Repair of naphthalene-induced acute tracheal injury by basal cells depends on β-catenin

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Objectives: Little is known about the role of Wnt/β-catenin in postnatal airway homeostasis and basal cell function. This study aimed to investigate the role of Wnt signaling in the self-renewal of basal cells and the involvement of β-catenin in tracheal repair after naphthalene-induced injury.

Methods: Mice were treated with naphthalene and injected with 4-hydroxytamoxifen. Injury and repair of the tracheal epithelium after naphthalene-mediated secretory cell depletion was assessed by a immunohistochemical study. The involvement of Wnt and β-catenin signaling in basal cell proliferation was investigated during in vitro expansion.

Results: Immunohistochemical analysis of tracheal epithelium in wild-type mice showed a reduction in the number of Clara cell secretory protein (CCSP+) and forkhead box transcription factor (Fox-J1+) cells on days 2 to 5 after naphthalene-induced injury; this cell population was regenerated by day 10. After flush labeling, bromodeoxyuridine-positive (BrdU+) cells and Ki67+ cells were observed in tracheal epithelium on days 2 to 5 but not on days 10 and 21. Confocal microscopy visualizing K5+ and BrdU+ cells showed that Wnt3a promotes proliferation of K5+ cells. Immunohistochemical analysis of K5+ and CCSP+ in tracheal epithelial cells from wild-type littermate and K5-Cre–mediated β-catenin knock-out mice showed that on day 3, the number of CCSP+ cells was decreased in all mice. On day 10, CCSP+ cells were present in wild-type littermate mice but absent in conditional knock-out mice.

Conclusions: Basal cells serve as stem cells in the tracheal epithelium, regenerating and maintaining tracheal epithelial cells in a mouse model of tracheal injury. β-Catenin is required for proliferation and self-renewal of tracheal epithelial cells. (J Thorac Cardiovasc Surg 2014;148:322-32)

Mouse tracheal epithelium is pseudostratified and composed of basal cells, secretory cells (Clara cells), and ciliated cells. Histologic markers to define tracheal epithelial cell types include keratin (K5, K14) for basal cells, Clara cell secretory protein (CCSP) for Clara cells, and forkhead box transcription factor (Fox-J1) for ciliated cells.[1] Basal cells (BCs) are relatively undifferentiated cells that make up approximately 30% of the pseudostratified mucociliary epithelium of the lung. BCs are present throughout the airways of the human lung, including small bronchioles.[2] In rodents, BCs are confined to the trachea, where they are interspersed among the ciliated, secretory, and neuroendocrine cells. Although murine tracheal BCs have been shown to play a role in maintaining homeostasis in the human lung, the precise mechanism is unknown.[3]

Using bromodeoxyuridine (BrdU) uptake, Borthwick and colleagues[4] showed that BCs make up 80% of the replicating cells during repair of tracheal epithelial damage induced by detergent or sulfur dioxide inhalation. Another study suggests that BCs do not contribute to columnar cell repopulation during repair but rather form an epithelial barrier to defend against further insult to the basement membrane.[5] Strong evidence implicates BCs as putative stem cells in the trachea and main bronchi. These observations were made in studies using in vivo genetic lineage labeling of K14+ or K5+ BCs and an in vitro clonal sphere-forming assay in which mouse K5+ BCs self-renew and generate luminal cells.[6]

To investigate the role of BCs in repairing airway injury, several models have been applied. Most of these models are dependent on the induction of Clara cell injuries by lung

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Abbreviations and Acronyms

BC = basal cells
BrdU = bromodeoxyuridine
BSA = bovine serum albumin
CCSP = Clara cell secretory protein
DAPI = 4,6-diamidino-2-phenylindole
DMSO = dimethylsulfoxide
Fox-J1 = forkhead box transcription factor
4-OHT = 4-hydroxytamoxifen
PBS = phosphate-buffered saline
PCNA = proliferating cell nuclear antigen
USG = Ultroser G serum substitute

toxins, followed by monitoring the appearance of CCSP+ cells.[7] Cytochrome P450 mixed-function oxidases serve to metabolize xenobiotics in the lungs and CCSP+ cells serve to protect the lungs against hyperoxic damage[8] and inflammation.[9] Because of the cellular preference and high levels of expression of cytochrome P450 monooxygenase isoform 2F2 (CYP2F2), CCSP+ cells have been the subject of numerous investigations to define their regulation and functional significance. Among the lung toxins administrated in rodent models, naphthalene is the one most often used for this purpose, not only because it is a harmful environmental toxin and can be found in ambient water, groundwater, and cigarette smoke[10] but also because its toxicity requires metabolic catalyzation by cytochrome P450 monooxygenases.[11] Because mouse Clara cells are the primary cellular site of CYP2F2 in the airway, murine Clara cells are more susceptible to naphthalene-induced injury than other types of airway epithelial cells.[12]

