Leptin-deficient ob/ob mice. Interestingly, a drastic reduction of the percentile of rMCs-Sca1\textsuperscript{Pos} is observed in ob/ob mice compared to wild type mice (27.7% vs 9%, respectively). We also functionally tested the rMCs-Sca1\textsuperscript{Pos} from these different mice by co-culturing them with sorted AT2 cells using the alveolosphere assay. When the rMCs-Sca1\textsuperscript{Pos} are isolated from ob/ob male mice presenting the two risk factors, obesity and male (Figure 6B), we observed a complete absence of organoid formation (n = 3). However, when rMCs-Sca1\textsuperscript{Pos} are isolated from female ob/ob mice presenting only obesity as a risk factor, a significant number of organoids are present (3% CFE, n = 3). This CFE is, however, lower than the one observed in non-obese C57BL6 female wild type mice (5% CFE, n = 3) indicating that obesity alone is already impacting the functionality of the rMCs-Sca1\textsuperscript{Pos}. Interestingly, non-obese C57BL6 male wild type mice display also a reduced CFE compared to non-obese C57BL6 female wild type mice (2% vs 5%, respectively, n = 3) indicating that the

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**FIGURE 5** Analysis and comparison of Fgf10 and Fgf7 mRNA expression in relation to pro-Sftpc\textsuperscript{Pos} cells in the adult mouse lung. A, In situ hybridization for Fgf10 mRNA (in red) and IF staining against for pro-Sftpc (in green). Low and high magnification. Quantification of Fgf10 expressing cells close to Pro-Sftpc\textsuperscript{Pos} cells. B, In situ hybridization for Fgf7 mRNA (in red) and IF staining against for pro-Sftpc (in green). Low and high magnification. Quantification of Fgf7 expressing cells close to Pro-Sftpc\textsuperscript{Pos} cells. C, Co-staining of Fgf10 and Fgf7 expressing cells. Quantification of Fgf7, Fgf10 as well as Fgf7/Fgf10 expressing cells compared to total cells. Scale bar for low magnification: 50 μm and Scale bar for high magnification 200 μm

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gender alone is also impacting the functionality of the rMCs-Sca1Pos. We also observed that age was also having the same effect than obesity and gender on the capacity of the rMCs-Sca1Pos to maintain the proliferation of the AT2 stem cells (data not shown).

4 | DISCUSSION

4.1 | rMC-Sca1PosFgf10Pos are different from the rMC-Sca1PosAxin2Pos

A major problem arising from key publications using flow cytometry as a primary experimental approach is the lack of independent studies reporting the reproducibility of the results. This is due in part to the type of flow sorter used, the sophisticated flow cytometry protocols, including the gating conditions used and the lack of comparison with other endogenous population at the time of the sort as internal controls. This is particularly crucial in the context of the lung mesenchyme which is still a huge black box both in human and in mice in spite of recent scRNA seq studies. In our study, we adopted an unbiased and reproducible FACS strategy to sort both epithelial and mesenchymal populations. We used Sca1 as a resident mesenchymal cell marker to refine the rMC population. In previous studies, Cd45NegCd31NegSca1Pos cells were detectable by flow cytometry in the postnatal lung coincidently with the transition from the saccular to the alveolar stage of lung development. This population also co-expressed Cd34, Thy1 (Cd90), as well as Pdgfrα and was proposed to mark LIFs. Interestingly, our gene array data shows that Cd90 is highly expressed in rMC-Sca1PosFgf10Pos compared to rMC-Sca1PosAxin2Pos (data not shown). In another study, it was shown
that Cd45negCd31negEpcamnegSca1pos (rMC-Sca1pos cells) could be refined into two main subpopulations, namely Cd166posCd90neg and Cd166negCd90pos. The Cd166negCd90pos subpopulation contained undifferentiated mesenchymal progenitors capable of differentiating toward the LIF and the MYF lineage while the Cd166posCd90neg subpopulation contained more differentiated mesenchymal cells already committed to the MYF lineage. Additionally, the Cd166negCd90pos expressed high levels of Fgf10. Further organoid-based experiments indicated that Cd166pos compared Cd166neg displayed higher activity in supporting the proliferation of epiSPC. In vitro culture of rMC-Sca1pos cell showed that these cells lost their capacity to maintain epiSPC proliferation over time. This activity could be completely rescued by co-culturing these rMC-Sca1pos cells with the TGFB1-inhibitor SB431542. Such treatment was associated with the upregulation of endogenous Fgf10 expression in in vitro cultured rMC-Sca1pos.14 Finally, a drastic increase in CFE was observed upon treatment of rMC-Sca1pos-epiSPC co-culture with recombinant FGF10. In these experimental conditions, FGF7 did not display such activity.15 Taken together, our results indicate that rMC-Sca1posFgf10pos correspond to a specifically enriched population of LIFs capable of maintaining AT2 stem cell proliferation and differentiation. A recent milestone study published in Cell described a mesenchymal cell subpopulation called MANC (Mesenchymal Alveolar Niche Cells), which was positive for Axin2, Pdgfra, Wnt2, Ii6, and Fgf7. MANCs are located close to AT2s and sustain in vitro the proliferation and differentiation of AT2 stem cells. The current knowledge, before this study was therefore that the MANC were considered at the top of a hierarchy of mesenchymal niche cells and were likely to be important for both homeostasis and repair after injury. The current study brings into light a novel challenger for this important role. Our results indicate that rMC-Sca1posFgf10pos are likely different from the rMC-Sca1posAxin2pos (aka the MANC, but isolated in our experimental conditions) but has, nonetheless a similar function in regards to the AT2 stem cells. In addition, we cannot exclude the possibility that AT2 stem/progenitor cells are also heterogeneous, and that rMC-Fgf10pos and rMC-Axin2pos (MANC) may be targeting different AT2 stem/progenitor cell subsets.

