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Repair and regeneration of tracheal surface epithelium and submucosal glands in a mouse model of hypoxic-ischemic injury

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Abstract

Background and objective—The heterotopic syngeneic tracheal transplant mouse model is an acute hypoxic-ischemic injury model that undergoes complete repair and regeneration. We hypothesized that the repair and regeneration process of the surface epithelium and submucosal glands would occur in a reproducible pattern that could be followed by the expression of specific markers of epithelial cell types.

Methods—We used the syngeneic heterotopic tracheal transplant model to develop a temporal and spatial map of cellular repair and regeneration by examining the tracheal grafts at post-transplant days 1, 3, 5, 7, 10 and 14. We used pulsed BrdU and immunofluorescent staining to identify and follow proliferating and repairing cell populations.

Results—We confirmed the reproducibility of the injury and repair in the model and we found a distinct sequence of reappearance of the various stem/ progenitor and differentiated cell populations of the tracheal surface epithelium and submucosal glands. In the initial phase, the basal and duct cells that survived the injury proliferated to re-epithelialize the basement membrane with K5 and K14 expressing cells. Then these cells proliferated further and differentiated to restore the function of the epithelium. During this repair process, TROP-2 marked all repairing submucosal gland tubules and ducts. Non-CCSP-expressing serous cells were found to differentiate 4–5 days before Clara, mucus and ciliated cells.

Conclusions—Improving our understanding of the reparative process of the airway epithelium will allow us to identify cell-specific mechanisms of repair that could be used as novel therapeutic approaches for abnormal repair leading to airway diseases.

Keywords

airway epithelium; lung stem cell; repair and regeneration; submucosal gland and duct

INTRODUCTION

The proximal airway epithelium consists of the surface epithelium (SE) and the submucosal glands (SMG) and ducts. The differentiated cell types of the proximal airway epithelium are highly specialized to act in host defense, with serous, mucus and ciliated cell types present to perform mucociliary clearance.^{1,2} Abnormalities in repair and regeneration of these cell types result in the poor clearance of mucus with pneumonia and bronchiectasis. It is therefore important to understand how the proximal airway epithelium repairs and regenerates in order to develop new therapies for diseases of the proximal airway epithelium.

The syngeneic heterotopic murine tracheal transplantation model has been well characterized as a model system to study normal airway epithelial repair and regeneration, usually as a control for studying allogeneic reactions after transplantation. Transplanted syngeneic tracheas in this model demonstrate normal regeneration of the pseudostratified airway epithelium by day 21 post-transplantation.³⁻⁵ This model is clinically relevant as there is evidence that ischemia/reperfusion injury to the airway occurs with endotoxin administration⁶ and also occurs after lung transplantation when the bronchial arterial circulation is severed and no direct re-anastomosis is performed.

We hypothesized that only stem cells would survive the initial injury/necrosis stage thereby allowing us to identify the surviving cell type(s) and niche(s) from which repair and regeneration occurs in this model.⁷ Then, by following the tracheal grafts chronologically and identifying the extent of proliferation and the sequence of reappearance of the various cellular populations in tracheal epithelium and SMGs, we can identify their behaviour in response to injury. Identifying the temporal and spatial patterns of repair and regeneration of the airway epithelial cell subtypes is important for increasing our understanding of the extent to which each cell population is capable of repair and allows us to further our understanding of the repair and regeneration capabilities of the SMG duct stem/progenitor cell population that we recently identified using this model.⁷

METHODS

Mouse tracheal transplant model

The Chancellor's Animal Research Committee at University of California, Los Angeles approved all protocols with respect to breeding mice, euthanizing mice and survival surgeries. Mice were housed and bred under the regulation of the Division of Laboratory Animal Medicine at the University of California, Los Angeles. We used a well-established, reproducible murine model of tracheal epithelial regeneration using syngeneic subcutaneous tracheal transplants from C57BL/6 into C57BL/6 mice.³⁻⁵ Briefly, tracheas were collected from euthanized donor animals. Care was taken to include the whole trachea by dissecting at or above the larynx so as not to lose or compromise any of the big bunches of SMGs above (cartilage) C1 down to the tracheal bifurcation. Recipient animals were anaesthetized and the hair was shaved off their upper back. Cleanly shaven skin was washed with alcohol followed by betadine. A superficial incision was made in the loose skin of the neck/scapular area that was widened with scissors into two deep pockets under the skin flaps of the incision. One trachea was placed inside each pocket. The incisions were then stapled and antibiotic ointment was applied to the closed incision site. Tracheal grafts were collected from euthanized mice at post-transplant days 1, 3, 5, 7, 10, 14 and 20. Twenty tracheal grafts were examined from each time point. Collected tracheas were fixed in paraformaldehyde and embedded in paraffin transversely to obtain longitudinal sections of the whole length of the trachea from above the first cartilaginous ring (C1) down to at least C7. From each tracheal block, 4-micron-thick slices were collected and then the block was trimmed to

