Persistency of Mesenchymal Stromal/Stem Cells in Lungs

Erica Ferrini

Università degli Studi di Parma Dipartimento di Scienze Medico-Veterinarie

Fabio Franco Stellari

Chiesi Farmaceutici Spa

Valentina Franceschi

Università degli Studi di Parma Dipartimento di Scienze Medico-Veterinarie

Francesca Macchi

Università degli Studi di Parma Dipartimento di Scienze Medico-Veterinarie

Luca Russo

Università degli Studi di Parma Dipartimento di Scienze Medico-Veterinarie

Alba Murgia

University of Modena and Reggio Emilia, Department of Medical and Surgical Sciences for Children and Adults: Università degli Studi di Modena e Reggio Emilia

Giulia Grisendi

University of Modena and Reggio Emilia, Department of Medical and Surgical Sciences for Children and adults

Gino Villetti

Chiesi Farmaceutici Spa

Massimo Dominici

university of Modena and Reggio Emilia, Department of Medical and Surgical Sciences for Children and Adults

Gaetano Donofrio (✉ gaetano.donofrio@unipr.it)

Università degli Studi di Parma Dipartimento di Scienze Medico-Veterinarie

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Abstract

Background. Mesenchymal stromal/stem Cells (MSCs) are a fibroblast-like cell population with high regenerative potential that can be isolated from many different tissues. Several data suggest MSCs as a therapeutic tool capable of migrating to a site of injury and guide tissue regeneration mainly through their secretome. Pulmonary first-pass effect occurs during intravenous administration of MSCs, where 50 to 80% of the cells tend to localize in the lungs. This phenomenon has been exploited to study MSCs potential therapeutic effects in several pre-clinical models of lung diseases. Data demonstrated that, regardless the lung disease severity and the delivery route, MSCs were not able to survive longer than 24h in the respiratory tract, but still surprisingly determining a therapeutic effect.

Methods. In this work, two different mouse bone marrow derived MSCs (mBM-MSCs) lines, stably transduced with a third-generation lentiviral vector expressing Luciferase and GFP reporter genes tracking MSCs *in vivo* biodistribution and persistency, have been generated.

Results. Cells within the engrafted lung were *in vivo* traced using the high throughput Bioluminescence Imaging (BLI) technique, with no invasiveness on animal, minimizing biological variations and costs. *In vivo* BLI analysis allowed the detection and monitoring of the mBM-MSC clones up to 28 days after implantation, independently from the delivery route.

Conclusions. This longer persistency, than previously observed (24 hours), could have a strong impact in terms of pharmacokinetic (PK) and pharmacodynamics (PD) of MSCs as a therapeutic tool.

Background

At the beginning of their discovery, 1970s, mesenchymal stromal/stem cells (MSCs) were described as a bone marrow population of cells with fibroblastic and clonogenic properties; for these reasons were defined as “colony forming unit fibroblasts” [1–3]. Currently MSCs can be obtained from many tissues and a minimal criteria for their definition was internationally established [4]. They should have: 1) fibroblastic-like morphology; 2) adhesion properties on plastic; 3) the capacity to differentiate down to chondrogenic, osteogenic and adipogenic lineages and 4) express CD105, CD73, CD44, CD90 and not express CD11b, CD79α/CD19 and HLA-DR surface markers [4].

MSCs have been shown to exert therapeutic potential through their secretome (comprehensive of a complex array of components ranging from soluble secreted factors to factors encapsulated in extracellular vesicles with anti-inflammatory as well as growth properties) and through the capability of migrating to the site of tissue injury, a characteristic that involves a contact dependent mechanism of action [5, 6]. Recently, it has been observed in a sepsis model that heat-inactivated and secretome-deficient MSCs were still capable of inducing monocyte recognition, phagocytosis, and selective apoptosis. Interestingly, these activities were passively enabled by the MSC structures [7]. MSCs exhibit a diverse array of effects and multifunctional mechanisms of action, with no single and overarching biological effect. Probably, a complicated network, orchestrated by MSCs and in relation with nearby

cells, of the injured tissue, generate a biological reaction out-coming in a “therapeutic effect”. Many strategies have been adopted to potentiate MSCs therapeutic profile and reduce the amount of cells administered with the aim of reducing procedure costs and increase their safety for the patient. These strategies include: optimising culture conditions, MSCs activation before administration and, probably the most important, alteration of MSCs biodistribution.

