

Developing cell therapy techniques for respiratory disease: Intratracheal delivery of genetically engineered stem cells in a murine model of airway injury

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Short title: Stem cell therapy for respiratory disease.

Abstract

Over the past decade, interest has increased in the use of exogenous stem cells to optimize lung repair and serve as carriers of a therapeutic gene for genetic airway disease such as cystic fibrosis. We investigated the survival and the engraftment of exogenous stem cells after intratracheal injection, in a murine model of acute epithelial airway injury already used in gene therapy experiments on cystic fibrosis. Embryonic stem cells and mesenchymal stem cells were intratracheally injected 24hr after 2% povidocanol administration, when epithelial airway injury was maximal. Stem cells were transfected with reporter genes immediately prior to administration. Reporter gene expression was analyzed in trachea-lungs and bronchoalveolar lavages using non-fluorescent, quantitative and sensitive methods. ELISA quantitative results showed that 0.4 to 5.5% stem cells survived in the injured airway. Importantly, no stem cells survived in healthy airway or in the epithelial lining fluid. Using X-Gal staining, transduced mesenchymal stem cells were detected in injured trachea and bronchi lumen. When the epithelium was spontaneously regenerated, the *in vivo* amount of engrafted mesenchymal stem cells from cell line decreased dramatically. No stem cells from primary culture were located within lungs at 7 days. This study demonstrated the feasibility of the intratracheal cell delivery for airway diseases with acute epithelial injury.

Introduction

Respiratory diseases remain one of the main causes of morbidity and mortality in the world. Interest has increased as to the possibility of optimizing the repair of the lung with the use of stem cells (SCs). In particular, combining SC ability to engraft into damaged lungs and their ability to serve as carriers of a therapeutic gene has a great potential for pulmonary fibrosis (Loebinger *et al.*, 2008) and for genetic airway disease such as cystic fibrosis. This developing therapeutic approach has been stimulated by early reports demonstrating that both embryonic stem cells and SCs derived from adult bone marrow, including mesenchymal stem cells, can differentiate into respiratory cells *in vitro*, thus acquiring phenotypic and functional markers of airway and alveolar epithelial cells (Coraux *et al.*, 2005; Wang *et al.*, 2005; Rippon *et al.*, 2006). Systemic administration of adult SCs from bone marrow in mice after total-body irradiation (Pereira *et al.*, 1995; Loebinger *et al.*, 2008; Sueblinvong *et al.*, 2008) and/or pollutant-reagent treatment have been reported recently (Beckett *et al.*, 2005; MacPherson *et al.*, 2006; Mei *et al.*, 2007; Serikov *et al.*, 2007; Xu *et al.*, 2007; Loebinger *et al.*, 2008) and showed that the administered SCs were mainly engrafted in alveolar spaces (Pereira *et al.*, 1995; Beckett *et al.*, 2005; Mei *et al.*, 2007; Serikov *et al.*, 2007; Xu *et al.*, 2007; Loebinger *et al.*, 2008; Sueblinvong *et al.*, 2008) and sometimes in conducting airway (Pereira *et al.*, 1995; Krause *et al.*, 2001; MacPherson *et al.*, 2005; Loi *et al.*, 2006; MacPherson *et al.*, 2006; Serikov *et al.*, 2007). The SC differentiation as pneumocytes (I or II) or airway epithelial cells was mostly evaluated using different fluorescence techniques. Reported engraftment rates of adult bone marrow SC-derived cells ranged from 0% (Wagers *et al.*, 2002) up to 20% (Krause *et al.*, 2001) in the lungs, and from 0.025% (Loi *et al.*, 2006) up to 4% (Krause *et al.*, 2001) in conducting airway. These discrepancies have been partly attributed to the different fluorescence techniques used to detect

the donor-derived epithelial cells, leading to significant artifacts (Loebinger *et al.*, 2008). Importantly, lung SC engraftment is currently estimated to be between 0.01% and 0.1% (Krause 2005; Bruscia *et al.*, 2006). Despite this low engraftment level, there is evidence that transplanted SC post-injury have some therapeutic effects (Loebinger *et al.*, 2008).

In most of these studies, systemic administration of SCs required total-body irradiation of the recipient to promote SC bone marrow engraftment, a difficult condition to apply in the clinical setting, especially in patients suffering from airway disease associated with chronic infections such as cystic fibrosis. Alternatively, considering the advantages of the intratracheal route to target the airway and the respiratory epithelium, more recent studies reported the intratracheal administration of adult SCs in reagent-injured lungs (Gupta *et al.*, 2007; Wong *et al.*, 2007). Using fluorescent techniques SC engraftment was enhanced with the intratracheal route as compared to the intravenous route (Wong *et al.*, 2007; Wong *et al.*, 2009) but remained 5-10% (Gupta *et al.*, 2007; Wong *et al.*, 2007; Wong *et al.*, 2009). The intratracheal route was also used for the administration of differentiated cells and showed both positive (Serrano-Mollar *et al.*, 2007) and negative results (Kuang *et al.*, 2005; Gupta *et al.*, 2007). SC or differentiated cell engraftment levels in these studies were mostly assessed using fluorescence techniques, which could explain these conflicting results.

The aims of our study were to evaluate the survival and the engraftment of different types of exogenous SCs and differentiated cells after intratracheal injection. We used a murine model presenting acute epithelial airway injury without total-body irradiation. This model was developed for gene therapy experiments on cystic fibrosis and consisted in intranasal injection of povidocanol detergent (Parsons *et al.*, 1998). Quantification of cell survival rates and cell location within lungs were performed using ELISA, biochemical assay and PCR.

Materials and methods

Animal model and intratracheal administration

Male, 8- to 10-week old Swiss mice were obtained from Janvier Laboratories (Le Genest St Isle, France). Animal experiments were performed under the approval of the *Guide for The Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996). Mice were anesthetized with an intraperitoneal injection of xylazine (15mg/kg) and ketamine (70mg/kg), and placed supine. Briefly, intratracheal administration was performed using a 22-gauge catheter placed within an intubation canula (Harvard Apparatus) that was removed immediately before injection. Airway injury was induced after anaesthesia by intratracheal administration of 25µl of 2% povidone iodine (PDI: polyoxyethylene 9-lauryl ether, Sigma, (Borthwick *et al.*, 2001; MacPherson *et al.*, 2005)) in phosphate-buffered saline (PBS) through the 22-gauge catheter. Cells (10⁶ cells/25µl) or PBS were intratracheally injected 24hr after PDI administration using the same protocol. Mice were sacrificed by an intraperitoneal lethal injection of nesdonal 24hr or 7 days after intratracheal administration.

Bronchoalveolar lavages

Bronchoalveolar lavages (BALs) were performed 24hr after CAT-transfected cell administration in healthy or injured airway. Mice were anesthetized and thoracic cavity was opened by careful dissection. The trachea was exposed, and a small transverse incision performed just below the level of the larynx. BAL was then performed using one dose of 1 ml of PBS, ensuring that both lungs inflated during the lavage process and that there was no leakage of lavage fluid from the trachea. BAL fluid was centrifuged at 400 g for 5 min. The supernatant was removed and stored at -80°C until ELISA. Cell pellet was suspended in lysis buffer and stored at -80°C until ELISA.

Cell cultures

Undifferentiated mouse embryonic stem cells (ESCs, R1 cell line, (Nagy *et al.*, 1993)) were cultured in dishes coated with 0.1% gelatine (Sigma). The culture medium was composed of Dulbecco minimum essential medium with 4.5g/l glucose (DMEM, Invitrogen) supplemented with 15% FCS (Invitrogen), 0.1% non essential amino acids (Invitrogen), 1mM sodium pyruvate (Invitrogen), 2mM L-Glutamine (Invitrogen), 10^{-7} M β mercaptoethanol (Sigma), penicillin/streptomycin (100U/ml and 100 μ g/ml respectively, Invitrogen) and 2000U/ml of LIF (Sigma).

Undifferentiated murine mesenchymal stem cells (BMC9s, (Dennis and Caplan 1996)) were cultured as previously described (Chateauvieux *et al.*, 2007) in alpha MEM medium with nucleosides (Invitrogen) supplemented with 10% FCS (Invitrogen), 2mM L-Glutamine (Invitrogen), penicillin/streptomycin (100U/ml and 100 μ g/ml respectively, Invitrogen).

