Constitutive expression of human keratin 14 gene in mouse lung induces premalignant lesions and squamous differentiation

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Squamous cell carcinoma accounts for 20% of all human lung cancers and is strongly linked to cigarette smoking. It develops through premalignant changes that are characterized by high levels of keratin 14 (K14) expression in the airway epithelium and evolve through basal cell hyperplasia, squamous metaplasia and dysplasia to carcinoma *in situ* and invasive carcinoma. In order to explore the impact of K14 in the pulmonary epithelium that normally lacks both squamous differentiation and K14 expression, human keratin 14 gene *hK14* was constitutively expressed in mouse airway progenitor cells using a mouse Clara cell specific 10 kDa protein (CC10) promoter. While the lungs of CC10-hK14 transgenic mice developed normally, we detected increased expression of K14 and the molecular markers of squamous differentiation program such as involucrin, loricrin, small proline-rich protein 1A, transglutaminase 1 and cholesterol sulfotransferase 2B1. In contrast, wild-type lungs were negative. Aging CC10-hK14 mice revealed multifocal airway cell hyperplasia, occasional squamous metaplasia and their lung tumors displayed evidence for multidirectional differentiation. We conclude that constitutive expression of *hK14* initiates squamous differentiation program in the mouse lung, but fails to promote squamous maturation. Our study provides a novel model for assessing the mechanisms of premalignant lesions *in vivo* by modifying differentiation and proliferation of airway progenitor cells.

Introduction

Squamous cell carcinoma (SQCA) of the lung accounts for ∼20% of human lung cancers in the USA and is strongly linked with cigarette smoking. Lung SQCA and its precursor lesions express high levels of keratin 14 (K14) (1,2). In many epithelia, including that of skin, esophagus and tongue, squamous differentiation represents a normal pathway of differentiation, whereas in other tissues, including the epithelial lining of conducting airways, it occurs under pathological conditions (3). Morphologic and biochemical studies have indicated that squamous differentiation is a multistage process and involves the sequential expression of many specific genes including keratins (3,4).

Keratins form a cytoplasmic network of 10–12 nm wide intermediate filaments and are classified into acidic type I keratins K9–K23

Abbreviations: CCE, cornified cell envelope; CC10, Clara cell specific 10 kDa protein; hK14, human keratin 14; IHC, immunohistochemistry; mCC10, mouse Clara cell 10 kDa protein; mRNA, messenger RNA; PCR, polymerase chain reaction; Q-RT–PCR, quantitative reverse transcription polymerase chain reaction; SQCA, squamous cell carcinoma; TG, transgenic; WT, wild-type.

and basic type II keratins K1–K8 (5). They occur as heterodimers, whose expression is dependent on the type of the epithelium. K14 is an epidermal keratin, which is paired with keratin 5 (K5) and located in the proliferating basal cell layer of stratified squamous epithelia. On the other hand, K8 and 18 are the keratins in non-cutaneous, single-layered epithelium including normal airways (1,5). There is emerging evidence that cytokeratins are important scaffolding proteins that may impact many cellular functions ranging from cell signaling to proliferation and apoptosis in addition to their more traditional role in structural support (6,7).

SQCA is the only human lung cancer type, whose premalignant lesions are well characterized (8–10). It is thought to develop from K14-positive basal cells through hyperplasia, squamous metaplasia and dysplasia to *in situ* and invasive carcinoma, whereas normal airway epithelium lacks any signs of squamous differentiation. In mouse lung, SQCA and K14-containing cells are exceedingly rare (supplementary Figure 1s is available at *Carcinogenesis* Online). In order to explore the role of K14 expression in pulmonary epithelium that normally lacks both squamous differentiation and K14 expression, human keratin 14 gene *hK14* was constitutively expressed in mouse lung using a Clara cell specific 10 kDa protein (CC10) promoter.