collect deeper slices and so on for at least five more sections so that there were representative slices throughout the tracheal thickness.

BrdU injections

All recipient mice with the heterotopic tracheal grafts received intra-peritoneal injection of 2 mg of BrdU 6 and 3 h prior to euthanasia.

Immunofluorescence

Tissue sections were deparaffinized in xylenes and rehydrated in graded ethanols, and microwave-based antigen retrieval was obtained by boiling in 10 nmol/L sodium citrate buffer for 10 min.

Non-serum protein block (Dako, Carpinteria, CA, USA) was applied for 30 min.

The primary antibodies used were rabbit K5 and K14 (Covance, Princeton, NJ, USA), mouse p63, goat K5, SCGB1A1, goat nerve growth factor receptor, goat and rabbit MUC1 and deleted in malignant brain tumour 1 (DMBT1) (Santa Cruz, Santa Cruz, CA, USA), rat Integrin- α 6, rabbit nerve growth factor receptor, E-cadherin, mouse and rabbit K14 (Abcam, Cambridge, MA, USA), goat TROP-2 and polymeric immunoglobulin receptor (R&D, Minneapolis, MN, USA), rat EpCAM-647 and Integrin- β 4 (Biolegend, San Diego, CA, USA), rabbit lactoferrin (Millipore, Billerica, MA, USA), rabbit lysozyme (kindly provided by Dr. Tom Ganz), mouse α -smooth muscle actin (α -SMA), acetylated β -tubulin (Sigma, St. Louis, MO, USA), rat Integrin- α 6 (e-Bioscience, San Diego, CA, USA), mouse MUC5AC (NeoMarkers, Fremont, CA, USA), rabbit annexin v (Abcam) and rat BrdU (Abcam). Negative controls were performed with immunoglobulin G from the species in which individual primary antibodies were made.

At least six tissue slices from each tracheal transplant were immunostained and then examined under a fluorescent microscope, and images were captured with the 10 $\times$, 20 $\times$ and 40 $\times$ lens objectives. Positively stained cells were counted in at least 10 fields from the whole thickness of each trachea and their percentages were calculated based on the total number of cells obtained by counting the 4',6-diamidino-2-phenylindole (DAPI) stained nuclei. Average and standard deviations of all percentages from the different images were calculated.

Removal of basal cells prior to tracheal transplantation

We determined that pronase digestion of the mouse tracheas for 4 h resulted in complete stripping of the SE and removed all basal cells, without removing duct cells.⁷ We performed tracheal transplants with these pronase-digested grafts to assess the role of duct cells in airway epithelial repair in the absence of basal cells.

RESULTS

Establishing cell-specific markers that identify the cellular populations of the steady-state tracheal epithelium

We examined a panel of markers identified from published gene expression array databases and previously described markers of complicated epithelia and glands in order to confirm previously identified markers and identify novel markers for each epithelial cellular subtype in tracheal SE and SMG. This was performed to allow us to examine each of the cell populations as they after hypoxic-ischemic injury. We performed immunofluorescence staining on longitudinal tracheal sections from naïve wild-type mice to confirm the morphology and distribution of each cell subtype of the SE and the SMG. Figure 1a

demonstrates a typical longitudinal section of the upper mouse trachea with the location of the SMGs and ducts.