A human hepatocellular carcinoma cell line (Hep 3B2.1-7, ATCC number HB-8064), transformed African green monkey kidney fibroblast cell line (COS-7, ATCC Number CRL-1651) and murine osteosarcoma cell line (mOS-J, (Joliat *et al.*, 2002)) were used for control experiments. Cells were cultured in DMEM medium with 4.5 g/l glucose supplemented with 15% FCS, 2 mM L-Glutamine, penicillin/streptomycin (100U/ml and 100 μ g/ml respectively).

Primary culture of adult mesenchymal stem cells

Total bone marrow was obtained from wild type adult male Swiss mice and male Rosa26 lacZ-mice (background C57Bl/6J x 129S2, kindly provided by Dr M.F. Gardahaut, Nantes, France) by flushing femurs and tibias with culture medium. Cells were plated at a density of 500 000 cells/cm² in medium composed of alpha MEM with nucleosides supplemented with 10% FCS, 2 mM L-Glutamine, penicillin/streptomycin (100U/ml and 100 μ g/ml respectively) and 2ng/ml of

human FGF2 (AbCys). The culture medium was changed at day 3 to remove non-adherent cells and was subsequently replaced weekly. The cells were grown for 2-3 weeks until confluence. Adherent cells were then detached by 0.5% trypsin-EDTA and plated at a density of 10 000 cells/cm². Subsequent passages and seeding of the cells were performed at a density of 5 000 cells/cm². From passage 8, MSC cultures were characterized by FACS analysis (FACS Calibur Instrument, BD Biosciences, CellQuestPro Software) after incubation with anti-CD45-PE, anti-CD90-PE, anti-CD29-CyChrome, anti-Sca1-PE, anti CD106-CyChrome (BD Biosciences) (Dominici *et al.*, 2006).

In vitro cell transfection with CAT or GFP reporter genes and evaluation of gene transfer system efficacy

ESCs, BMC9s and differentiated cells were transfected with pCIK-CAT (4.7 Kb) and pEGFP-C1 (4.7 Kb) plasmids encoding for Chloramphenicol AcetylTransferase (CAT) protein and GFP, using synthetic vectors just prior to intratracheal administration (Pitard *et al.*, 2001). DNA plasmids were complexed with ICAfectin® 441 according to the manufacturer's instruction (In-Cell-Art, Nantes, France). After 2hr, transfection complexes were removed by changing growth medium and cells were lysed for dosages or kept in culture for 24hr, 7 days, or injected intratracheally as indicated above.

MSCs from Swiss mice were transfected with pCIK-CAT and pEGFP-C1 plasmids using nucleofection (Nucleofector Solution, Amaxa Biosystem) just prior to intratracheal administration (Aluigi *et al.*, 2006). After 2hr, growth medium was changed and cells were lysed for dosages or kept in culture for 24hr, 7 days, or injected intratracheally as indicated above. The percentage of GFP-expressing cells was evaluated 24hr after GFP nucleofection using FACS analysis.

The efficacy of gene transfer systems was evaluated using cytometry for GFP-expressing cells and ELISA for CAT-expressing cells. The percentage of GFP-expressing cells was evaluated 24hr after GFP transfection using FACS analysis (FACS Calibur Instrument, BD Biosciences, CellQuestPro Software). CAT protein quantity per stem cell at 24hr was then calculated using *in vitro* data: CAT protein quantity in cell lysis buffer was divided by the cell number and by the percentage of GFP-expressing cells (obtained by FACS analysis). Finally, this value was used to determine the minimum number of CAT-expressing stem cells required to be detected by ELISA *in vitro* or *in vivo* considering that CAT ELISA detection threshold was 50pg CAT protein (see below).

In vitro stem cell transduction with nls-lacZ reporter gene

BMC9s were transduced with an amphotropic recombinant murine retrovirus vector containing the nls-LacZ reporter gene (LacZ gene encoding β galactosidase (β gal) coupled to a nuclear localization signal (nls), kindly provided by Dr. N. Ferry, Nantes, France). This retrovirus vector was obtained from the human producer cell line TELCeB6AF7 derived from the Te671 cell line as described in (Aubert *et al.*, 2002). 24hr recombinant retroviral supernatant was harvested from the confluent producer cell line and filtered through a 0.45 μ m membrane. The titer of the β gal supernatant, determined by endpoint dilution using Te671 target cells, was 2×10^8 transducing particles/ml. After BMC9 transduction, retrovirus vector was removed by changing growth medium and cells were lysed for dosages or kept in culture or injected intratracheally as indicated above.

To evaluate the percentage of β gal-transduced BMC9s, cells were fixed in 4% paraformaldehyde, washed with PBS and incubated in 5mM K₄Fe(CN)₆, 5mM K₃Fe₃(CN)₆ and 2mM MgCl₂ in PBS containing 0.5mg/ml X-Gal ([5-bromo-4-chloro-3-indolyl- β -D-

galactopyranoside] dissolved in N,N-dimethylformamide at 20mg/ml before dilution into the reaction mixture, Sigma) for 2hr, at 37°C. Cells were identified as positive for β gal activity by blue nuclear staining after X-Gal reaction. The percentage of β gal expressing cells *in vitro* was obtained by counting the number of nuclear-blue cells in a total of 200 cells.

MSCs from Swiss mice were transduced with a lentivirus vector containing the nls-lacZ reporter gene. The vector was derived from pHR' LacZ (Zufferey *et al.*, 1997) in which a nls from the SV40 T Ag was cloned in frame 5' to the LacZ cDNA. The lentivirus vector was produced on human embryonic kidney 293T cells as previously described (Selig *et al.*, 1999). After transduction with 50 MOI (Multiplicity Of Infection), lentivirus vector was removed by changing growth medium and cells were lysed for dosages or kept in culture or injected intratracheally as indicated above. The percentage of β gal-transduced MSCs was evaluated by *in vitro* X-Gal staining and counting as described above.

Reporter gene assay and estimation of survival rate

CAT-transfected cells, BAL cells and BAL fluid, or whole frozen trachea-lungs were homogenized in reporter lysis buffer (Roche Diagnostics) supplemented with protease inhibitors (Roche Diagnostics). After centrifugation at 10 000 rpm for 5min, CAT quantity was measured in supernatant with a VICTOR² multilabel counter (PerkinElmer), using a CAT enzyme-linked immunosorbent assay (ELISA) kit according to the instructions of the supplier (Roche Diagnostics). Each sample was analyzed in duplicate. CAT detection threshold was 50pg. Protein content was measured with a bicinchoninic acid (BCA) protein assay kit (Pierce).

To estimate cell survival rate 24hr after intratracheal administration, we first calculated CAT protein quantity per CAT-expressing cell, using *in vitro* data: CAT quantity in cell lysis buffer was divided by the cell number and by the percentage of GFP-expressing cells (obtained by

FACS analysis). Finally, the total *in vivo* CAT protein quantity per animal was divided by this *in vitro* value, in order to estimate the number of CAT-expressing cells per animal and subsequently the cell survival rate.

BMC9s and Rosa26 MSCs or whole frozen murine trachea-lungs were homogenized in 1ml reporter lysis buffer (Roche Diagnostics) supplemented with a protease inhibitor cocktail (Roche Diagnostics). After centrifugation at 10 000 rpm for 5min, β gal activity was determined by enzymatic fluorimetric assay using 4MUG (4-methylumbelliferyl- β -D-galactoside, Sigma) as a fluorescent substrate. Each cell and trachea-lung samples were analyzed in duplicate. β gal activity detection threshold was 230pg. Protein content was measured with a BCA protein assay kit. Additional experiments were done with lysates of β gal-transduced BMC9s or Rosa26 MSCs after three cycles of freezing and thawing. Viability was ascertained by trypan blue exclusion.

Detection of β galactosidase activity

Tissues were fixed in 4% paraformaldehyde (at room temperature, 20min) and rinsed 3 times with PBS. Immediately after fixation, trachea-lungs were stained by intratracheal infusion (though a 19-gauge needle, total volume = 5ml) and immersion in 5mM K₄Fe(CN)₆, 5mM K₃Fe₃(CN)₆ and 2mM MgCl₂ in PBS containing 0.5mg/ml X-Gal (as indicated above) for 6hr, at 30°C to avoid the detection of endogenous β gal activity (Weiss *et al.*, 1997). Tissues were again washed with PBS and immediately embedded in paraffin. Tissues were identified as positive for β galactosidase activity by nuclear blue staining after X-Gal reaction. Sections (4 μ m thick) were stained with nuclear fast red (Sigma).

