Expression and role of Oct3/4, Nanog and Sox2 in regeneration of rat tracheal epithelium

N. Song*, X.-S. Jia*†, L.-L. Jia*, X.-B. Ma*, F. Li‡, E.-H. Wang*† and X. Li*

*Department of Pathology, The First Affiliated Hospital of China Medical University, Shenyang, China, †Department of Pathology, College of Basic Medical Sciences, China Medical University, Shenyang, China, and ‡Department of Biology, Old Dominion University, Norfolk, VA, USA

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Abstract

Objectives: To explore the role of Oct3/4, Nanog and Sox2 in regeneration of rat tracheal epithelium.

Materials and methods: An *ex vivo* model of rat tracheal epithelial regeneration using 5-fluorouracil (5-FU) was developed, to induce injury. Expression levels of Oct3/4, Nanog and Sox2 were examined using Western blot analysis, RT-PCR or microscopically observed immunofluorescence, and cell morphological changes were observed using HE staining, during the recovery process.

Results: Oct3/4, Nanog and Sox2 were not detectable in normal tracheal epithelium. After treatment with 5-FU, the normally proliferating tracheal epithelium desquamated and only a few cells in G0 phase of the cell cycle were left on the basement membrane and Oct3/4, Nanog and Sox2 could be observed at this time. Thereafter, the number of Oct3/4-, Nanog- and Sox2-positive cells increased gradually. When the cells differentiated into ciliated cells, mucous cells or basal cells, and restored pseudostratified mucociliary epithelium, the number of Oct3/4-, Nanog- and Sox2-positive cells decreased and gradually disappeared.

Conclusions: G0 phase cells with resistance to 5-FU damage expressed Oct3/4, Nanog and Sox2. This indicated that these cells were undifferentiated, but had the ability to terminally differentiate into downstream-type cells. They possessed stem cell properties. The results are consistent with Oct3/4, Nanog and Sox2-expressing cells being considered as tracheal stem cells.

Introduction

A concept of a correlative genome for embryonic stem (ES) cells has been proposed, recently, which is expressed only in the ES cells, but not in mature somatic cells. This plays an important role in maintaining the undifferentiated state and pluripotency of stem cells. *Oct3/4*, *Nanog* and *Sox2* genes are typical examples of this genome. *Oct3/4* is a synonym for *Pou5f1* which encodes Pou5f1 protein, a member of the POU family of transcription factors (1–3). A marker of totipotency, it can promote formation of inner cell mass (ICM) and maintain the undifferentiated state of ES cells (4–6). *Oct3/4* can be expressed in many kinds of totipotent cells including oocytes, archaeocytes, pre-implantation embryos, primitive ectoderm, ICM and ES cells (7–10). However, it is never expressed in differentiated daughter cells (11–13).

Recently, several co-factors of Oct3/4 have been identified including ELA, Sox2 (Sry-related factor) and Rox-1 (14,15). ELA was thought to be a bridging factor between Oct3/4 and the basic transcription machinery and Sox2 can activate *Ulf1* and *Fgf-4* genes (16,17). These target genes of Oct3/4 are expressed in different embryonic tissues and regulating expression of these genes maintains normal development of embryos (5,18). Nanog is a newly identified transcriptional factor and evidence has shown that it is expressed specifically in ICM, ES cells and EG cells (19,20), but not in differentiated, mature somatic cells (21,22). Nanog-deficient ES cells lose their pluripotency and begin to differentiate, as Nanog is an important regulator for maintaining pluripotent and self-renewal capacities of ES cells (23). Remarkably, these pluripotency factors, Oct3/4, Sox2 and Nanog, have also been shown to participate in reprogramming of differentiated cells back to the pluripotential state (24,25).