EpCAM and E-cadherin expression were present on all epithelial cells in the SE and SMGs (Fig. 1b i,ii). We identified that TROP-2, which is a marker of prostate basal cells,¹⁰ was expressed on all SE and SMG duct cells, but not on SMG tubules (Fig. 1b ii).⁷ SE basal cells were identified by their morphology (low pyramidal to cuboidal cells that reside on the basement membrane (BM), and their positive staining for cytokeratin (K)5,¹¹ Integrin- α 6¹² and nerve growth factor receptor¹² (Fig. 1b iii,iv,v,ix). They also expressed Integrin- β 4 on their basolateral surface (Fig. 1b vi). Secretory cells (Clara and serous cells) were identified by their columnar shape and expression of Clara cell secretory protein and polymeric immunoglobulin receptor, respectively (Fig. 1b vii,viii). Ciliated cells in the SE and the proximal part of SMG ducts were identified by their characteristic columnar shape and cilia that stained positively for acetylated β -tubulin (Fig. 1b ix).

SMGs consist of serous and mucus tubules, which are lined with polyhedral serous and mucus cells with rounded basal nuclei. Serous cells express lactoferrin, lysozyme and the polymeric immunoglobulin receptor, while mucus cells stain for MUC5AC, MUC1 and DMBT1, and mucins can also be visualized with Alcian Blue-Periodic Acid Schiff staining (Fig. 1c i–v). The SMG tubules are surrounded by elongated myoepithelial cells (MEC), which expressed K5, K14 and α -SMA (Fig. 1c vi). SMG tubules converge into the SMG ducts that are lined with a thin layer of stratified epithelium. The basal cells of the SMG duct cells express K5, K14, Integrin- α 6 and nerve growth factor receptor in addition to TROP-2 (Fig. 1b iii–v,ix and c vi).

Reproducibility and consistency of the hypoxic-ischemic injury in the tracheal transplant model

The severity of injury and stages of repair were consistently the same at day 1 and day 3 post-transplant in all tracheas examined. At the later time points post-transplant, the repair stages were consistent among tracheas, except for one to two tracheas per group, which showed a failure of re-epithelialization of the trachea. In these tracheas, no living epithelial cells were seen on the BM or in the SMG bed.

Repair of the SE, SMGs and SMG ducts occurs in a reproducible spatio-temporal pattern

To assess the time course of the *in vivo* regeneration of the mouse trachea SE, SMG and SMG ducts after severe hypoxic-ischemic injury, we performed syngeneic heterotopic murine tracheal transplants. This model^{3-5,13} allowed us to examine the surviving cell populations after injury and to study the temporal and spatial repair of the SE, SMG and SMG ducts.

At day 1, many SE cells expressed annexin v and sloughed into the lumen of trachea or SMG duct. Cells that remained attached to BM were $94.5 \pm 2.1\%$ K5+ basal cells of which only $4.7 \pm 4.8\%$ were also K14+, $4.9 \pm 1.2\%$ were CC10+ Clara cells and there were no ciliated cells. $55.5 \pm 3.2\%$ of the cells that remained attached to the BM were also annexin v+. In SMGs, $7.1 \pm 7.2\%$ of duct cells and $80.5 \pm 7\%$ of tubular cells were annexin v+ (Fig. 2 i–iii).

At day 3 post-transplantation, wide areas of the BM were denuded and all cells that remained attached to BM were K5+ basal cells with no CC10+ Clara cells or ciliated cells. About $12.9 \pm 4.2\%$ of the K5+ basal cells also expressed K14. Some of these K5+ cells still had rounded or low pyramidal nuclei, while other cells were found to have elongated nuclei with elongated cytoplasmic processes. Only $8.1 \pm 1.8\%$ of the cells that remained attached to BM were annexin v+. In the SMGs, there were no annexin v+ duct cells. Most of the SMG

beds located deeper in the submucosa than the ducts showed no nuclei, with only halos of dead cells. Of the few remaining nuclei, $95.1 \pm 6.8\%$ of cells were annexin v+ (Fig. 2 iv–vi).