Detection of *nls-lacZ* reporter gene

Total DNA from whole murine trachea-lungs was isolated for PCR analysis. Tissues were digested overnight with lysis buffer containing proteinase K (10mg/ml, Sigma). Total DNA was

extracted with phenol/chloroform/isoamyl alcohol (Sigma) method and then precipitated in ethanol. Total DNA from 10^6 transduced MSCs was also extracted for PCR experiments. PCR were performed using primers that specifically bind the nls sequence and the lacZ gene encoded in the nls-lacZ lentivirus vector: 5'- GTA ACA ACT CCG CCC CAT T -3' and 5'- GAC AGT ATC GGC CTC AGG AA -3'.

Statistical analysis

Data are expressed as means \pm S.E.M. Statistical significance was evaluated using the analysis of variance (ANOVA) method to compare control and treatment groups of 3 or more. Tukey's test was subsequently used for pair-wise comparisons. A non parametric Mann and Whitney test was performed to compare two groups. Statistical significance was set at $p < 0.05$.

Results

Transient epithelial airway injury induced by polidocanol intratracheal administration

Intratracheal administration of 2% PDOC induced macroscopic lung injury at 24hr with haemorrhage (Figs. 1b,k,n). The major histological findings at 24hr were acute injury of the murine airway epithelium with focal shedding areas observed in the epithelial surface, with only a remaining layer of basal cells or a total denudation of the basement membrane (Figs. 1e,h). However, in some regions, the luminal layer of epithelial cells was just disrupted or sloughed whereas the basal cell layer and the basement membrane remained intact (not shown), as already described after intratracheal or intranasal administration (Driscoll *et al.*, 2000 and Southam *et al.*, 2002, respectively). Inflammation was present in pulmonary parenchyma (Figs. 1k,n, arrow heads). Trachea-lung sections at 7 days after PDOC administration demonstrated significant improvement of haemorrhage (Figs. 1c,l) and of airway injury with epithelial surface again covered by cilia (Figs. 1f,i). PDOC effect did not alter significantly animal survival (Table S1 in supplementary data).

ESC and BMC9 survival in murine airway with acute epithelial airway injury

In a first set of experiments, embryonic stem cells (ESCs) and adult mesenchymal stem cells (BMC9s) from well-characterized cell lines were intratracheally injected. ESCs and BMC9s were injected 24hr after PDOC administration, when epithelial airway injury induced by PDOC was acute (MacPherson *et al.*, 2005). Stem cell survival was evaluated 24hr after stem cell administration. To calculate the survival rate, we used a quantitative method based on *in vivo* expression of reporter genes by transplanted cells. Stem cells were transfected *in vitro* with plasmids encoding CAT or GFP reporter genes 2hr prior to intratracheal injection. CAT-transfected stem cells were then intratracheally injected, at a time where they did not yet express

the foreign genes (see below). We hypothesized that only surviving stem cells would express *in vivo* CAT protein in trachea-lungs 24hr after intratracheal cell injection.

Two hours after the beginning of cell incubation with both plasmids and synthetic vectors, transfection complexes were removed and reporter gene expression was evaluated. Neither CAT protein nor cell fluorescence was detected in stem cells using ELISA (Fig. 2b) and microscopy (not shown), demonstrating that stem cells did not express CAT nor GFP protein at the time of *in vivo* injection. *In vitro* reporter gene expression was also evaluated 24hr and 7 days after incubation with plasmids and synthetic vectors. As evaluated by FACS analysis, $19.4 \pm 0.2\%$ ESCs (n = 6) and $37.0 \pm 4.4\%$ BMC9s (n = 6) expressed GFP *in vitro* at 24hr (Fig. 2a). However, CAT and GFP were not detected at 7 days after transfection (Fig. 2b), suggesting that transgene expression was transient. Using CAT ELISA quantitative results and GFP-expressing cell rates, we calculated that CAT-expressing ESCs and CAT-expressing BMC9s contained at 24hr $0.03 \pm 0.01\text{pgCAT/cell}$ (n = 10) and $0.08 \pm 0.02\text{pgCAT/cell}$ (n = 6), respectively. Cell values were further used to evaluate the number of *in vivo* surviving cells after intratracheal injection.

For intratracheal administration, CAT-transfected ESCs and CAT-transfected BMC9s were harvested 2hr after the beginning of transfection and injected intratracheally. Mice were sacrificed 24hr after intratracheal administration; trachea-lungs were used *in toto* to quantify CAT protein by ELISA (Fig. 2c). No CAT protein was detected in control animals or animals injected with PDOC only. Importantly, CAT protein was detected in 61% (19/31) animals intratracheally injected with PDOC and ESCs and in 81% (17/21) animals intratracheally injected with PDOC and BMC9s, suggesting that ESCs and BMC9s survived within lungs and expressed reporter genes 24hr after intratracheal injection. To estimate the number of surviving cells within the lungs at 24hr, total CAT protein quantity per animal was divided by CAT

quantity per cell *in vitro*. The survival rate of 10^6 injected stem cells was $3.69 \pm 0.86\%$ ESCs ($n = 19$), whereas this survival rate was significantly less with BMC9s ($0.43 \pm 0.12\%$, $n = 17$, Tukey's test, $p = 0.02$). CAT cell quantities were also used to calculate the *in vivo* cell detection threshold. A minimum of $10 \pm 1.2 \times 10^3$ ESCs and $4.3 \pm 1.1 \times 10^3$ BMC9s could be detected in one animal *in vivo*, corresponding to 0.4-1% injected cells. These results suggest that even if the percentage of cells expressing reporter gene *in vitro* was low, this was still a sensitive method for *in vivo* detection of a small cell number.

Influence of differentiation state and airway environment on ESC and BMC9 survival

To determine whether cell survival ability in murine injured airway was specific to stem cells, differentiated cells from various origins (Hep3B, mOS-J and COS-7 cells) were transfected *in vitro* with CAT plasmids in conditions and results similar to that of ESCs and BMC9s (not shown). CAT-transfected differentiated cells were then intratracheally injected 2hr after the beginning of incubation with CAT plasmid. No CAT protein was detected in any animal injected with PDOC and differentiated cells at 24hr (Fig. 2c), suggesting that only stem cells survived after intratracheal injection in injured airway. CAT-transfected ESCs and BMC9s were injected in control healthy animals. No CAT protein was detected in any animal with healthy airway 24hr after stem cell injection (Fig. 2c), suggesting that stem cell survival was favoured by airway injury.

Cell survival in the epithelial lining fluid

To evaluate whether cells expressing CAT reporter protein were present within the epithelial lining fluid or after phagocytosis by alveolar macrophages, bronchoalveolar lavages (BALs) were performed at 24hr in *in vivo* conditions when phagocytosis was expected maximal, e.g. when CAT ELISA results were negative: mesenchymal stem cells (BMC9s) in healthy airway

and murine differentiated cells (mOS-J) in injured airway. As further controls, BALs were also performed in control and PDOC animals and in animals intratracheally injected with PDOC and BMC9s. No CAT protein was detected in any BAL fluid nor BAL cell sample from any animal (Fig. 2d), suggesting that if stem cells or differentiated cells were phagocytosed by alveolar macrophages, such phagocytosis did not alter CAT protein measurement nor cell survival detection. Importantly, in animals intratracheally injected with PDOC and BMC9s, high levels of CAT protein were measured in trachea and lung homogenates whereas no CAT protein was detected in BAL fluid nor BAL cell samples, suggesting that CAT-expressing cells were mostly present within the lungs and did not survive in the epithelial lining fluid (Fig. 2d).