We postulated that mice rarely develop pulmonary SQCA because of the lack or relative sparsity of K14 in the airways. Here, we show evidence that constitutive expression of *hK14* initiates squamous differentiation pathway in CC10-hK14 transgenic (TG) mouse lung as evidenced by the expression of squamous differentiation markers of the cornified cell envelope (CCE) formation (11,12). TG animals displayed multifocal airway cell hyperplasia, squamous metaplasia and lung tumors with increasing age. While there was no histological evidence for overt squamous maturation, tumors displayed immunohistochemical evidence for multidirectional differentiation, suggesting that *hK14* modulates divergent pathways in the lung.

Materials and methods

Preparation of hK14 construct

We cloned *hK14* from human epidermal keratinocytes (ScienceCell Research Laboratories, San Diego, CA) by polymerase chain reaction (PCR) using high-fidelity enzyme *pfu* (Stratagene, La Jolla, CA; supplementary Figure 2s is available at *Carcinogenesis* Online). The *hK14* fragment (1.4 kb) was cloned into pcDNA3.1 (Invitrogen, San Diego, CA) and into 0.8 and 2.1 kb of upstream 5′-flanking sequences of the mouse Clara cell 10 kDa protein (mCC10) promoter (13). The promoter was a gift from Dr Francisco DeMayo, Baylor College of Medicine, Houston, TX. An *XbaI* fragment containing the transgene was gel purified using Qiaex (Qiagen, Valencia, CA) resin and eluted into 60 μl of modified Tris–ethylenediaminetetraacetic acid.

Cell lines and transfection experiments

The human immortalized normal bronchial epithelial BEAS-2B and lung adenocarcinoma (NCI-H441) cell lines were obtained from American Type Culture Collection (Manassas, VA) and propagated in BEGM® (Lonza, Walkersville, MD) and RPMI (Invitrogen), respectively. Transient transfections using liposome technique were carried out with a total of 5 μg DNA per well for six-well plates for 16 h.

Animals and genotyping

The CC10-hK14 transgene was microinjected into one-cell fertilized FVB/N mouse embryos at a concentration of 2 ng/μl as described previously (14). All animals were housed and handled in a human manner following the guidelines by an National Cancer Institute Animal Care and Use Committee

(NCI-ACUC)-approved protocol. Incorporation of the transgene was confirmed by Southern blot analysis of 10 µg of tail DNA digested with *BamHI* (15) and was probed with CC10-hK14 fragment labeled with ^{32}P by random priming with (^{32}P)dCTP (ICN, Irvine, CA) (16). The hybridization conditions were as described by Church *et al.* (17). TG pups were identified using a PCR analysis of tail biopsies taken after weaning. DNA was extracted from the specimens using Red-Extract (Sigma–Aldrich, St Louis, MO). The strategy and primers are illustrated in Figure 1C and Table I, respectively. Samples were amplified following denaturation at 94°C for 2 min by 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min and extension at 72°C for 1 min, with a final extension of 72°C (Bio-Rad iCycler, Hercules, VA).

RNA isolation and quantitative reverse transcription poymerase chain reaction analysis

Total RNA was isolated from cell lines and mouse tissue using RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. For real time PCR, complementary DNA was initially generated from 1.5 µg of RNA (previously treated by DNAase-I from Roche Diagnostics, Indianapolis, IN) extracted from cell lines, lungs and tracheas, using Script cDNA Synthesis Kit (Ambion, Austin, TX). PCR conditions for keratins and CCE proteins (Table I) were as follows: 95°C for 5 min, 25 cycles at 95°C for 1 min, 58°C for 1 min and 72°C for 1 min, with an extension step of 7 min at 72°C at the end of the last cycle. For quantitative reverse transcription polymerase chain reaction (Q-RT–PCR), QuantiTect SYBR® Green PCR Kit (Qiagen) and PCR amplification in MyiQ (Bio-Rad, Richmond, CA) were used according to the protocol provided by the manufacturer. Conditions for PCR included 50°C for 2 min, 95°C for 15 min and 45 cycles of 95°C for 30 s and 60°C for 30 s. Each reaction was run in triplicate in a 96-well plate. Relative expression of the target gene was calculated using the 2ΔCT method described previously (18). Relative expression $= 2^{-\Delta CT}$, where $\Delta CT = CT^{(target\ gene)} - CT^{(endogenous\ control\ gene)}$ where mouse ribosomal 15 and 18 (S15, S18) were used as the endogenous control gene.