On day 5 post-transplantation, apart from the sloughed cells in the lumen of the trachea or SMG ducts, no annexin v+ cells were detectable. The BM was completely covered with $90.3 \pm 3.1\%$ of SE cells being K5+, $8.5 \pm 2.7\%$ were CC10+, $8.2 \pm 1.6\%$ were lysozyme+ and there were no tubulin positive cells. $88 \pm 3.5\%$ of the K5+ cells also expressed K14. $92.7 \pm 3.4\%$ of SMG cells were K5+, $16.5 \pm 5.9\%$ were α -SMA+ and $14 \pm 5.6\%$ expressed lysozyme (Fig. 3 i–iv). Scattered areas of the tracheal SE were two to three cell layers thick with round to oval-shaped cells. TROP-2,¹⁰ a previously described marker of prostate basal cells, was detected in all the SE cells and was also detectable in all the SMG-like structures that were forming in the SMG area (Fig. 3 ii). This is in contrast to steady-state tracheas, where TROP-2 is present in all SE cells but only in SMG duct cells and not in SMG tubules (Fig. 1b ii). $16.5 \pm 5.9\%$ of the TROP-2+ SMG-like structures developed two to three layers of epithelial cells in which the outer cells expressed α -SMA, in addition to K5 and K14 (Fig. 3 iii).

$14 \pm 5.6\%$ of the inner cells of the SMG-like structures expressed lysozyme, lactoferrin and polymeric immunoglobulin receptor (all markers of serous cells) that were now also detectable in secretions within the lumen of the SMG-like structures (Fig. 3 iv and data not shown). Although mucus secretions as determined by DMBT1 expression were not present (Fig. 3 iv), AB-PAS staining detected neutral mucus secretions in the lumen of the healing SMG ducts, indicating at least initial differentiation towards mucus cells (Fig. 3 i).

The repair continued through day 7, where the BM was completely covered with hyperplastic SE consisting of three to five cell layers of stratified to pseudostratified epithelium (Fig. 3 v–viii). TROP-2 was still present in all the SMG-like structures in the submucosa (Fig. 3 vi). About $85 \pm 4.6\%$ of the cells of the SE were K5+ and of these $79.1 \pm 9\%$ also expressed K14 (Fig. 3 vii). Of the remainder of the cells on the SE, $14.4 \pm 1.9\%$ were CC10+, $8.3 \pm 2.3\%$ were lysozyme+ and no tubulin positive cells were seen. Many of the cells that expressed these differentiation markers in SE and SMG were also positive for K5.

The epithelial hyperplasia extended into the SMG ducts too. $82.1 \pm 6.9\%$ of all the cells of the SMG ducts still expressed K5 and K14, while other ducts expressed K5/K14 in cells in the basal layer only, as is seen in steady-state ducts (data not shown). In the SMGs, $43.8 \pm 5.5\%$ were α -SMA+/K5+/K14+ MECs but were still rounded in morphology and not elongated, as in the fully repaired state (Fig. 3 vii). About $20.2 \pm 2.7\%$ of the SMG cells expressed lysozyme, but the mucus cell marker DMBT1 was still undetectable (Fig. 3 viii). Similar to the day 5 post-transplant time point, neutral mucus was detected by AB-PAS staining, indicating the presence of some functional mucus cells (Fig. 3 v).

By day 10 post-transplantation, $89 \pm 3\%$ of the cells of the SE were K5+ and of these $51.3 \pm 3.5\%$ still expressed K14. $18.6 \pm 2.2\%$ of SE cells were CC10+, $8.5 \pm 2.4\%$ were lysozyme+ and $13.3 \pm 4.1\%$ were tubulin positive ciliated cells. Many of the differentiation markers in SE and SMGs were also positive for K5 (Fig. 4 ii,iii). The SMGs now had distinct duct-like and tubule-like structures. TROP-2 was detected in all the SE, SMG ducts and in all the tubule-like structures, including the MECs (Fig. 4 ii). The α -SMA+/K5+/K14+ MECs represented $14.9 \pm 8.8\%$ of all SMG cells and were now thinner and longer (Fig. 4 iii). $10 \pm 3.4\%$ of the differentiated cells of the SE were serous secretory cells (expressing lysozyme). No DMBT1 expression was seen yet (Fig. 4 iv), although both neutral and acidic mucus were detectable with AB-PAS staining, indicating the development of differentiated mucus cells (Fig. 4 i).

By 14 days post-transplantation, there was near complete return of SE to its normal pseudostratified appearance with $37.8 \pm 6\%$ of SE cells expressing K5, which were mostly basal cells and K14+ cells represented only $15.4 \pm 4.3\%$ of all cells of the SE (Fig. 4 vi,vii). About $31.4 \pm 5.4\%$ of SE cells were CC10+, $14.8 \pm 1.8\%$ were lysozyme+ and $20.4 \pm 3.4\%$ were tubulin positive. Clara, serous and ciliated cells were therefore present in the SE in numbers similar to the steady state.