BMC9 location at 24hr and 7 days

Next, we investigated BMC9 location at 7 days when the airway epithelium was known to be spontaneously regenerating (MacPherson *et al.*, 2005). As CAT expression using synthetic vectors was transient, we used integrative virus vectors allowing long-term foreign gene expression. BMC9s were transduced with a retrovirus vector encoding nls-lacZ gene. As evaluated by X-Gal staining, $57.2 \pm 0.1\%$ BMC9s expressed nuclear β galactosidase (β gal) when they were injected (Fig. 3a, n = 6). β gal activity in transduced BMC9s was significantly increased just prior to injection as compared to endogenous β gal activity in BMC9s (Fig. 3b, Mann and Whitney test, p = 0.02) and remained stable at 7 days (not shown). Animals were assessed for β gal activity 24hr and 7 days after intratracheal administration, using the 4 MUG method (Fig. 3c). In contrast to previous experiments, β gal-transduced BMC9s expressed nls-lacZ gene at the time of intratracheal injections. Therefore, as a further control, we injected lysates of β gal-transduced BMC9s, and no significant increase in β gal activity was observed as compared to endogenous β gal activity (Tukey's test, p = 0.98). In animals intratracheally

injected with PDOC and β gal-transduced BMC9s, 24hr after injection β gal activity significantly increased as compared to that of control animals (Tukey's test, $p = 0.003$) and to animals injected with PDOC and lysate of β gal-transduced BMC9s (Tukey's test, $p = 0.004$), confirming results with CAT gene transfer. However, 7 days after stem cell injection β gal activity was similar to that of control animals (Tukey's test, $p = 0.98$) and animals injected with PDOC and lysate of β gal-transduced BMC9s (Tukey's test, $p = 0.94$), suggesting that the number of surviving BMC9s in the airway epithelium decreased significantly between 24hr and 7 days.

To locate BMC9s after intratracheal injection into injured airway, we performed X-Gal staining of *in toto* trachea-lungs at 24hr and 7 days (Fig. 4). No blue staining was observed in trachea-lungs (Fig. 4a) and histological sections (Figs. 4b-d) from any control animal (Fig. 4a, $n = 3$), animal injected with PDOC (Fig. 4b, $n = 3$), or animal intratracheally injected with β gal-transduced BMC9s only (Fig. 4c, $n = 5$). No cell with nuclear blue staining was observed in any animal injected with PDOC and lysate of β gal-transduced BMC9s (Fig. 4d, $n = 5$), suggesting that cells with nuclear blue staining expressed the nls-lacZ gene *de novo*. In contrast, macroscopic and microscopic strong nuclear blue staining was observed in trachea and pulmonary lobes at 24hr (Figs. 4e-m, $n = 16$) and 7 days (Figs. 4n-p, $n = 7$) in each animal intratracheally injected with PDOC and β gal-transduced BMC9s. At 24hr, macroscopic analyses showed blue spots (≤ 5 spots) in trachea from 8/16 animals (Figs. 4e,f) and blue spots in one (15/16, Figs. 4g,h) or two pulmonary lobes (1/16, Figs. 4i,j). Blue spots were also observed in large bronchi from 5/16 animals (Figs. 4i,j). Histological analyses confirmed the macroscopic result for each sample, showing β -gal positive cells on histological sections where blue spots had been observed. Clusters of cells with blue nuclei were observed in the lumen of injured trachea (Fig. 4k) and injured bronchi or bronchioles (Figs. 4l,m), but not in lung parenchyma (Fig. 4l).

At 7 days, blue spots were observed in 7/7 animals, in the lumen of bronchioles (Figs. 4n-p). No blue cell was observed in trachea or in pulmonary parenchyma (not shown). Clusters of blue cells were sometimes located in polyp-like structures, located in bronchioles and large bronchi. Polyp-like structures were not observed in any animal receiving PDOC only. Further quantitative and statistical analyses demonstrated that the development of polyp-like structures at 7 days was not due to stem cell administration but rather to a second intratracheal administration (PBS or BMC9) 24hr after PDOC injection (Table S2 in supplementary data). Histological analyses at high magnification showed that β gal-expressing cells did not have a respiratory phenotype, with no cilia at 24hr or 7 days (not shown).

MSC survival in murine airway with acute epithelial airway injury

In a second set of experiments, to avoid *in vivo* cell survival linked to cell line transformation, we repeated previous experiments with the same experimental design, using murine MSCs from primary cultures. MSC cultures were characterised according to (Dominici *et al.*, 2006) and (Chateauvieux *et al.*, 2007). FACS analysis demonstrated that MSCs were CD45⁻ (Fig. 5a, $0.4 \pm 0.1\%$, n = 4), CD90⁻ ($0.43 \pm 0.2\%$, n = 6) and CD29⁺, Sca-1⁺ (Fig. 5a, $96.9 \pm 1.3\%$ and $98.1 \pm 0.7\%$ respectively, n = 4) and CD106⁺ ($96 \pm 0.8\%$, n = 4).

MSCs were transiently transfected *in vitro* with plasmids encoding CAT or GFP reporter genes. In contrast to ESCs and BMC9s, MSC transfection with synthetic vectors was ineffective (not shown), therefore nucleofection was used to obtain significant gene transfer rate. As evaluated by FACS analysis, $56.8 \pm 2.9\%$ MSCs expressed GFP *in vitro* at 24hr (Fig. 5c, n = 3), but CAT and GFP were not detected 7 days after nucleofection (Fig. 5b), suggesting that foreign gene expression was again transient and that nucleofection could not be used for 7 days *in vivo* experiments. CAT-transfected MSCs contained 0.35 ± 0.02 pg CAT/cell at 24hr (n = 6). CAT-

transfected MSCs were then intratracheally injected 2hr after the beginning of incubation with CAT plasmid. In control mice and mice injected with PDOC only, no CAT protein was detected at 24hr (Fig. 5d). Importantly, CAT protein was detected in each animal injected with PDOC and MSCs (Fig. 5d, n = 8, Tukey's test, $p < 0.001$), suggesting that MSCs from primary cultures survived in injured airway and expressed CAT protein after intratracheal administration. MSC survival rate was $5.52 \pm 1.9\%$ (n = 8), a minimum of 710 ± 45 CAT-expressing MSCs could be detected in one animal *in vivo*, corresponding to 0.07% injected cells, confirming CAT ELISA sensitivity for *in vivo* experiments.

MSC location at 24hr and 7 days

To investigate whether MSCs could be located into the airway epithelium 7 days after cell injection, we first used MSCs in primary cultures from β gal+ transgenic (Rosa26) mice, thus expressing continuously cytoplasmic β gal (Fig. 6a). Using the 4 MUG method (Fig. 6c), *in vitro* β gal activity was significantly increased in Rosa26 MSCs as compared to MSCs from Swiss mice (Figure 6c, Mann and Whitney test, $p = 0.03$). Mice were assessed for β gal activity 24hr and 7 days after intratracheal administration of Rosa26 MSCs or of a lysate of Rosa26 MSCs (Fig. 6d). As observed in previous experiments with β gal-transduced BMC9s, β gal activity was similar in murine airway from control animals and animals intratracheally injected with lysates of Rosa26 MSCs (Tukey's test, $p = 0.68$). In animals intratracheally injected with PDOC and Rosa26 MSCs, β gal activity was significantly increased as compared to that of both control groups (Tukey's test, $p = 0.01$). However, in contrast to previous experiments with β gal-transduced BMC9s, no blue staining was observed in trachea-lungs from animals injected intratracheally with PDOC and Rosa26 MSCs at 24hr, and Rosa26 MSCs could not be located on

histological slides (not shown). Finally, β gal activity in murine injured airway returned to baseline at 7 days after Rosa26 MSC intratracheal injection (Tukey's test, $p = 0.85$).

In a last set of experiments, as previously performed with BMC9s we overexpressed nls-lacZ gene in MSCs from wild-type Swiss mice, to locate them *in vivo* at 24hr and 7 days. MSC transduction with the β gal retrovirus vector remained ineffective. Therefore, MSCs from Swiss mice were transduced prior to intratracheal injection with a lentivirus vector encoding the nls-lacZ gene (Conrad *et al.*, 2007). After lentiviral transduction, $20.1 \pm 3.0\%$ MSCs expressed β gal (Fig. 6b, $n = 4$). Nevertheless, no blue staining was observed in trachea-lungs from animals injected intratracheally with PDOC and β gal-transduced MSCs at 7 days (not shown). Since rapid down regulation of foreign gene expression under cytomegalovirus promoter has been described in lungs (Pringle *et al.*, 2005), we concurrently used PCR to detect the presence of nls-lacZ gene even without gene expression. PCR experiments using primers specific to nls-lacZ gene were performed 24hr and 7 days after β gal-transduced MSC injection (Fig. 6e). At 24hr, PCR was positive in trachea-lungs from each animal injected with PDOC and β gal-transduced MSCs (Fig. 6e, $n = 7$), confirming that β gal-transduced MSCs were present 24hr after injection in injured airway. However, no nls-lacZ gene was detected in animals at 7 days after β gal-transduced MSC injection, suggesting that no β gal-transduced MSCs from primary culture survived at this time once the epithelium is regenerated (Fig. 6e, $n = 6$).