Western blot analysis and immunoprecipitation

Cells and tissues were washed twice with phosphate-buffered saline and lysed in radio-immuno precipitation assay (RIPA) buffer. Protein concentration was
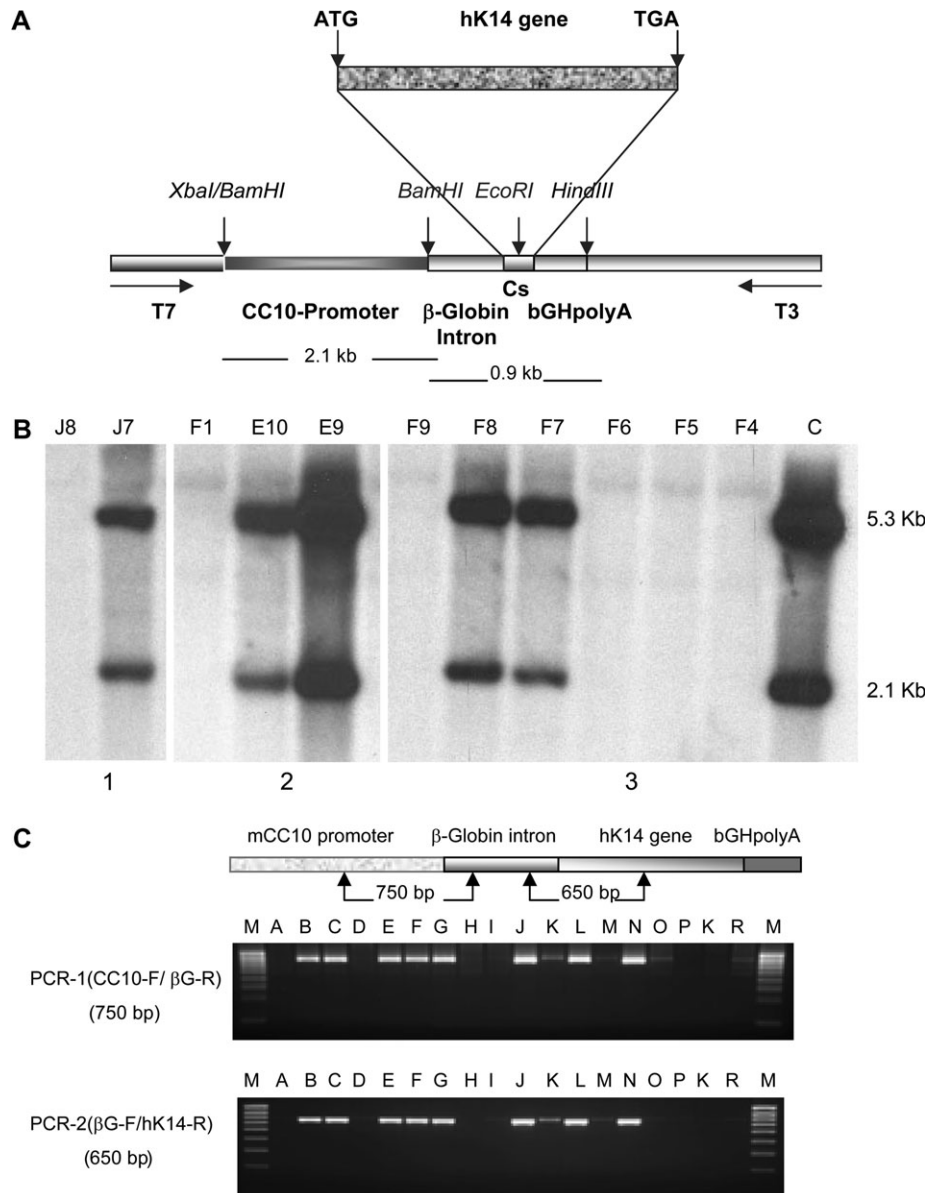