$71.5 \pm 5.5\%$ of SMG cells were K5+ and $11.5 \pm 2.7\%$ were lysozyme+, and DMBT1 was detectable and stained $6.3 \pm 3.5\%$ of the SMG cells (Fig. 4 vii,viii). Many of the cells that expressed differentiated markers in the SE and SMG also expressed K5. Neutral and acidic mucus were now detectable in the SMGs and in SE, indicating the development of mucus tubules and goblet cells (Fig. 4 v). TROP-2 was still detected in the SE, SMG ducts and in all the tubule-like structures, including the MECs (Fig. 4 vi). The α -SMA+/K5+/K14+ MECs represented $20.8 \pm 7.1\%$ of cells and were elongated in morphology (Fig. 2c vii). We found that repair of the SE and SMG was almost complete by 14 days post-transplantation and that tracheas collected at 21 days post-transplantation were similar to the 14-day tracheas but their lumens, SMG ducts and tubules were distended with secretions, which probably reflects the lack of proper drainage of secretions in the grafts placed in a heterotopic position (data not shown). A graphical representation of the quantitative immunostaining data is shown in Figure 5 and demonstrates the temporal change in cell populations during injury and repair.

Identification of the proliferating cell type and distribution

We performed pulsing studies with BrdU to delineate the proliferating cell populations. All recipient mice with the heterotopic tracheal grafts received two doses of BrdU 6 and 3 h prior to euthanasia. At day 1, no BrdU+ cells were seen in the SE or SMG (Fig. 6 i). At day 3, $53.4 \pm 9.1\%$ of cells on the SE were BrdU+ and K5+ basal cells. $7.7 \pm 4.9\%$ of cells in SMG ducts were BrdU+. No BrdU+ cells were seen in SMG tubules (Fig. 6 ii). At day 5, there was a marked increase in the number of BrdU+ cells both on the SE and in the SMGs. In the SE, $22 \pm 9.8\%$ of cells were BrdU+ and K5+. No cells were double positive for BrdU and the differentiation markers CC10, lysozyme or tubulin. In the SMGs, $29.4 \pm 8.1\%$ of cells in the repairing ducts were BrdU+ and K5+ with $5 \pm 2.2\%$ also staining for α -SMA. No BrdU+ cells were seen in the deeper portions of the SMG bed. Some unidentified parenchymal cells were also BrdU positive (Fig. 6 iii). At day 7, $24 \pm 17.3\%$ of cells on the SE were BrdU+ and K5+ with $4.2 \pm 2\%$ also staining for CC10 or lysozyme but not tubulin. In the SMGs, $39.9 \pm 14.6\%$ of cells in SMG ducts were BrdU+ and K5+ with $12.3 \pm 4.4\%$ also staining for α -SMA. BrdU+ cells were seen throughout the SMG tubules (Fig. 6 iv). At day 10, $2.9 \pm 1.4\%$ of cells on the SE were BrdU+ and K5+ with no cells being double positive for BrdU with CC10, lysozyme or tubulin. In the SMGs, $8.9 \pm 4.2\%$ of cells were BrdU+ and K5+ with $4.6 \pm 3.5\%$ also staining for α -SMA (Fig. 6 v). At day 14, only $0.5 \pm 0.3\%$ of cells on the SE were BrdU+ and K5+ with no cells being double positive for BrdU with CC10, lysozyme or tubulin. In the SMGs, $5.8 \pm 2.6\%$ of cells were BrdU+ and K5+ with $2.7 \pm 1.5\%$ also staining for α -SMA (Fig. 6 vi). These data collectively indicate that the surviving K5+ basal and duct cells proliferate and then begin to differentiate at days 3–5. α -SMA+ differentiating MEC cells in the SMGs also start to proliferate from day 5 onward, while CC10+ differentiating secretory cells in the SE briefly participate in proliferation only at around day 7 post-transplantation.