When MSCs are intravenously administrated, most of them (~ 50–80%) tend to localize to the lung (first-pass effect) [8–11] due to their large size (~ 20–30 μm of diameter) [10, 12, 13], larger than circulating immune cells and lung capillary [14]. Furthermore, the number of trapped cells decreased with the administration of a vasodilator [10, 12, 13], supporting the hypothesis that MSC size is a major contributor to the first-pass effect.

This lung trapping, although considered an obstacle when MSCs needs to be delivered to organs different than lung, was exploited for repair, remodelling and/or regenerating damaged lungs. Preclinical therapeutic models exploiting MSCs for different lung diseases, such as Idiopathic Pulmonary Fibrosis (IPF) [15], Bronchopulmonary Dysplasia (BPD) [16], Cystic Fibrosis (CF) [17], Chronic Lower Respiratory Disease (CLRD) [18] and Acute Respiratory Distress Syndrome (ARDS) [19] have been generated. For each of the model accounted, regardless to the route of delivery (intravenous, intratracheal, intraperitoneal and intranasal), cells reaching the lung did not survive longer than 24 hours [20, 21], after which were cleared and, although in some case limited, a therapeutic effect was surprisingly and still achieved. Based on these data, in the present work, MSCs persistency in the lung was revised and a longer persistency was observed.

Methods

Cell cultures

Bone marrow-derived murine MSCs (mBM-MSCs) clones were isolated from C57BL/6 mice. Whole bone marrow cells were cultured at a density of $1 \times 10^6/\text{cm}^2$ and the plastic adherent fraction were defined as MSCs. These mBM-MSCs were characterized by the expression of typical MSC markers as: CD29, CD49e, CD90, CD105, and Sca-1, whereas they lacked the expression of CD45. Cells were grown in complete Eagle's Minimal Essential Medium (EMEM, Euroclone) containing 1 mM of Sodium Pyruvate (Gibco), 2 mM of L-glutamine (Sigma), 100 IU/mL of penicillin (Sigma), 100 $\mu\text{g}/\text{mL}$ of streptomycin (Sigma) and 2.5 $\mu\text{g}/\text{mL}$ of Amphotericin B (Sigma), supplemented with 10% Fetal Bovine Serum (FBS) and incubated at 37°C, 5% CO₂. Every 3–4 days mMSCs were passaged with 0.05% Trypsin-EDTA (Gibco) when they reached the 80% confluence. In this study we used mMSCs between passage 5 and 10.

Lentiviral construct preparation and lentivirus reconstitution

The lentiviral construct pLuc/GFP was generated sub-cloning the Luciferase ORF, excised from the commercial vector pGL3basic (Promega) through HindIII/XbaI digestion and after a T4 polymerase blunting treatment, in the lentiviral third generation pWPI vector (Trono Lab), opened with PmeI. pWPI is a

bicistronic transfer vector, able to efficiently deliver heterologous ORFs, in this case the Luciferase ORF, upstream of an IRES sequence (Internal Ribosomal Entry Site), followed by the Green Fluorescent Protein (GFP) ORF and a WPRE regulatory element (Woodchuck Hepatitis Virus (WHP) Post-transcriptional Regulatory Element).