Recent studies have shown that Oct3/4 is expressed in a variety of somatic stem cells and in multipotent somatic progenitor cells, where it has been suggested that it

Correspondence: Prof. X.-S. Jia, Department of Pathology, College of Basic Medical Sciences, China Medical University, Shenyang 110001, China. Tel.: +86-24-23256666; Fax: +86-24-22515706; E-mail: xinshanjia@hotmail.com

functions in a manner analogous to its role in ES cells (26–28); Sox2 and Nanog may regulate expression of Oct3/4 (29). An *ex vivo* model of rat tracheal epithelial regeneration using 5-fluorouracil (5-FU) was developed to induce tissue injury (30). In the trachea, there are tracheal stem cells which when in the G0 phase have resistance to 5-FU. To identify new markers of tracheal stem cells, the same model was used in the present study and expression of Oct3/4, Nanog and Sox2 detected to study their roles in this tissue.

Materials and Methods

Model for tracheal epithelial regeneration

Male and female Wistar rats (~200 g) were used in accordance with the guidelines of the Animal Care Committee of the China Medical University. Material and reagents for tissue culture were purchased from Invitrogen (Carlsbad, CA, USA). Animals were killed and tracheas were excised under sterile conditions and then cultured in 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 medium (DMEM/F12) containing 120 mg/mL 5-FU and 10% foetal bovine serum (FBS), for 12 h at 37 °C. After washing off the 5-FU, tracheas were continued to be cultured in DMEM/F12 containing 10% FBS. After 0, 3, 6, 12, 24 and 48 h in culture, the tracheas were collected. For RT-PCR and Western blot analyses, tracheal epithelia was digested and stored at –80 °C until further use. For immunofluorescence analysis, tracheas were fixed in 4% paraformaldehyde and prepared for paraffin-embedded tissue sections (3 µm thick) with haematoxylin–eosin (HE) staining and immunofluorescence investigation.

Isolation of tracheal epithelial cells

Epithelial cells were isolated from treated and untreated tracheas. Tracheas were digested by filling lumina of ligated trachea with 1 mg/mL proteinase XIV solution (Sigma-Aldrich, St Louis, MO, USA) in DMEM/F12 without serum, at 4 °C overnight. Enzymatic digestion was terminated by adding 10 mL of DMEM/F12 containing 10% FBS. Cell aggregates were collected, a subset was stored at –80 °C for further analysis using RT-PCR and Western blotting, while other fractions were pooled and centrifuged at 1500 *g* for 5 min at room temperature. After removal of supernatants, pellets were resuspended and washed in 10 mL of phosphate-buffered saline (PBS), centrifuged at 1500 *g* for 5 min and resuspended in 0.5 mL of PBS. Cells were labelled while in the PBS with 5.0 µg/mL Hoechst33342 dye, the method being basically as described previously (31).

Indirect immunofluorescence

Indirect immunofluorescence staining was performed using Oct3/4, Nanog and Sox2 antibodies, on serial tissue sections (3 µm thickness) of tracheas during the recovery from injury period (Santa Cruz Biotechnology, Santa Cruz, CA, USA), using an experimental protocol as described previously (30). Briefly, goat anti-Oct3/4, rabbit anti-Nanog and goat anti-Sox2 (dilution 1:100) were used as primary antibodies. Rhodamine isothiocyanate (TRITC)-conjugated rabbit antigoat IgG and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (IgG) (dilution 1:100; HuaMei, Beijing, China) were used as secondary antibodies, which were diluted with 1% bovine serum albumin (BSA)–PBS. After treatment with the secondary antibody, specimens were incubated with 0.5 µg/mL of 4,6-diamidino-2-phenylindole (DAPI; Sigma) for nuclear counterstaining. Specimens were examined using an epillumination fluorescence microscope BX50 (Olympus, Tokyo, Japan). For serum controls, 1% BSA–PBS instead of the primary antibody was used as a negative control.

Western blotting and densitometric analyses

Total cell homogenates were prepared by lysing cells in NP40 lysis buffer consisting of 1% NP40, 10% glycerol, 20 mM Tris–HCl pH 8.0, 137 mM NaCl and 4% complete protease inhibitor cocktail mix (Roche, Mannheim, Germany). Eighty micrograms of total protein was used for sodium dodecyl sulphate–polyacrylamide gel electrophoresis, followed by transferring blotting to Polyvinylidene fluoride (PVDF) (Immobilon; Millipore Corp., Billerica, MA, USA). Membranes were blocked with 5% non-fat dried milk in PBS for 1 h with gentle shaking, and were then washed three times, 10 min each wash, with PBS containing 0.1% Tween 20. Membranes were incubated with primary antibody (Table 1, all antibodies from Santa Cruz) in 1% BSA in PBS overnight at 4 °C with shaking. Membranes were washed as described earlier and incubated with secondary antibodies (Table 1) for 2 h at room temperature. They were then washed again and incubated with 3,3'-diaminobenzidine (DAB) for 2–3 min at room temperature. When bands reached the desired darkness (2–5 min), membranes were washed briefly in water followed with PBS. Finally, membranes were dried and photographed. After scanning, densitometric analysis was performed using IMAGEJ 1.33 software (National Institutes of Health, Bethesda, MD, USA).