SMG duct cells that survived the severe hypoxic-ischemic injury can regenerate the SE

The hypoxic-ischemic injury leaves behind some SE basal cells that are the presumed source of the regenerated SE after tracheal transplantation. To examine whether SMG duct cells have the ability to regenerate the SE when basal cells are absent, we incubated dissected

tracheas in pronase for 4 h, an enzymatic digestion that we showed previously removes all basal cells, leaving SMG duct and SMG cells in place.⁷ Then we performed heterotopic syngeneic transplants with the pronase-digested tracheas and compared them with heterotopic syngeneic tracheal transplants, which were not pronase digested and had basal cells. We found that the remnants of the SMGs and their ducts could regenerate pronase-denuded SE (Fig. 7a v,vi). However, notably, the SE of the pronase-digested tracheas repaired more slowly than the non-pronase-digested tracheas, with a delay in repair of the SE of about 7 days. The BM was still denuded at 7 days post-transplant in the pronase-digested tracheal grafts compared with the non-pronase-digested grafts that were lined by several layers of K5+K14+ epithelial cells (Fig. 7a i vs ii,iii). K5+K14+ epithelial cells were seen lining the BM at 14 days post-transplant in the pronase-digested tracheal grafts (Fig. 7a v,vi) but were delayed in repair as compared with the epithelium of non-pronase-digested tracheal grafts (Fig. 7a iv). The repair of the SMGs was not delayed after pronase digestion and the return of the different SMG and SMG duct cell types remained the same (Fig. 7b). These data suggest that in the absence of basal cells, SMG duct cells may have the ability to regenerate the SE.

DISCUSSION

Here, we show that the repair and regeneration of the mouse tracheal SE and SMG occurs in a set pattern with specific differentiated cell types that repair in a set order over a period of time. The initial phase, as expected, is to epithelialize the BM with K5 and K14 expressing cells. Thereafter, pseudostratification of the airway proceeds with gradual loss of K14 expression, as described previously.⁸ Our data suggest that the SMG tubules arise from the SMG ducts during the repair process in this model, as the SMG tubules express TROP-2 during airway repair, unlike at steady state where TROP-2 is only expressed in SMG ducts. The pattern of α -SMA expression is initially coincident with the TROP-2 expressing tubule cells, but at later time point begins to delineate the developing MECs from the cell types inside the tubules.

No previous work has been performed to demonstrate the exact order of repair of the cell types of the proximal airway epithelium, including the SMGs after hypoxic-ischemic injury. Genden *et al.* examined tracheal re-epithelialization after orthotopic allogeneic tracheal transplantation in mice and reported on re-epithelialization from recipient airway surface epithelial cells, which improved with immunosuppression.¹⁴ They noted the late appearance of ciliated cells but did not examine the possible contribution to repair and regeneration of the tracheal epithelium from endogenous cell populations and did not report on SMG repair.

The BrdU studies indicate that the initial proliferation and repair is from the stem/progenitor cells located in the SMG ducts and basal cells. They also show that the MECs contribute to repair of the SMG tubules through proliferation and we speculate that MECs may differentiate into the distal tubular cells although these data cannot address this question. It also indicates that Clara cells on the SE participate in repair by proliferating at around day 7 post-transplant. The Clara cells are possibly selfrenewing and/or differentiating into ciliated cells as seen in other injury models.¹⁵

This severe hypoxic-ischemic model is clinically relevant as in lung transplantation the blood supply is cut and the proximal and distal airways remain hypoxic for many hours and develop an ischemic injury. While the pulmonary circulation is restored at the time of transplantation, the bronchial vessels are usually not re-anastomosed and this further exacerbates the hypoxic-ischemic injury of the proximal airways until neovascularization occurs.

The contribution of the few basal cells left after injury to repair of the airway epithelium, as compared with the contribution of duct cells to repair, is important to identify. We therefore removed the basal cells to study this question. The repair of the SE occurred but was slower and indicates that the basal cells of the SE are likely the ‘first responders’ after the injury and that presumably the duct cells take about 5-7 days to move to the surface of the BM to help with SE repair. The duct cells give rise to all the SMG tubular cells, including MECs, and we believe that this is their primary reparative function. We therefore speculate that they are multi-potent cells in the correct setting.

In summary, we have shown the order of repair and regeneration of the cell populations of the large airways after hypoxic-ischemic injury. This is important for understanding abnormal repair and regeneration that can lead to airway diseases.

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Figure 1.