Luc/GFP lentivirus has been reconstituted on HEK 293T cells in T175 cm² flask. Cells were co-transfected with 25 µg of pLuc/GFP transfer vector, 15 µg of p8.74 packaging vector, 13 µg of pMD2 pseudotyping vector and 5µg of pREV, diluted in 3 mL of complete DMEM (Euroclone) without serum and 145 µL of PEI (Polysciences, Inc.) (ratio 1:2.5 DNA-PEI). The transfection mixture was incubated for 15 minutes at room temperature, added with 4 times volume of complete DMEM without serum and transferred to the cell monolayer.

After 6 hours incubation at 37°C with 5% CO₂ the transfection medium was replaced with 25 mL of fresh complete medium and the cells were 48 hours incubated at 37°C, 5% CO₂. The flask was then frozen-thawed at - 80°C, Transfected Cells Supernatant (TCS) containing Luc/GFP lentivirus was clarified via centrifugation at 3,500 rpm for 5 minutes at 4°C, filtered through a 0.45 µm filter (Millipore), aliquoted, tittered by limited dilution and stored at -80°C.

Creation of stably transduced mouse bone-marrow derived mesenchymal stem/stromal cells (mBM-MSCs)

mBM-MSCs clone #2 and #11 were seeded at a concentration of 10⁵ cells in a 25 cm² flask with 5 mL of complete EMEM with 10% FBS for 4 hours at 37°C in an atmosphere with 5% CO₂. When the cells were attached, they were transduced with 5 ml of non ultra-centrifuged, Luc/GFP lentivirus. After 24 hours the medium was replaced with 5 mL of fresh complete medium, incubated at 37°C and split when they reached the confluence. The transduction efficiency of mBM-MSC and the percentage of GFP-positive/green fluorescent cells were evaluated by flow cytometry analysis using a FACS Canto II (BD Bioscience). Fluorescence intensity was determined with 50,000 cells per sample, using a gated strategy for GFP signals based on the background signal from the non-transduced cells. Data acquisition and analysis were carried out with Diva 7 software (BD Bioscience). Luciferase expression was also assayed for the characterization of different transduced MSCs clones before *in vivo* studies. *In vitro* titration of bioluminescence signals was obtained diluting cells in PBS (1:2) starting from 10'000 cells/well. 5 µL of luciferin per well were added, and photons emitted from transduced cells were immediately measured using IVIS imaging system (PerkinElmer Inc.) and quantified with the software Living Image® version 4.3.1. (PerkinElmer Inc.). An average of photons/cells was reported.

MTT assay

Parental clone #2Luc/GFP and #2LucRi/GFP MSCs were seeded 1,5x10⁴ cells/well in 0.5 mL of complete medium in 48 multi-well plates. After 24, 48, 72 and 96 hours from cell seeding, culture medium was replaced with 0.2 mL of MTT powder (0.5 mg/mL Thiazolyl Blue Tetrazolium Bromide, Sigma) diluted in serum-free culture medium. Plates were then incubated for 4 hours at 37°C. The deposition of violet crystals in the bottom well reveals the presence of metabolic active cells. The solution was then removed

and crystals dissolved in 0.2 mL of DMSO. Absorbance was immediately quantified using a plate reader (xMark Microplate Spectrophotometer, Bio-Rad) at 570 nm wavelength and optical densities (O.D.) quantified through the Microplate Manager Software 6 version 6.3 (Bio-Rad). At each time point of observation, the proliferation assay was measured in triplicate for both the cell lines and the assay was repeated at least twice with similar results.

In vivo studies

This study was conducted using female inbred C57BL/6 mice from Envigo (San Pietro al Natisone, Udine, Italy) and Charles River Laboratories (Calco, Lecco, Italy) aged 7 to 8 weeks. Prior to use, mice were acclimatized for at least 5 days to the local vivarium conditions (20–24°C room temperature; 40–70% relative humidity; 12 hours of light-dark cycle), having free access to standard rodent chow and softened tap water. All animal experiments described herein were authorized by the official competent authority and approved by the intramural animal-welfare committee for animal experimentation of Chiesi Farmaceutici under protocol number: 733/2019-PR and comply with the European Directive 2010/63 UE, Italian D.Lgs 26/2014 and the revised “Guide for the Care and Use of Laboratory Animals.”