RT-PCR analysis

Total RNA was extracted from cells harvested using TRIzol reagent (Invitrogen). RT-PCR was performed with

Table 1. Antibodies used in Western blot analysis

Primary antibody				Secondary antibody (peroxidase-conjugated)		
Name	Source	Dilution	Product no.	Name	Dilution	Product no.
Oct3/4	Goat	1:200	sc-8628	Rabbit anti-goat IgG	1:2000	sc-2768
Sox2		1:100	sc-17320			
Nanog	Rabbit	1:100	sc-33760	Goat anti-rabbit IgG	1:2000	sc-2004
β -actin		1:200	sc-7210			

Table 2. RT-PCR primer sequences and product sizes

Gene	Primer sequence (5' → 3')	Accession number	Size (bp)	Annealing temp. (°C)
Oct3/4	agg cag gag cac gag tgg a cga agc ggc aga tgg ttg t	NM_001009178	264	58.3
Nanog	tct cct ccg cct tcc tct ttg cct ctg aaa cct atc ctt g	NM_001100781	204	53.1
Sox2	ggg ctc tgt ggt caa gtc tag tcg gca tca cgg ttt	NM_001109181	435	62.1
β -actin	cca agg cca acc gcg aga aga tga c agg gta cat ggt ggt gcc gcc aga c	NM_031144	587	58

the TaKaRa RNA PCR Kit (AMV) version 3.0 (Takara Bio, Otsu, Japan) according to the manufacturer's protocol. PCR primers were designed span exons, to exclude the possibility of genomic contamination (Table 2), and β -actin was used as internal control. PCR conditions were as follows: 94 °C for 2 min, 94 °C for 30 s, variable temperature (see Table 2) for 40 s, and 72 °C for 1 min, for 35 cycles. Reverse transcription reactions lacking reverse transcriptase served as negative controls. PCR products were visualized by ethidium bromide staining on 2% agarose gels using a gel scanner (UVP, Cambridge, UK).

Statistical analysis

Data from at least three independent experiments were used for statistical analysis by the SPSS 11.5 (SPSS Inc.,

Chicago, IL, USA). All values were expressed as mean $\pm$ SD. Statistical analyses were performed using one-way ANOVA, where $P < 0.05$ was considered significant.

Results

Morphological changes in rat tracheal epithelium after ex vivo injury induced by 5-FU

Normal rat trachea and rat tracheas treated with 5-FU were analysed using HE staining, to investigate morphological changes in rat tracheal epithelium (Fig. 1). Immediately after 5-FU treatment (0 h), only a few cells with less cytoplasm were observed on the basement membrane. Three hours later, cytoplasm extended along

Figure 1. Morphological changes in rat tracheal epithelium after injury induced by 5-FU as observed by HE staining. N) Normal rat tracheal epithelium is pseudostratified. 0 h) Only a few cells with less cytoplasm than normal tracheal epithelial cells remained on the basement membrane immediately after 5-FU treatment. 3 h) Cytoplasm of these cells extended along the basement membrane and cells became to look flat. 6 h) The number of flat cells increased and they had less cytoplasm. 12 h) Cells appeared cuboidal. 24 h) The number of cells increased sharply. 48 h) Cilia appeared on the luminal side of the trachea, tracheal epithelium became pseudostratified and appeared to be almost completely recovered (original magnification $\times 400$).