Location and expression of markers of the different cell types of the mouse tracheal SE and SMG and ducts. (a) Longitudinal section of the mouse trachea demonstrating the normal location of SMGs, with the largest group being between the larynx and C1 and smaller groups being located between C1 and C2; and C2 and C3. (b) Expression of markers of the SE and SMG ducts in the naïve trachea. E-cadherin and EpCAM are expressed on all the epithelial cells of the SE and SMG and SMG ducts (i, ii). TROP-2 was expressed on all cells of the SE and the SMG ducts but not in the SMG tubules (ii–iv). K14 was strongly expressed in SMG ducts and also in MECs (iii). K14 was expressed on only a few cells of the SE (iii).^{7–9} NGFR and ITGA6 were expressed in the basal cells of the SE and the basal cells of the SMG ducts (iv, v). ITGB4 was expressed on the basal surface of the basal cells (vi). CCSP and pIgR were expressed in the SE differentiated cells, while acetylated β -tubulin was expressed in ciliated cells of SE and proximal part of SMG (arrows) (vii–ix). (c) Expression of markers of SMG tubules in the naïve trachea. Lysozyme is a known marker of serous tubules and DMBT1 is a novel marker of mucus tubules (i, iv). They were both expressed strongly in the SMGs, while some SE cells expressed lysozyme, pIgR and lactoferrin (i.e. serous cells) (i–iv). AB/PAS staining detected neutral mucins (blue) and acidic mucins (magenta) in the SMG tubules (v). No positive mucin staining was seen in the SE, as there are no goblet cells in the naïve mouse SE (i, ii, iv, v). MEC express K5, K14 and α -SMA. SMG ducts were K5+/K14+ (white arrows). Occasional basal cells expressed K14 (green arrow) (vi). AB/PAS, Alcian Blue-Periodic Acid Schiff; α -SMA, α -smooth muscle actin; CCSP, Clara cell secretory protein; 4',6-diamidino-2-phenylindole DAPI, ●●; DMBT1, deleted in malignant brain tumour 1; ITGA6, Integrin- α 6; ITGB4, Integrin- β 4; MEC, myoepithelial cell; NGFR, nerve growth factor receptor; pIgR, polymeric immunoglobulin receptor; SE, surface epithelium; SMG, submucosal gland.

Figure 2.

Repair and regeneration of the mouse trachea at days 1 and 3 post-tracheal transplant. At day 1 post-transplantation (i–iii), initial injury and necrosis of all epithelial tissues were seen by haematoxylin and eosin (i). Most surface epithelial cells sloughed off into the lumen and most cells that remained attached to the BM were K5 positive basal cells. K5-expressing SMG duct cells were also seen (white arrows in iii). Widespread expression of annexin v was seen in the sloughed surface epithelial cells (ii). At day 3 (iv–vi), the cell death reached its maximum with only SMG duct cells and a few scattered basal cells on the SE surviving the injury. Wide areas of the BM were denuded and the few cells that remained attached to the BM were K5 positive. SMG ducts were lined with K5+ cells, while SMG tubules and MEC were necrotic (v, vi). Annexin v expression was seen in the sloughed cells in the lumen, SMG tubules and ducts (v). BM, basement membrane; 4',6-diamidino-2-phenylindole DAPI, ••; SE, surface epithelium; SMG, submucosal gland.

Figure 3.

Repair and regeneration of the mouse trachea at days 5 and 7 post-tracheal transplant. On day 5 post-transplantation (i–iv), the denuded basement membrane was now completely covered with TROP-2+K14+ cells (ii, iii). Most areas of the tracheal surface were two to three cell layers thick with round to oval cells (i–iv). TROP-2 was also found in all the SMG-like structures that were now starting to be seen in the submucosal area (ii). Annexin v expression was almost absent (ii). Some of the TROP-2+ SMG-like structures developed two to three layers of epithelial cells in which the outer cells started to express α -SMA, in addition to K5 and K14 (iii). No DMBT1 was expressed yet, but some cells of the regenerating SMG expressed lysozyme (iv). By day 7, the basement membrane was completely covered with three to five cell layers of epithelial cells of stratified to pseudostratified epithelium (v–viii). TROP-2 was still present in all the SMG-like structures in the submucosa as well as all the SE (vi). Most cells were K14+ although a few suprabasal cells were K14- (vii). In the submucosa, more α -SMA+/K14+ MEC were visible but were still rounded in morphology and not elongated, as in the fully repaired state (vii). DMBT1 was still not expressed, and cells of the regenerating SMGs continued to express lysozyme (iv). α -SMA, α -smooth muscle actin; 4',6-diamidino-2-phenylindole DAPI, ••; DMBT1, deleted in malignant brain tumour 1; MEC, myoepithelial cell; SE, surface epithelium.