Discussion

Using different and independent methods based on reporter gene transfer we demonstrated the short term survival of different types of exogenous stem cells, including embryonic and adult mesenchymal stem cells, after intratracheal injection in murine airway presenting acute epithelial airway injury and without total-body irradiation. In contrast to differentiated cells, 0.43 to 5.5 % stem cells were capable of surviving within the injured lungs at 24hr. Importantly no SCs survived in healthy airway or in the epithelial lining fluid. Biochemical staining showed that transduced MSCs were located in the lumen of conducting airway at 24hr and at 7 days. However, the *in vivo* amount of engrafted MSCs decreased dramatically with time. No primary MSCs were located within lungs at 7 days.

In our study, several gene transfer systems were used, according to our aims and to cell types. Using careful timing, non-viral vectors were useful to allow expression of the foreign gene only after intratracheal cell injection. Gene delivery with viral vectors was used for *in vivo* studies at 7 days, but we had to use different viral vectors for transducing MSCs from cell lines and primary MSCs. Depending on the gene transfer system and the cell type, gene transfer efficacy was variable *in vitro*. Studies with retrovirus vectors showed high efficacy and stable MSC transduction (up to 97%, (Sales *et al.*, 2007)) whereas studies with lentivirus vectors showed variable efficacy between murine MSCs (up to 50%, (Ricks *et al.*, 2008; Santoni de Sio *et al.*, 2008; Xu *et al.*, 2008)) and human MSCs (up to 93%, (Chan *et al.*, 2005)). These data confirmed that primary murine MSCs are more difficult to transduce than human MSCs.

Engraftment of exogenous stem cells into the lungs has been reported in several *in vivo* studies, but cell engraftment rate remains controversial (Loebinger *et al.*, 2008). Our results highlight the main advantage in using ELISA and biochemical methods to detect and quantify a small number

of administered cells in the whole organ. Although lacZ gene is widely used to locate stem cells, some reports have shown that standard protocols for X-Gal staining can lead, especially in the lungs, both to false positive results as well as to failure to adequately detect β gal expressing cells (Weiss *et al.*, 1997). In our study, while β gal activity significantly increased after intratracheal injection of MSCs from Rosa26 mice, surviving Rosa26 MSCs could not be located *in vivo* on histological slides, as already described in another report (MacPherson *et al.*, 2005). The lacZ gene coupled with a nuclear localization signal had already been used in several studies from our group (Lemarchand *et al.*, 1994; Chapelier *et al.*, 1996) and others (Mastrangeli *et al.*, 1993) and was shown to allow easy detection of endogenous from exogenous β gal activity. Using nls-LacZ gene, we were able to detect and locate nuclear β gal-expressing cells, even when β gal activity in the whole lungs was not significantly different from baseline.

We investigated whether stem cells would be capable of locating specifically on the epithelial side of airway after intratracheal administration. BAL experiments showed that stem cells expressing reporter protein did not survive in the epithelial lining fluid. X-Gal staining showed that stem cells were localised into conducting airway *in vivo*. By injecting stem cells in healthy airway, we also confirmed the major role of injured environment on the engraftment (MacPherson *et al.*, 2005). Stem cells may not engraft into healthy airway because of mucociliary clearance mechanism that could impede access to airway epithelium, as described for *in vivo* gene transfer to the lungs (Weiss 2002). We speculated that mucociliary clearance would be disrupted in polidocanol (PDOC)-treated airway and that the freshly denuded basement membrane may also promote specifically stem cell adherence and engraftment in the airway epithelium (Engelhardt *et al.*, 1992; Nikolova *et al.*, 2006). In the first set of experiments, murine ESCs were intratracheally injected and a significant survival cell rate was observed, although *in*

vitro ESC transfection rate was low. This confirms the great potential of ESC pluripotency for regenerative medicine and in particular airway injury. Nevertheless, ESCs for cell-based therapy have met with ethical, moral and political challenges, and with inherent risks associated with immune rejection (Chidgey *et al.*, 2008). In further experiments, we focused our attention on MSCs because they are already used in the clinical setting (Uccelli *et al.*, 2008). Our ELISA data indicated that MSC survival rate varied according to immortalized or primary cultures, probably because of the different gene transfer systems we used. Survival rates (from 0.4% to 5.5%) 24hr after intratracheal administration were higher than those described after intravenous administration (from 0.01% to 0.1%) and in agreement with those usually described in stem cell therapy protocols, including cardiology (Robey *et al.*, 2008) or diabetology (Bottino *et al.*, 2003) protocols, and lung injury models (Gupta *et al.*, 2007; Wong *et al.*, 2007; Wong *et al.*, 2009).

We hypothesized that MSC incorporation in the lungs at 7 days could be masked by a down regulation of transgene expression into the lungs because of the common cytomegalovirus enhancer/promoter element in the virus vector constructs (Alton *et al.*, 1999; Pringle *et al.*, 2005). The negative results of PCR experiments on nls-lacZ gene at 7 days demonstrated that this was not the case. Allogeneic rejection seems unlikely to be responsible for the lack of survival of differentiated cells at 24hr since ESCs, although known to be targeted for rejection by the immune system (Chidgey *et al.*, 2008), survived at 24hr. Nevertheless, allogeneic MSC rejection cannot be ruled out at 7 days.

Another hypothesis to explain MSC negative results at 7 days is the spontaneous regeneration of the airway epithelium that may have hampered MSC incorporation into epithelial airway. At 24hr, e.g. when there is a desquamation of the epithelial surface and a denuded basement membrane, the PDOC murine model is a good model to evaluate stem cell survival and presence

in injured airway but stem cell differentiation into airway epithelial cells is unlikely at this time. At 7 days after PDOC administration, the epithelium was spontaneously regenerated, demonstrating that PDOC did not alter endogenous lung capability to regenerate. Therefore, the PDOC model may not be an adequate model to evaluate stem cell capability in regenerating lung epithelium, since the endogenous repair mechanisms may impede MSC contribution to achieve structural lung regeneration. This limitation was described in (Wong *et al.*, 2007) study in which the naphthalene-injured airway epithelium was still undergoing rapid cell turnover and regeneration and the number of administered stem cells also decreased with time. A more appropriate animal model to study stem cell contribution to structural lung regeneration would be a model with chronic epithelial airway injury and a recurrent loss of the epithelial surface, due to a lack or an exhaustion of endogenous progenitor cells. This hypothesis was evaluated in animal models with permanent retinal epithelial degeneration such as the RCS rat model (D'Cruz *et al.*, 2000) and the rhodopsin knock-out mouse (Humphries *et al.*, 1997). MSC engraftment and epithelial regeneration involving MSCs were observed in these animal models (Arnhold *et al.*, 2007; Inoue *et al.*, 2007). For lung tissue, a recent murine model overexpressing the beta-subunit of epithelial Na⁺ channel (ENaC^{+/+}) and showing epithelial degeneration in newborn animals (Mall *et al.*, 2008) may be more appropriate to evaluate MSC contribution to lung regeneration. Finally, an important limitation of our study was also the lack of phenotypic characterisation of surviving cells. This characterisation of stem cell-derived epithelial cells has been hampered by methodological problems, in the lungs as in many other organs such as the heart (Murry *et al.*, 2004) or the brain (Castro *et al.*, 2002), and remains difficult and controversial. In this regard, additional studies using complex transgenic models with reporter genes under the control of lung-specific promoters *in vivo* will be essential.

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No competing financial interests exist.

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Figure legends

Figure 1: Epithelial damage induced by intratracheal administration of polidocanol (PDOC). **(a-c)** Trachea-lungs from **(a)** control animal, **(b)** animals injected with PDOC at 24hr and **(c)** 7 days after PDOC administration. **(d-i)** tracheal and **(j-o)** bronchial sections from **(d,g,j,m)** control animal, **(e,h,k,n)** animals injected with PDOC at 24hr and **(f,i,l,o)** at 7 days. **(k)** Magnification 4x. **(d-f)** Magnification 10x. **(j,l)** Magnification 20x. **(g-i,m-o)** Magnification 100x. Haematoxylin counterstaining, airway epithelial damage (arrows), ciliated cells (white arrow), inflammation areas (arrow heads), haemorrhagic areas (white arrow heads). Areas marked by *asterisks* were magnified in the adjacent pictures.