Parental clone #11Luc/GFP and #2LucRi/GFP MSC cells were firstly administered via intravenous (IV) or intratracheally (IT), 5×10^5 cells/mouse and 10^6 cells/mouse, respectively, in 4 different groups (n=5) of C57BL/6 female mice (Envigo).

Subsequently, 10^6 #2Luc/GFP cells/mouse were IT implanted in 5 C57BL/6 mice (Envigo). After 14 days from instillation mice were culled, lungs were isolated and homogenized. #2Luc/GFP cells were sorted by FACS and expanded. After 24h incubation with growth medium supplemented either with FBS (Gibco) (n = 5) or homologous serum (Sigma) (n = 5), they were lung re-implanted (named #2LucRi/ GFP) in C57BL/6 female mice (Envigo) via IT (10^6 cells/mouse).

Finally, a comparison between two different animal producers (Envigo and CRL) was conducted: 10^6 #2Luc/GFP cells cultured with FBS were intratracheally administered in Envigo (n = 5) and CRL (n = 5) C57BL/6 female mice.

For In vivo and *ex vivo* bioluminescence detection, animals were slightly anesthetized with 2.5% isoflurane (IsoFlo, Zoetis) and shaved, to allow longitudinal detection of *in vivo* Bioluminescent Imaging (BLI) signal. At day 0, 7, 14, 21 and 28 from cells implantation, mice were intraperitoneally injected (IP) with 200 µL/mouse luciferin substrate (15 mg/mL in saline, PerkinElmer Inc.) and slightly anesthetized with isoflurane (2.5%). After 15 minutes they were imaged in supine position using IVIS imaging system. Bioluminescence from the chest of the mice was quantified in photons/sec/cm² using the software Living Image® version 4.3.1. (PerkinElmer Inc.). At the endpoint experiment, after the *in vivo* imaging, mice were culled with an overdose of anaesthetic followed by bleeding from abdominal aorta, lungs were harvested and imaged *ex vivo* using IVIS imaging system.

Sorting of bright clone #2GFP/Luc mBM-MSC from lung homogenates

Mice lungs (n = 8) were enzymatically digested at 37°C for 90 min with 0.4 mg/ml of collagenase P. Enzyme activity was neutralized with Dulbecco's modified Eagle's medium (DMEM), containing 10% FBS and centrifuged at 1200 g for 10 min to obtain a cell suspension. Cells were then resuspended in PBS at final concentration of 1×10^6 cells/ml and #2GFP/Luc mBM-MSC were sorted by FACSARIA III flow cytometer (BD Biosciences) equipped with two air-cooled lasers at 488 and 633 nm wavelengths. Data were analysed by Diva software (BD Biosciences).

Results

Stable labelling for *in vivo* tracking of mMSCs

Before to attempt mouse MSCs (mMSCs) lung localization *in vivo*, two different clones (#2 and #11) of C57BL/6 mBM-MSCs were stably transduced/transgenized with two different reporter genes to easy track their bio-distribution *in vivo*. A third generation lentiviral vector delivering Luciferase and GFP in a bicistronic transfer vector, pWPI (Addgene; <https://www.addgene.org/>), was generated by sub-cloning the Luciferase ORF upstream of an IRES (Internal Ribosomal Entry Site) followed by the GFP ORF and WPRE (Woodchuck Hepatitis Virus (WHP) Post-transcriptional Regulatory Element) (Fig. 1A). VSVg pseudotyped lentiviral vector particles were reconstituted in HEK293T cells and mBM-MSCs cells transduced with an efficiency close to 100% of GFP expression, measured by Flow cytometry analysis (Fig. 1B). Further, both clones nicely express Luciferase, as monitored by IVIS, with the clone #2 (#2Luc/GFP) displaying a stronger signal intensity (2037 photons/cell) respect to the clone #11 (#11Luc/GFP) (1298 photons/cell) (Fig. 1C and D). However, this difference could not be attributed to general biological differences between the two clones, but probably only limited to transgene expression due to a different site of integration of the provirus genome into the host genome or different provirus genome copy number integrated. Although this is an interesting issue but because out of the purpose of this work, it was not further investigated.