Figure 4.

Repair and regeneration of the mouse trachea at days 10 and 14 post-tracheal transplant. By day 10 post-transplantation, there were more areas of pseudostratified SE (i–iv). Many cells were still K14+ (ii). The SMGs now had distinct duct-like and tubule-like structures. TROP-2 was detected in all the SE, SMG ducts and in all the tubule-like structures, including the MECs (ii). The α -SMA+/K14+ MECs were now thinner and longer (iii). A few serous secretory cells (expressing lysozyme) were detected in the SE (iv). By 14 days post-transplantation, there was near complete return of SE to its normal pseudostratified appearance (v–viii), with K14 expression disappearing from more basal cells (vi). MECs, SMG tubules and ducts all expressed K14 (vi, vii). TROP-2 was still detected in the SE, SMG ducts and in all the tubule-like structures, including the MECs (vi). DMBT1 expression was now found and lysozyme continued to be expressed in the SMG tubules (viii). α -SMA, α -smooth muscle actin; 4',6-diamidino-2-phenylindole DAPI, $\bullet\bullet$; DMBT1, deleted in malignant brain tumour 1; MEC, myoepithelial cell; SE, surface epithelium; SMG, submucosal gland.

Figure 5.
A graphical representation of the quantitative immunostaining data., annexin;, K5;, K14; , secretory cells;, tubulin.

Figure 6.

BrdU expression at time points post-tracheal transplantation. All recipient mice with the heterotopic tracheas from all time points received 2 mg of BrdU 6 and 3 h prior to sacrifice. At day 1, no BrdU+ cells were seen in the SE or SMGs (i). At day 3, BrdU+ cells are seen in the surviving basal and duct cells (ii). At day 5, there was a marked increase in the number of BrdU+ cells both in SE and SMG duct cells. No BrdU+ cells were seen in the SMG bed. Some unidentified parenchymal cells were also BrdU positive (iii). At day 7, the number of BrdU+ cells continued to be high both in the SE and in the SMG duct cells. BrdU+ cells were seen all over the SMG bed (iv). At day 10, there was a marked drop in BrdU+ cells in the SE and to a lesser extent in the SMGs. BrdU+ cells in the SMGs tended to be in the deeper portion of the gland bed. At day 14, very few BrdU+ cells were seen in both the SE and SMGs. DAPI, **; SE, surface epithelium; SMG, submucosal gland.

Figure 7.

Repair and regeneration of the mouse SE, SMGs and SMG ducts after severe hypoxic-ischemic injury after removal of basal cells. (a) Delayed repair and regeneration of the SE after pronase digestion of tracheal grafts. The basement membrane was still denuded at 7 days post-transplant in the pronase-digested tracheal grafts compared with the non-pronase-digested grafts that were lined by several layers of K5+K14+ epithelial cells (i vs ii, iii). K5+K14+ epithelial cells were seen lining the basement membrane at 14 days post-transplant in the pronase-digested tracheal grafts (vi) but were delayed in repair as compared with the epithelium of non-pronase-digested tracheal grafts (iv). SMG ducts and SMGs were not delayed in their repair and regeneration (i vs iii). (b) The cell types of the repairing tracheal grafts were not altered by pronase digestion of the grafts. SMG ducts expressed K5, K14 and TROP-2 in the typical pattern at 14 days post-transplant (i, ii, v) and SMG ducts (yellow arrows) were clearly distinct from tubules (white arrows) (ii). Myoepithelial, ciliated, mucous and serous cell types were also present in the usual expression pattern (ii–v). AB/PAS, Alcian Blue-Periodic Acid Schiff; α-SMA, α-smooth muscle actin; BM, basement membrane; 4',6-diamidino-2-phenylindole DAPI, **; SE, surface epithelium; SMG, submucosal gland.