Table 3. Cell number of different cell types in tracheal epithelium per five fields ($\times 400$) during regeneration

Time	Type	N	0 h	3 h	6 h	12 h	24 h	48 h
Oct3/4(+)	Number	0	10	19	63	31	12	5
	Ratio*	0	0.400	0.475	0.830	0.221	0.070	0.020
Nanog(+)	Number	0	10	21	65	30	10	4
	Ratio*	0	0.400	0.525	0.867	0.214	0.059	0.016
Sox2(+)	Number	0	9	18	62	33	10	5
	Ratio*	0	0.360	0.450	0.827	0.236	0.059	0.020
Total		252	25	40	75	140	170	249

*Ratio = Cell number of different cell types/total cell number.

basement membranes and cells appeared flattened. After 6 h, the number of flattened cells increased and they appeared to become cuboidal by 12 h. The number of flattened cells increased sharply at 24 h. At roughly 48 h, cilia were observed on the surface of the tracheal epithelium, which appeared pseudostratified and almost completely normal.

Expression of Oct3/4, Nanog and Sox2 in tracheal epithelium as detected by indirect immunofluorescence

To examine localization of Oct3/4, Nanog and Sox2, immunofluorescence staining was performed on serial sections of the tracheal epithelium with and without 5-FU treatment. Bright green fluorescence in cells indicated Nanog positive expression, and bright red fluorescence indicated Oct3/4 and Sox2 positive expression.

It was observed that Oct3/4 was negative in normal tracheal epithelium. Immediately after 5-FU treatment, very few Oct3/4-positive cells were detected. Subsequently, the number of Oct3/4-positive cells increased gradually and reached the maximal level at around 6 h (Table 3). Then its expression began to decrease and returned to baseline levels after 48 h. Expression patterns of Nanog and Sox2 were similar to those of Oct3/4, and all three were all localized in the same cells (Fig. 2).

Expression of Oct3/4, Nanog and Sox2 in tracheal epithelium as detected by Western blot analysis

To confirm further changes in Oct3/4, Nanog and Sox2 during regeneration of tracheal epithelium, the protein levels of Oct3/4, Nanog and Sox2 (Fig. 3a) were examined in after 5-FU treatment, using Western blot analysis. Results showed similar expression trends for all Oct3/4, Nanog and Sox2. However, Oct3/4, Nanog and Sox2 were not detectable in normal rat tracheal epithelium. After treatment with 5-FU, expression levels increased and reached the maximal level at around 6 h, and decreased gradually to return to very low levels by

approximately 48 h (Fig. 3b). This indicated that changes in Oct3/4, Nanog and Sox2 levels were consistent with results obtained by immunofluorescence staining.

Figure 2. Immunofluorescence staining of Oct3/4, Nanog and Sox2 during recovery from injury induced by 5-FU. (a) In untreated rat tracheal epithelium, Oct3/4 was not expressed. After treatment with 5-FU, the number of Oct3/4-positive cells increased gradually, reaching its peak at about 6 h, then decreased gradually and returned to its normal levels at about 48 h. (b,c) Expression tendency of Nanog and Sox2 was similar to that of Oct3/4, as they all are located in the same cells (original magnification $\times 400$).

Figure 4. Expression of Oct3/4, Nanog and Sox2 in the rat tracheal epithelium. (a) mRNA levels of Oct3/4, Nanog and Sox2 in the rat tracheal epithelium before and after 5-FU treatment, were examined using RT-PCR. (b) Expression levels of Oct3/4, Nanog and Sox2 relative to β -actin. Data are presented as mean $\pm$ SD of three independent experiments.

Expression levels of Oct3/4, Nanog and Sox2 in tracheal epithelium as detected by RT-PCR analysis

Change in trend of Oct3/4, Nanog and Sox2 levels is in accordance with results obtained by Western blotting (Fig. 4a). There was no detectable Oct3/4, Nanog and Sox2 mRNA in normal tracheal epithelium. After treatment with 5-FU, expression levels increased and reached peak levels at about 6 h, and decreased gradually thereafter to return to very low levels by about 48 h (Fig. 4b).

Hoechst33342 labelling in the tracheal epithelium cells

To detect tracheal stem cells existing in the epithelium after treatment with 5-FU, we stained them with Hoechst33342 dye, which has been proved to provide dull staining in stem cells (32). As shown in Fig. 5, cells with no staining with Hoechst33342 under UV excitation, but with clear round cellular morphology under the bright visual field might be tracheal stem cells.