Stable transduction of mMSCs allows *in vivo* direct monitoring of mMSCs lung persistency by BLI

Although the clone #2Luc/GFP displayed a better luciferase expression respect to the clone #11Luc/GFP, both clones were used for lung *in vivo* engraftment and to investigate if clonal selection could impact on cell engraftment. #11Luc/GFP and #2Luc/GFP cells were transplanted via IV (5×10^5 cells) or IT (10^6 cells) in 4 different groups (n=5) of C57BL/6 female mice and monitored by IVIS at different times (0, 1, 5, 7, 14, 21, 28 days) post implantation. In all groups of mice, independently from the route of administration and the cellular clone employed, cells localization into the lung was successful. #2Luc/GFP IT implanted cells were better detected in the lung (up to 7 days) respect to #11Luc/GFP IT cells or #2Luc/GFP IV cells (Fig. 2A and B) and well visualized, at least *ex vivo*, up to 28 days post implantation (Fig. 2C and D).

Transduced mBM-MSCs pre-adaptation into the lung does not enhance tissue persistency

Since #2Luc/GFP IT lung implanted cells could be better detected, with a decrease of signal intensity through the time for up to 28 days, it was of interest to investigate whether pre-adaptation of the cells in the lung could select a cellular phenotype, due to biochemical stimuli received from the environment, able to survive longer when reimplanted. Fourteen days post #2Luc/GFP IT lung implantation, cells were isolated by lung tissue dissociation and green cells sorted by Fluorescent Activated Cell Sorting (FACS). These cells, defined as #2LucRi/GFP, were first *in vitro* expanded and then characterized in terms of MSC markers/purity, proliferation potential and differentiative capabilities/mesenchymal characteristics, compared to the parental clone #2Luc/GFP (data not shown). #2LucRi/GFP cells maintained MSC characteristics/purity and stable lentiviral transduction (**Supplementary Fig. 1A and B**), besides a proliferating activity identical to #2GFP/Luc (**Supplementary Fig. 1C**). Further, when #2LucRi/GFP cells were transplanted IT into the mice lung, they survived for the same period of time as the parental #2Luc/GFP cells (**Fig. 3A and B**). Therefore, pre-adaptation of #2GFP/Luc into the lung did not impact their survival.

Homologous serum does not impact cell persistence into the lung

mBM-MSCs are grown in the presence of FBS and cells tend to unspecifically endocytose serum proteins. These bovine proteins, hence antigens, could be released and activate a strong immune response when implanted into the mice lung, thus compromising cell survival. #2Luc/GFP cells were incubated with medium containing murine homologous serum for 24 hours in order to eliminate heterologous traces and then IT lung implanted. As shown in **Fig. 3C and D**, although slight differences could be visually observed, no statistically significant differences in terms of engraftment were observed between #2Luc/GFP cells incubated with homologous serum, respect to those incubated with FBS. This was confirmed also between #2Luc/GFP and #2LucRi/GFP (data not shown).

Host genetic background does not impact cell persistency into the lung

Since the C57BL/6 host genetic background was syngenic with that of the transplanted cells, it was wondered if host mice, derived from different producers (Charles River Laboratories (CRL) and Envigo (ENV)), could create a difference in terms of MSCs persistency. This might be due to very small genetic differences such as gene copy-number variants or Single Nucleotide Polymorphisms (SNP). #2Luc/GFP cells were transplanted IT into two groups of mice lung (CRL = 5 and ENV = 5) and, as observed in **Fig. 4**, no differences in terms of persistency were observed between the two groups.