Figure 2: *In vitro* cell transfection with reporter genes and *in vivo* CAT gene expression into injured airway 24hr after intratracheal stem cell administration. To follow stem cell survival *in vivo*, murine embryonic stem cells (ESCs) or adult mesenchymal stem cells (BMC9s) were transfected *in vitro* with plasmids encoding CAT or GFP reporter genes just prior to intratracheal administration. (a) Representative histograms of FACS analysis of GFP-expressing ESCs or BMC9s 24hr after *in vitro* transfection. (b) CAT protein quantification in ESC and BMC9 lysates using ELISA, 2hr, 24hr or 7 days after incubation with plasmids and synthetic vectors. (c) CAT protein quantification in whole trachea-lung homogenates using ELISA, 24hr after ESC or BMC9 intratracheal injection. Evaluated groups included animals that were intratracheally injected with the followings: no injection (control), PDOC only (PDOC), ESCs or BMC9s only (ESC, BMC9), PDOC and ESCs or BMC9s 24hr later (PDOC + ESC, BMC9), PDOC and differentiated cells 24hr later (PDOC + Hep3B, mOS-J, COS-7). (d) CAT protein quantification in bronchoalveolar lavages and whole trachea-lung homogenates using ELISA, 24hr after BMC9 or murine differentiated cells (mOS-J) intratracheal injection. Evaluated groups included animals that were intratracheally injected with the followings: no injection (control), PDOC only (PDOC), BMC9s only (BMC9), PDOC and mOS-Js 24hr later (PDOC + mOS-J), PDOC and BMC9s 24hr later (PDOC + BMC9). Dotted line depicts control threshold. * $p < 0.05$.

Figure 3: *In vitro* and *in vivo* detection of β gal activity 24hr or 7 days after β gal-transduced BMC9 intratracheal administration. BMC9s were transduced *in vitro* with a retrovirus vector encoding nls-lacZ gene prior to *in vivo* administration. (a) Representative example of β gal-transduced BMC9s just prior to injection, after X-Gal staining. Magnification 20x. (b) *In vitro* β gal activity in BMC9s and β gal-transduced BMC9s just prior to injection. (c) β gal activity in whole trachea-lung homogenates, 24hr or 7 days after BMC9 intratracheal injection. Evaluated groups included animals that were intratracheally injected with the followings: no injection (control), PDOC and lysate of β gal-transduced BMC9s (PDOC + BMC9 lysate), PDOC and β gal-transduced BMC9s (PDOC + BMC9). Dotted line depicts control threshold. * $p < 0.05$.

Figure 4: BMC9 location 24hr and 7 days after intratracheal injection, using X-Gal staining. **(a-d)** Trachea-lungs or histological sections at 24hr after X-Gal staining from **(a)** control animal, **(b)** animal intratracheally injected with PDOC or **(c)** β gal-transduced BMC9s or **(d)** with PDOC and 24hr later β gal-transduced BMC9 lysate. No blue staining was observed in any control animals. **(e-j)** Trachea-lungs, **(k)** tracheal and **(l,m)** bronchial sections from animals intratracheally injected with PDOC and 24hr later β gal-transduced BMC9s. Animals were sacrificed 24hr after BMC9 injection and stained using X-Gal. Blue spots were observed **(e,f)** in trachea and **(g-j)** in pulmonary lobes. **(f,h,i)** Higher power views of the area marked by *asterisks* in Figs. 4e,g,j, respectively. **(m)** Higher power view of the area marked by *asterisk* in Fig. 4l. **(n-p)** Trachea-lungs and histological sections from animal intratracheally injected with PDOC and β gal-transduced BMC9s, sacrificed 7 days after BMC9 injection and stained using X Gal. Blue spots were observed in the lumen of bronchioles. **(p)** Higher power view of the area marked by *asterisk* in Fig.4o. **(c,d,o)** Magnification 4x. **(b,l)** Magnification 10x. **(k)** Magnification 20x. **(m,p)** Magnification 40x. Nuclear fast red counterstaining.

Figure 5: *In vitro* cell transfection with reporter genes and *in vivo* CAT gene expression 24hr after intratracheal administration into injured airway of murine adult mesenchymal stem cells (MSCs) from primary cultures. (a) MSC morphology and representative histograms of CD45, CD29 and Sca-1 labeling, using FACS analysis just prior to intratracheal injection. (b-d) To follow MSC survival *in vivo*, MSCs were transfected *in vitro* with plasmids encoding CAT or GFP reporter genes using nucleofection, just prior to intratracheal injection. (b) CAT protein quantification in MSCs using ELISA, 2hr, 24hr or 7 days after incubation with plasmid and nucleofection solution. (c) FACS analysis of GFP-expressing MSCs 24hr after incubation with GFP plasmid and nucleofection solution. (d) CAT protein quantification in whole trachea-lung homogenates using ELISA, 24hr after CAT-transduced MSC intratracheal injection. Evaluated groups included animals that were intratracheally injected with the followings: no injection (control), PDOC only (PDOC), PDOC and 24hr later CAT-transduced MSCs (PDOC + MSC). Dotted line depicts control threshold.

Figure 6: *In vitro* and *in vivo* detection of Rosa26 MSCs and β gal-transduced MSCs 24hr or 7 days after administration. MSCs from wild type mice were transduced with a lentivirus vector encoding nls-lacZ gene. **(a,b)** Representative example of **(a)** Rosa26 MSCs or **(b)** β gal-transduced MSCs after *in vitro* X-Gal staining just prior to intratracheal injection. Magnification 20x. **(c)** *In vitro* β gal activity in MSCs and Rosa26 MSCs just prior to intratracheal injection. **(d)** β gal activity in whole trachea-lungs homogenates, 24hr or 7 days after Rosa26 MSC intratracheal administration. Evaluated groups included animals that were intratracheally injected with the followings: no injection (control), PDOC and lysate of Rosa26 MSCs (PDOC + Rosa26 MSC lysate), PDOC and Rosa26 MSCs (PDOC + Rosa26 MSC). **(e)** *In vivo* detection of the nls-lacZ gene by PCR 24hr and 7 days after intratracheal injection of β gal-transduced MSCs. Dotted line depicts control threshold. * $p < 0.05$.

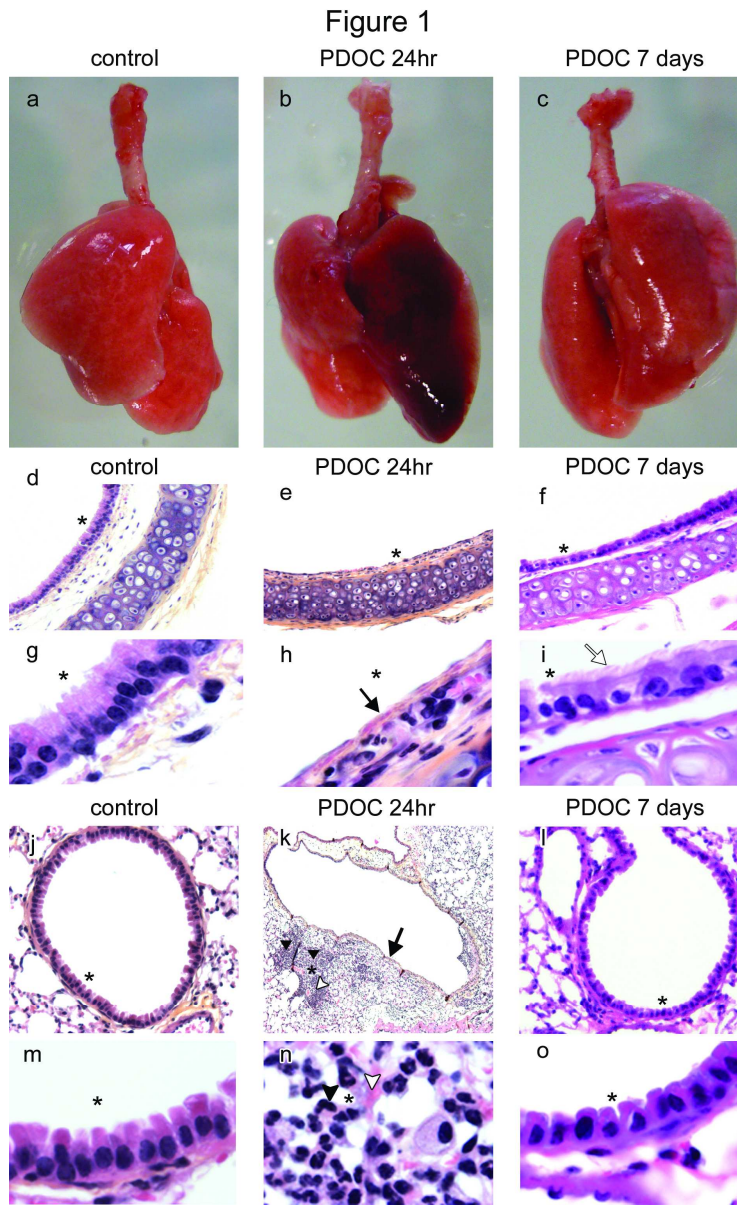


Figure 1: Epithelial damage induced by intratracheal administration of polidocanol (PDOC).
160x260mm (300 x 300 DPI)