Discussion

Borthwick *et al.* (33) have shown that bromodeoxyuridine (BrdUrd) label-retaining cells (LRC) represent the tracheal stem cell compartment, which are localized to glands/-ducts in the upper trachea and to systematically arrayed foci in the lower trachea, where the BrdUrd LRC labelling index exceeded 30%. However, Kiel *et al.* (34) confirmed that less than 6% of haematopoietic stem cells (HSCs)

retain BrdUrd and less than 0.5% of all BrdUrd-retaining haematopoietic cells were HSCs, revealing that BrdUrd has poor specificity and poor sensitivity as an HSC marker. Therefore reliability of BrdUrd label-retaining tracheal stem cells remains to be elucidated. Recent reports have indicated that more than 95% HSCs (35,36) or epidermal stem cells (37) are in the G0 phase of the cell cycle, which provided an opportunity for us to investigate stem cells in G0 phase cells. A regeneration model of *ex vivo* trachea damaged by 5-FU has been constructed by us previously (30). 5-FU is a member of the antimetabolite

Figure 5. Tracheal epithelial cell smears with Hoechst 33342. (a) After UV excitation, blue nuclei could be seen; (b) cell shapes under bright field visual illumination compared with the same cells with no Hoechst33342 dye combination (arrow).

drug family and after treatment with 5-FU, normally proliferating tracheal epithelium desquamated, leaving only a few cells in G0 phase at the basement membrane (38–40). These cells were PCNA negative, indicating that they were in G0. In this study, we have repeatedly adjusted the concentration of 5-FU to maintain integrity of the basement membrane, this was indicated by silver staining (data not shown). After removal of 5-FU, the tracheal epithelium became flat then cuboidal, then recovered into pseudostratified epithelium. This process was only achieved by viable G0 cells left on the basement membrane. Our results confirmed that these cells were Hoechst 33342 negative and ABCG2 positive (30). This process also occurred in the *in vivo* model (41). Our results suggested that these cells might be tracheal stem cells.

The transcriptional regulator Oct3/4 is a Pou domain-containing protein expressed in pluripotent embryonic cells and cells of the germline, where its inactivation results in loss of pluripotency and apoptosis respectively (42,43). Oct3/4 interacts with other embryonic regulators, such as Sox2 and Nanog, to oversee a vast regulatory network that maintains pluripotency and inhibits cell differentiation (44,45). In this study, it was observed that Oct3/4, Nanog and Sox2 were not expressed in normal tracheal epithelium. After treatment with 5-FU, Oct3/4, Nanog and Sox2 were all expressed in G0 phase cells. When the cells differentiated into ciliated cells, mucous cells or basal cells, Oct3/4-, Nanog- and Sox2-positive cells decreased and gradually disappeared. Western blotting and RT-PCR data confirmed our observation. Oct3/4 gene expression in the adult has been reported in stem cells from a variety of tissues including kidney (46), mammary epithelium (47), pancreatic islets (48), mesenchymal stem cells (26–28) and liver (49). Upon induction of differentiation, expression of Oct3/4 decreased and disappeared. Oct3/4 may play an important role in self-renewal of somatic stem cells, and Sox2 and Nanog may regulate expression of Oct3/4; our results were consistent with these studies. Therefore, expression of Oct3/4, Nanog and Sox2 may be used as tracheal stem cell markers. Our study has suggested that Oct3/4, Nanog and Sox2 may not only be crucial for maintenance of pluripotency in embryonic cells, but may also play an important role in self-renewal of tracheal stem cells and in maintaining tissue homeostasis.

Recent reports have shown that Oct3/4, Nanog and Sox2 were expressed in ES cells. After differentiation, expression of Oct3/4, Nanog and Sox2 decreased and disappeared. These changes were regulated by effects of DNA methylation (50), histone acetylation or microRNAs (51). Thus, expression changes of Oct3/4, Nanog and Sox2 in regeneration of rat tracheal epithelium might use mechanisms similar to those of ES cells and further investigation is needed.

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