Discussion

In the present work, mBM-MSCs localization and persistency in the mouse lung was examined. mBM-MSCs were labelled via stable transduction with a replicating incompetent lentiviral vector delivering an expression cassette for luciferase and GFP. Since the luciferase ORF was in bicistronic form with GFP by an IRES sequence [22, 23], the level of GFP expression in the transduced cells, should reflect the expression level of the ORF upstream to the IRES which, in this specific case, was luciferase. However, in most of the cases, the luciferase expression level is even higher respect to that of the downstream GFP ORF [22, 23]. Using this strategy, mBM-MSCs expressing the highest amount of luciferase could be

selected by simply sorting cells with the highest GFP expression. Thus, increasing the sensitivity of the system since BLI was employed for detection.

BLI is a powerful technique based on the detection of visible light produced during luciferase-mediated oxidation of a molecular substrate in the presence of the enzyme resulting from its expression *in vivo* as a molecular reporter. Bioluminescence arising from luciferase can be imaged as deep as several millimeters within tissues, allowing at least organ-level resolution. Being simple to execute and minimally invasive, BLI enables monitoring and serial quantification of biological processes without sacrificing the experimental animal. This powerful technique can therefore reduce the number of animals required for experimentation because multiple measurements can be made in the same animal over time, thus also minimizing the effects of biological variations. Whole animal bioluminescent imaging is progressively becoming more widely applied by investigators with diverse backgrounds because of its low cost, high throughput, and relative ease of operation in visualizing a wide variety of *in vivo* cellular events. In addition, the ability to continually monitor a single individual reduces the amount of inter-animal variation and can reduce errors, leading to higher resolution and less data loss. In addition, the constant progress in the hardware and software, required for this technique, facilitates its application by researchers with little background in molecular imaging on living animals [24, 25]. Taking advantages of an integrated bioluminescent over-expressed reporter gene into the genome of transduced mBM-MSCs and of BLI, we have been able to localize mBM-MSCs in the lung in real time up to 28 days, independently from the route of administration and from the amount of cells administered. There are two main methods to introduce cells into the lung: systemic delivery, IV and local delivery, IT. Although IV represents the simplest delivery way, it rises a thromboembolic potential risk directly correlated to the number and the concentration of cells injected. In particular, concentration above 10^7 cells/ml was shown, at least in mice, to generate a significant increase of pulmonary embolism and consequent mortality [26]. In our case, a single dose of $10^5/200$ μ L cells was chosen, because, after several attempts, this amount of cells showed to be the best compromise between risk of embolism and signal detection. Alternatively to the IV route, IT route was employed too. The lung is the only corporeal district where compounds can be directly delivered through the airways. Although previous works reported positive [27–29] and negative [27, 30] results, cell survival levels in these studies were performed using fluorescent techniques, which could justify these strong discrepancies. In our study, to determine if the airway might be a more effective route of mMSCs delivery, $10^5/50$ μ L cells were administered. Although the number of IT delivered cells was larger respect the IV ones, the cell persistency and signal kinetic we obtained in the lung were similar, even if a fraction of IT cells is generally coughed out by mucociliary clearance.

Even if with very low efficiency, it was shown that mMSCs derived from cytokeratin-18 (K18) promoter driven GFP (K18GFP) transgenic mice, when delivered to the lung of wild-type recipient, could engraft and transdifferentiate in epithelial committed cells showing K18GFP transgene *in vivo* up regulation but not *in vitro* [28, 31]. This data suggests that the lung environment is responsible for lineage commitment and perhaps for long term survival of mMSCs [28, 31]. Based on this assumption, mBM-MSCs were pre-

adapted in the lung, re-isolated and IT re-delivered to the lung. Even though, cell survival or at least their detectability did not vary, still persisting for 28 days.