Figure 2

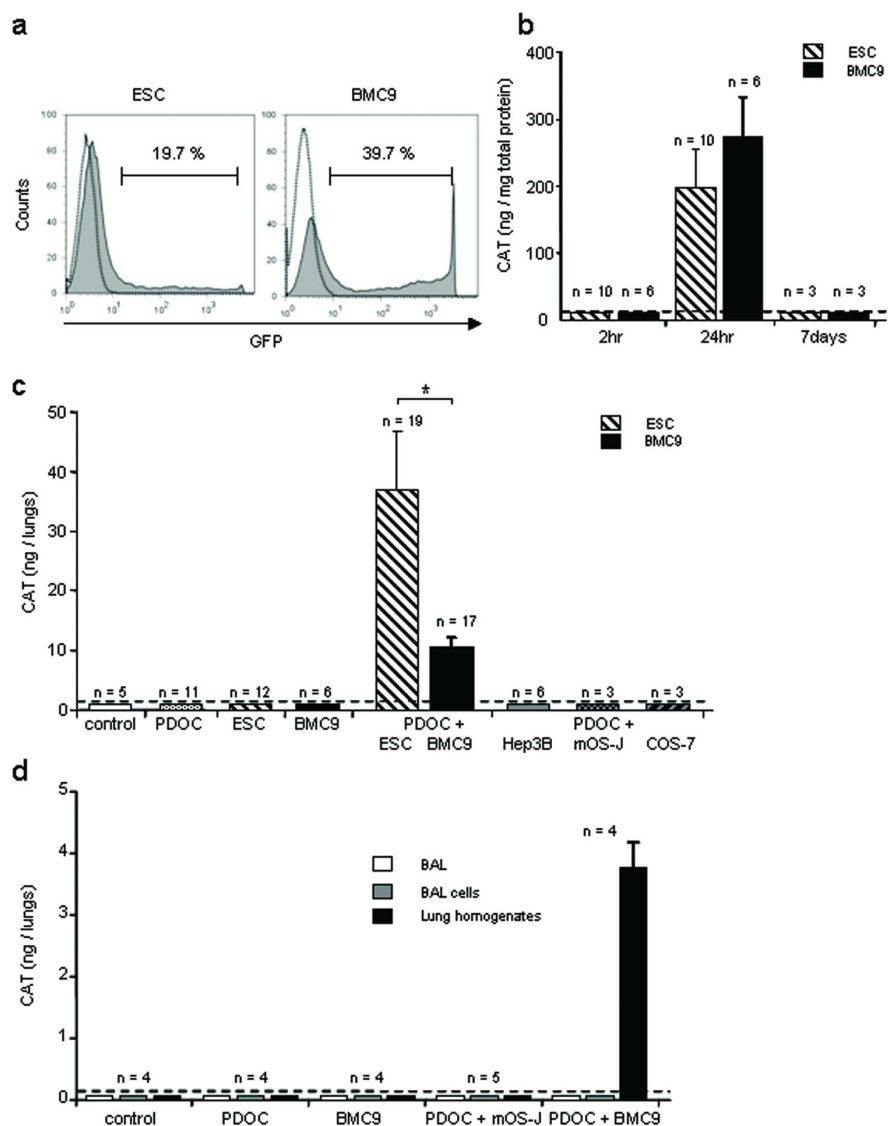


Figure 2: In vitro cell transfection with reporter genes and in vivo CAT gene expression into injured airway 24hr after intratracheal stem cell administration.

Figure 3

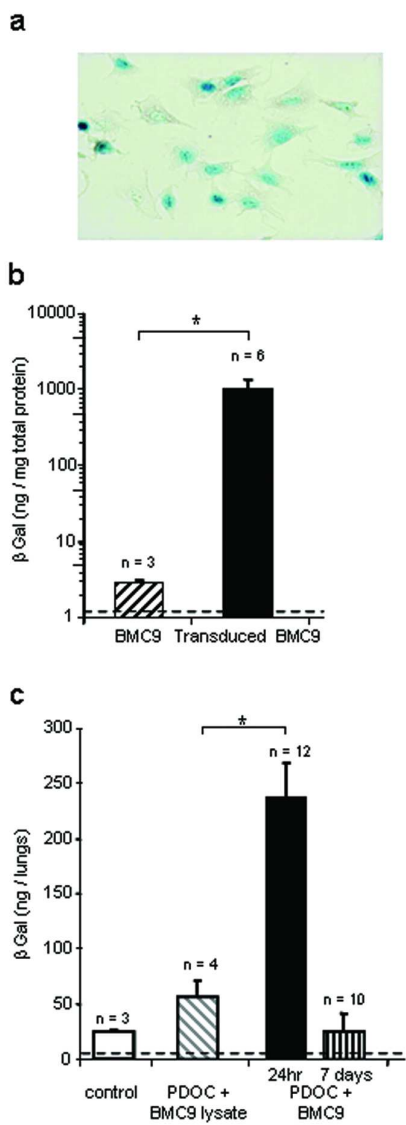


Figure 3: In vitro and in vivo detection of β gal activity 24hr or 7 days after β gal-transduced BMC9 intratracheal administration.

Figure 4

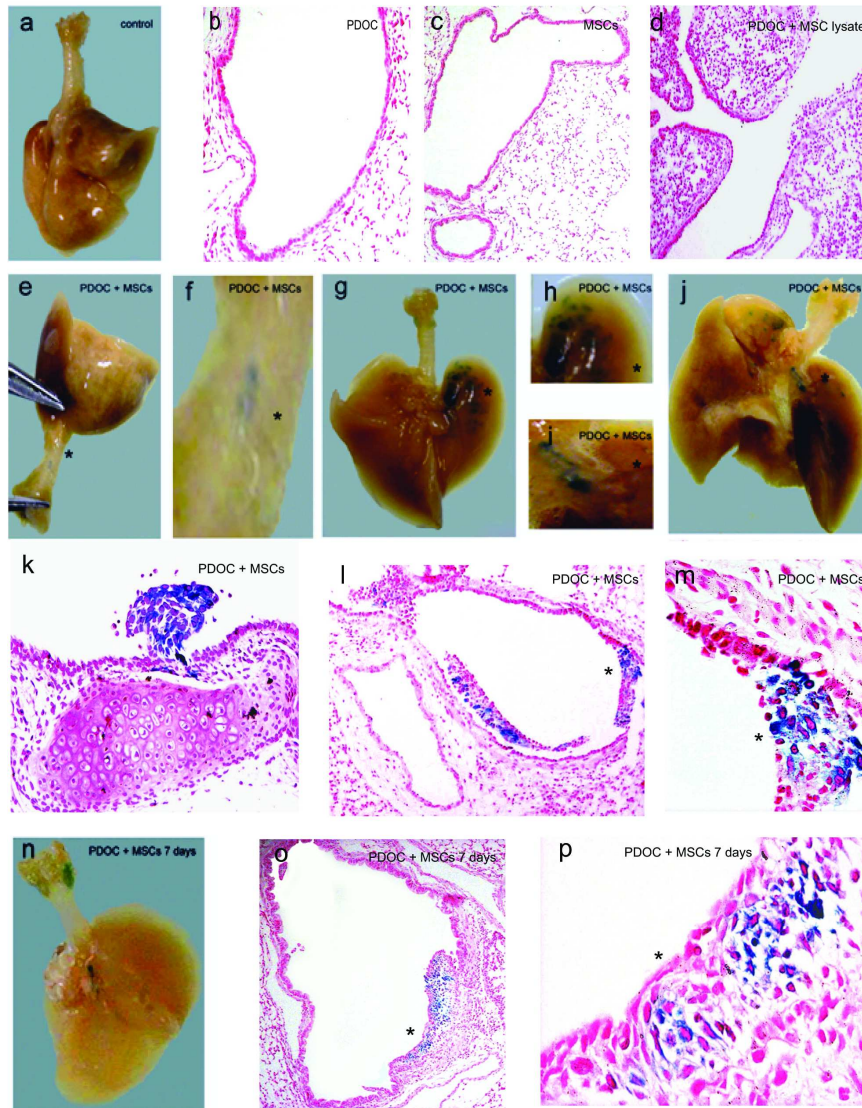


Figure 4: BMC9 location 24hr and 7 days after intratracheal injection, using X-Gal staining.
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Figure 5