4.2 Are the rMC-Sca1posFgf10pos more relevant than the rMC-Sca1posAxin2pos for the repair process after injury?

Given the fact that both subpopulations appear to sustain the proliferation and differentiation of AT2 stem cells in vitro, a natural question is therefore whether they have redundant functions or whether one population appears to be more crucial than the other. From the angle of Fgf signaling and based on the consequence of Fgf7 vs Fgf10 gene inactivation in mice, we can conclude that rMC-Sca1posFgf10pos are likely important. Fgf10 inactivation leads to lung agenesis21 while Fgf7 null mice are viable and display no obvious lung phenotype.22 Changes in endogenous Fgf10 expression has been correlated with disease progression and/or repair after lung injury both in mice and humans.23-25 Evidence for such a role for Fgf7 in the embryonic or adult lung in human or mice are still lacking in spite of the fact that Fgf7 was discovered 7 years before Fgf10.26-27 Another important question is whether it matters if mesenchymal niche cells express Fgf7 vs Fgf10 as they are both ligands acting through Fgfr2b. Indeed, it does matter as Fgf7 and Fgf10, although belonging to the same Fgf subfamily of paracrine growth factors elicit different biological activities on isolated lung epithelium grown in Matrigel.4 During the process of lung branching, Fgf10 induces the formation of new buds by a process of chemotaxis, while Fgf7 triggers the proliferation of the epithelium leading to the formation of a cyst-like structure.

4.3 The activity of the rMC-Sca1pos cells is impacted by obesity and gender

In the context of lung fibrosis, the accumulation of activated MYF-producing extracellular matrix components modifies the lung structure and negatively impacts gas exchange. The LIF to MYF reversible differentiation switch appears to be a key process in fibrosis formation and resolution. Moreover, this transition was also shown in vitro in response to hyperoxia, as a key event in bronchopulmonary dysplasia (BPD). AT2 cells express angiotensin-converting enzyme II (ACEII), a main receptor for SARS-CoV-2.30 Interestingly, SARS-CoV-2 induces the expression of transforming growth factor β (TGF-β),31 which has been described to trigger the LIF to activated MYF transition. Such transition can be reversed by the administration of a PPARγ agonist (Rosiglitazone) as well as by the antidiabetic drug metformin.7,8 Interestingly, metabolic dysregulation such as the one observed in obese patients has been associated with a worst prognostic in case of COVID-19. A similar conclusion has been reached for the gender as well as for the age. Indeed, we can detect the impact of obesity and gender on the functionality of the rMC-Sca1pos cells to sustain AT2 stem cell proliferation and differentiation. Our findings open the way to screen for drugs capable of restoring the stromal niche capabilities.

5 Conclusion

In this study, we have shown that rMC-Sca1posFgf10pos/LTpos is an important rMC subset allowing the proliferation of AT2 stem cells. We propose that this activity can be negatively impacted by many factors such as obesity and gender. In the future, restoring rMC function may be instrumental to optimize repair after injury.
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CONFLICT OF INTEREST
The authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS
S.T.: performed all experiments, conceived the project and wrote the manuscript, contributed to the article and approved the submitted version; M.H and A.I.V.A.: performed FACS analysis and sorting for samples, contributed to the article and approved the submitted version; J.W.: carried out the bioinformatics analysis, contributed to the article and approved the submitted version; S.H. and C.C.: contributed to the article and approved the submitted version; J.Z. and S.B.: conceived the project and wrote the manuscript, contributed to the article and approved the submitted version.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are openly available in GEO accession reference number GSE162859.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

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