Wnt signals are transduced in a context-dependent manner to the canonical Wnt pathway for cell-fate determination.[13] Wnt proteins activate the canonical Wnt pathway through interaction with their Frizzled family receptors and LRP5/6 family coreceptors.[14] This interaction stabilizes β-catenin levels and affects the subcellular localization of β-catenin. Wnt signaling is endogenously activated in undifferentiated embryonic stem cells and is downregulated on differentiation. In addition, activation of Wnt signaling by a pharmacologic GSK-3-specific inhibitor can maintain pluripotency in human and mouse embryonic stem cells.[15] Previous studies also indicate that the self-renewal of hematopoietic stem cells in vitro can be promoted by activation of the canonical Wnt signaling pathway.[16] FZD2 and FZD6 are expressed on mesenchymal stem cells, in which Wnt10B activates the canonical Wnt signaling cascade to inhibit their differentiation into adipocytes.[17]

The role of Wnt/β-catenin in postnatal airway homeostasis and BC function is unknown. To investigate these questions, we generated a transgenic mouse line in this study that allowed us to genetically manipulate K5+ BCs and trace their lineage. We also investigated the kinase activation profile of BCs during in vitro expansion. We have shown that Wnt signaling plays an important role in the self-renewal of BCs and that β-catenin is necessary for in vitro proliferation and in vivo tracheal repair. The study design included immunohistochemical analysis performed to evaluate the expression of the markers of tracheal epithelial cell types after naphthalene-induced injury. An in vitro study was carried out to identify the Wnt/β-catenin pathway as the key signaling pathway necessary for basal cell proliferation. A confocal immunofluorescence study was performed to evaluate if there is nuclear translocation of β-catenin. Using transgenic mice, we investigated if β-catenin is required for tracheal repair after naphthalene-induced injury in vivo.

MATERIALS AND METHODS

Mice, Transgenic Mice, and Animal Husbandry

Colonies of either wild-type or genetically modified mice were maintained as in-house breeding colonies under specific pathogen-free conditions in the Laboratory Animal Facility of Taipei Veterans General Hospital. Animals received humane care and the protocol was approved by the Animal Committee of Taipei Veterans General Hospital. *Tg(BK5-CreER^T)*-inducible (I) mice bearing the *BK5-CreER^T* transgene were generated by pronuclear microinjection and further maintained on a C57BL/6J background. ROSA26 Cre reporter mice (Gt[ROSA]26Sor^{tm1Sor}; Jackson Laboratory, Bar Harbor, Maine) on a C57BL/6:129 mix background are referred to as R26R mice in this report. *Tg(BK5-CreER^T):R26R* bigenic mice were generated by breeding BK5-CreER^T mice with *ROSA26 Cre* reporter (*R26R*) mice as described previously.[18] B6.129-Ctnnb1^{tm2Kem}/*KnwJ (β-catenin^{FL})* mice were generated as previously described[19] and are available at Jackson Laboratory. *BK5-CreER^T;β-catenin$^{floxdel/+}$* mice were mated with *β-catenin$^{FL/FL}$* mice to generate *BK5-CreER^T;β-catenin$^{floxdel/FL}$* embryos (referred to generally as mutant embryos). Littermate *β-catenin$^{floxdel/FL}$* mice served as controls. The genotype was determined by polymerase chain reaction amplification of genomic DNA prepared from a 1-cm tail biopsy. For genotyping, the primers and cycling parameters have been described elsewhere.[17] For detecting *lacZ*, the following primers were used: 5′-GCA GAC CGT TTT CGC TCG G-3′ and 5′-CGA CCG CAT GGT CAG AAG C-3′.

Naphthalene and 4-Hydroxytamoxifen Treatments

Mice aged between 8 and 12 weeks were treated with naphthalene at a dose of 275 mg/kg body weight. Groups of 4 to 6 mice were allowed to recover for 0, 2, 3, 5, 10, or 21 days and injected intraperitoneally (i.p.) with 50 mg/kg body weight of BrdU (Sigma, St Louis, MO) 2 hours before being killed. Parenteral administration of 4-hydroxytamoxifen (4-OHT; Sigma) was used for conditional introduction of lineage tags within *Tg(BK5-CreER^T):R26R* bigenic mice. 4-OHT treatment was used as an inductive agent in *BK5-CreER^T* transgenic mice to activate the expression of Cre recombinase. 4-OHT was prepared as a 40 mg/mL suspension in sunflower seed oil and 100 μL of a 40 mg/mL solution was injected i.p. each day on days −1, 0, and 1 after naphthalene exposure. Groups of naphthalene/4-OHT−treated mice were then killed on day 0 and day 45 after naphthalene exposure for evaluation of LacZ reporter gene expression.

β-Galactosidase Histochemistry

The cell type specificity and ligand-dependence of cre-mediated recombination was assessed for each K5 line using systemic administration of 4-OHT (100 μg/mouse) in sunflower oil (250 μL/mouse i.p.) to the

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