A generally relevant aspect following MSCs allogeneic or xenogeneic transplantation is the recipient immune response against the transplanted cells. T-cells and antibodies production has been shown not only against the cells but also against FBS derived antigens present in the cell media used to culture the cells [32, 33]. We did substitute FBS in cell culture medium with mouse autologous serum 24 hours previous cell delivering into the lung. However, no substantial differences in terms of cell survival, were observed.

C57BL/6 mice genetic background are largely used for adoptive transfer experiments and a large number of sublines with genetic polymorphisms are used around the world [34] showing defined immune-phenotypic differences [35]. Although it is difficult to define the impact that a genetic drift of a mouse specific subline could have on mMSCs survival when delivered to the lung, we limited our investigation to two sublines coming from two different mouse breeders, without observing significant differences.

Conclusions

Over the last 10 years, MSCs therapy approach for lung diseases constantly progressed, probably slower than expected and respect to the starting enthusiasms. Initially, a certain number of works, accompanied by a small body of noteworthy literature, suggested that exogenous MSCs, as well as other type of cells with a residual pluripotency, could engraft and differentiate into airway, alveolar epithelial, pulmonary vascular and/or pulmonary interstitial cells [27–29]. It was hoped that when MSCs are delivered and engraftment takes place, they will differentiate, mature and integrate into the target organs, inducing/resulting in tissue regeneration. Thanks to sophisticated technologies, researchers have been able to demonstrate that although MSCs can be induced *in vitro* to partially differentiate and express specific markers of alveolar or epithelial cells, this was a very rare or absent event *in vivo* [36]. Since the initial idea of engraftment and differentiation, has been largely abandoned and substituted with MSCs immunomodulatory and paracrine actions, basic knowledge such as time of persistency of MSCs into the lung following systemic or local administration is still debated. This issue has strong implication in terms of pharmacokinetic (PK) and pharmacodynamics (PD) for MSCs with relevant therapeutic impact in their regenerative potential [37–39].

List Of Abbreviations

ARDS (Acute Respiratory Distress Syndrome); BLI (Bioluminescence Imaging); BM-MSCs (Bone Marrow Mesenchymal Stem Cells); BPD (Bronchopulmonary Dysplasia); CF (Cystic Fibrosis); CLRD (Chronic Lower Respiratory Disease); CRL (Charles River Laboratories); DMEM (Dulbecco's modified Eagle's medium); EMEM (Eagle's Minimal Essential Medium); ENV (Envigo); FACS (Fluorescent Activated Cell Sorting); FBS (Fetal Bovine Serum); GFP (Green Fluorescent Protein); K18 (cytokeratin-18); K18GFP (cytokeratin-18 promoter driven GFP); IP (intraperitoneally injected); IPF (Idiopathic Pulmonary Fibrosis);

IRES (Internal Ribosomal Entry Site); IT (intratracheal); IV (intravenous); Luc (Luciferase); mBM-MSCs (mouse Bone Marrow Mesenchymal Stem Cells); MSCs (Mesenchymal Stem Cells); MTT (Thiazolyl Blue Tetrazolium Bromide); ORF (Open Reading Frame); PD (pharmacodynamics); PK (pharmacokinetic); SNP (Single Nucleotide Polymorphisms); TCS (Transfected Cells Supernatant); WPRE regulatory element (Woodchuck Hepatitis Virus (WHP) Post-transcriptional Regulatory Element).

Declarations

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Authors' contributions: GD, MD and FFS conceived the experiments. EF, VF, FM, LR, AM, GG, FFS and GD performed the experiments. GD wrote the paper and MD edited the final version.