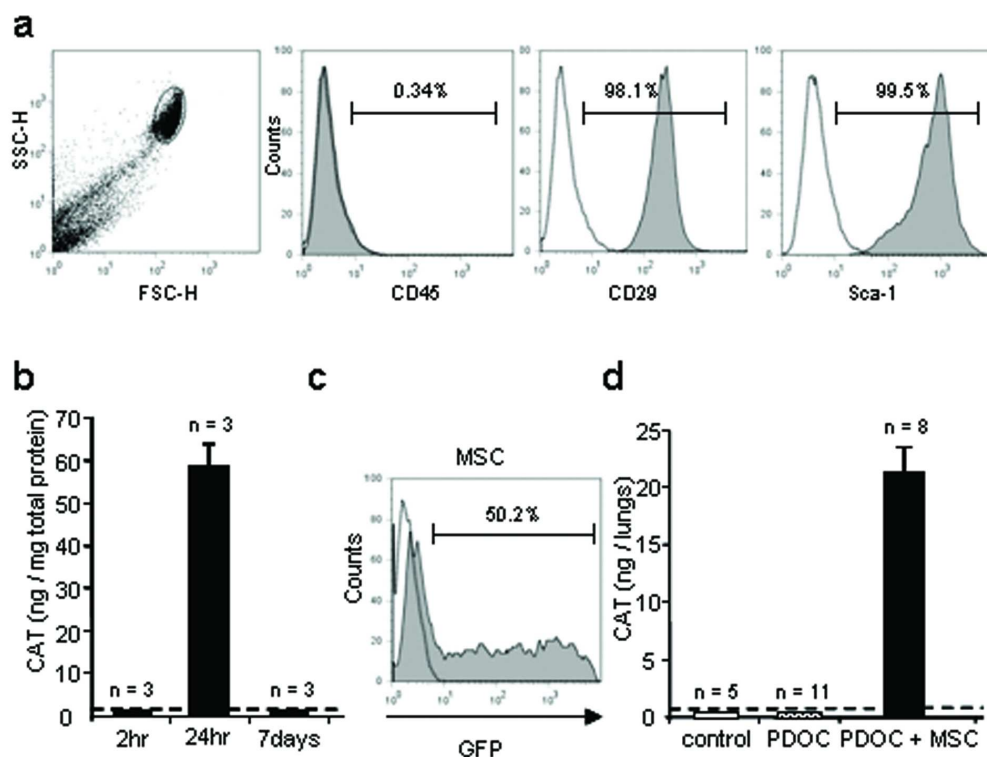
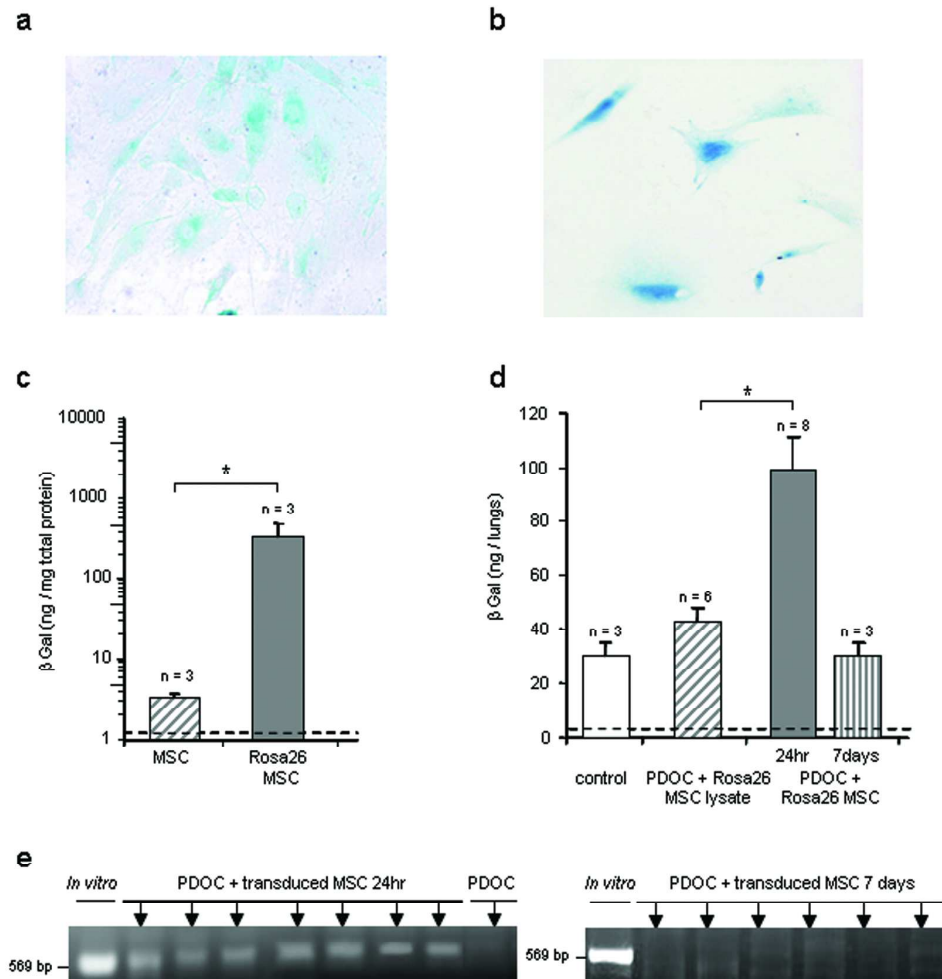


Figure 5: In vitro cell transfection with reporter genes and in vivo CAT gene expression 24hr after intratracheal administration into injured airway of murine adult mesenchymal stem cells (MSCs) from primary cultures.

Figure 6



In vitro and in vivo detection of Rosa26 MSCs and β gal-transduced MSCs 24hr or 7 days after administration.

Table S1: Animal survival survey.

intratracheal administration(s)	% survival at 24hr (n = animal number, number of experiments)
PBS only	94.4 ± 20.6 % (n = 10, 2)
2% PDOC only	90.1 ± 16.2 % (n = 812, 30)
PBS and 24hr later PBS	80.2 ± 13.5 % (n = 10, 2)
2% PDOC and 24hr later PBS	91.7 ± 8.3 % (n = 19, 2)
2% PDOC and 24hr later stem cells	96.3 ± 1.7 % (n = 96, 5)

Data are expressed in means ± S.E.M. No significant difference was observed between the different groups (ANOVA, p = 0.30). “2%PDOC and 24hr later stem cells” group includes experiments with ESCs, BMC9s, MSCs and Rosa26 MSCs.

Table S2: Quantification of polyp-like structures in murine airway at 7 days.

	Animal number	Slide number	Animal number with polyp-like structures in airway (percentage)	
			Trachea and large bronchi	Bronchioles
2 % PDOC only	4	25	0	0/4 (0%)
2% PDOC and 24hr later PBS	11	73	0	8/11 (72%)
2% PDOC and 24hr later nls-LacZ BMC9	7	50	0	5/7 (71%)

No significance was observed between the “2% PDOC and 24hr later PBS” group and “2% PDOC and 24hr later nls-LacZ BMC9”.