Availability of data and materials: Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

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Figures

Figure 1

Generation and *in vitro* characterization of stably transduced mBM-MSCs clones. A) Schematic representation not to scale of the pLuc/GFP third generation lentiviral construct carrying an expression cassette containing Luciferase (Luc, colored in grey) and Enhanced Green Fluorescent Protein (EGFP, colored in green) Open Reading Frames (ORFs) linked by an Internal Ribosome Entry Site (IRES, colored in red) sequence and transcriptionally regulated by human Elongation Factor 1 alpha (hEF1 α) promoter, along with a synthetic intron. B) Assessment of GFP expression by FACS analysis of clone #2 and #11Luc/GFP lentivirus transduced mBM-MSCs. Non-transduced cells are used as negative control (Ctrl-). C) *In vitro* titration of bioluminescence emission from clone #2 and #11 by IVIS bioluminescence imaging system. Clone #2 emits higher photon titers compared to clone #11. D) Plot of linear correlation between total photon emission (in y axis) and cell numbers (in x axis). Quantification of photons emitted per single cell allowed the identification of clone #2 as the brighter clone.

Figure 2

In vivo and ex vivo Imaging of mBM-MSCs in mice lung. A-B) In vivo Bioluminescence Imaging (BLI) of four groups of mice (n=5 each) intratracheally (IT) and intravenously (IV) inoculated with mBM-MSCs clone #2 and #11 (B and A, respectively). Mice were weekly monitored up to four weeks post inoculation. At each time point mice were intraperitoneally injected (IP) with 200 μ L/mouse luciferin substrate to allow longitudinal detection of In vivo BLI. At day 28 mice were anesthetized and lungs were harvested for ex vivo imaging using IVIS bioluminescence imaging system. C-D) BLI signal from mice chest of each group was quantified for seven times, expressed as photons/sec/cm² and plotted as mean $\pm$ SEM (Standard Error Mean) using the software Living Image® version 4.3.1. BLI signal at each time point represents the mean $\pm$ standard deviation of five animals.

Figure 3

In vivo and ex vivo imaging of IT injected mBM-MSCs. A) In vivo BLI of four groups of mice (n=5 each) intratracheally (IT) inoculated with mBM-MSCs clone #2Luc/GFP or clone #2LucRi/GFP. Mice were weekly monitored up to four weeks post inoculation. At each time point mice were longitudinally detected for in vivo BLI. At day 28 mice were anesthetized and lungs were harvested for ex vivo imaging using IVIS bioluminescence imaging system. No significant differences in survival were detected between mice transplanted with clone #2LucRi/GFP and mice transplanted with parental clone #2Luc/GFP. B) BLI signal from mice chest of each group was quantified for six times, expressed as photons/sec/cm² and plotted as mean $\pm$ SEM (Standard Error Mean) using the software Living Image® version 4.3.1. BLI signal at each time point represents the mean $\pm$ standard deviation of five animals. C) In vivo longitudinal BLI quantification comparison between IT inoculated mice with clone #2LucRi/GFP differentially cultured with murine or bovine serum. D) BLI signal from mice chest of each group was quantified for five times, expressed as photons/sec/cm² and plotted as mean $\pm$ SEM (Standard Error Mean) using the software Living Image® version 4.3.1. BLI signal at each time point represents the mean $\pm$ standard deviation of

five animals. No significant differences were detected between the two clones coltured in murine or bovine serum.

Figure 4

In vivo and ex vivo imaging of IT injected mBM-MSCs from different sources. A) In vivo BLI of IT mBM-MSCs clone #2LucRi/GFP inoculated CRL and ENV mice. Mice were weekly monitored up to four weeks post inoculation. At day 28 mice were anesthetized and lungs were harvested for ex vivo imaging using IVIS bioluminescence imaging system. No significant differences were detected between the two strains of mice thus host genetic background does not impact cell persistency into the lung. B) BLI signal from each mouse was quantified for five times, expressed as photons/sec/cm² and plotted as mean $\pm$ SEM. BLI signal at each time point represents the mean $\pm$ standard deviation of five animals.

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