Fig. 1. Generation of CC10-hK14 TG mice. (**A**) *hK14* complementary DNA was cloned into a plasmid that contains a 2.1 kb mouse CC10 (mCC10) promoter, β-globin intron as enhancer and bovine growth hormone (bGH) polyA. Sequencing was done using T7 and T3 primers. (**B**) Southern blot analysis of CC10-hK14 TG mouse founders. Lanes J7, E10, E9, F8 and F7 revealed the expected band size (2.1 kb) for *hK14* transgene. The positive control (lane C) included a mixture of plasmid DNA (CC10-hK14) of transgene and WT tail DNA digested with *BamHI*. Results composed of three separate gels (1–3). (**C**) Genotyping of CC10-hK14 TG mice by PCR. Two sets of primers were used as indicated in a schematic illustration (top). Amplification of both fragments of tail DNA in lanes B, C, E–G, J–O and R indicated the contiguity of the incorporated transgene. First and last lanes (M) correspond to molecular markers.

Table I. Sequences of the primers

PCR systems	mRNA	Primer name	Sequences (5′–3′)
Conventional PCR	Mouse Clara cell specific 10 kDa protein	CC10-F	CAGGTACTCAAGGGCTGCTC
	Mouse β globin intron	βG-R	GGGTCCATGGTGATACAAGG
		βG-F	GGGTGTTGTGCTGTCTCATC
	Human K14	K14-R1	ATCTGCAGAAGGACATTGGC
		K14-F2	AGGTGACCATGCAGAACCTC
		K14-R2	GTCCACTGTGGCTGTGAGAA
	Mouse K14	K14-F1	CTGGTGGGCAGTGAGAAAGT
		K14-R1	CCAGGATCTTGCTCTTCAGG
	T7 promoter	T7	TAATACGACTCACTATAGGG
	T3 promoter	T3	ATTAACCCTCACTAAAG
Real-time PCR	Human K14	K14-F3	TGAGAAGGTGACCATGCAGA
		K14-R3	ATTGTCCACTGTGGCTGTGA
	Mouse K14	K14-F2	CAGCCCCTACTTCAAGACCA
		K14-R2	GGCTCTCAATCTGCATCTCC
	Mouse involucrin	INV-F	CAGAACTGCCTCTGGGAAAG
		INV-R	GGGTCAGGTGACTCCTGGTA
	Mouse loricrin	LOR-F	TCCTATGGAGGTGGTTCCAG
		LOR-R	CCACCTCCGGAGTACTTGAC
	Mouse small proline-rich-protein 1A (SPRR 1A)	SPRR-F	ATGAGTTCCCACCAGCAGAA
		SPRR-R	AGGCTCTGGTGCCTTAGGTT
	Mouse cholestrol sulfotransferase 2B1 (SULT2B1)	SULT2B1-F	TACACCCGACCAGTTCCTTC
		SULT2B1-R	CAGCAGCGAGTAGTTGGACA
	Mouse transglutaminase 1 (TGase 1)	TGase 1-F	GACTGGCGGCAAGAATATGT
		TGase 1-R	TGAGCAGGATCTCCACACTG
	Mouse ribosomal S15	S15-F	TTCCGCAAGTTCACCTACC
		S15-R	CGGGCCGGCCATGCTTTACG
	Mouse ribosomal S18	S18-F	TCCCATCCTTCACATCCTTC
		S18-R	TGTGGTGTTGAGGAAAGCAG

determined using Bio-Rad DC® protein assay with bovine immunoglobin G as the standard. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Bio-Rad) and transferred to an Immobilon-P membrane (Millipore, Bedford, MA). Anti-K14 polyclonal antibody (The Binding Site, San Diego, CA) was used at dilution of 1:500. Internal expression controls were done by using β-actin (Sigma-Aldrich). The protein expression of the *hK14* transgene in mouse tissues was determined by immunoprecipitation. Tissue lysates were incubated overnight with the mouse anti-hK14 antibody (Clone 4A30; US-Biological, Swampscott, MA) and then precipitated with protein G-agarose beads (Pierce, Rockford, IL) as described previously (19,20). Washed immunoprecipitates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and visualized on nitrocellulose using streptavidin-peroxidase method and enhanced chemiluminescence.

Morphology and immunohistochemistry

For morphologic analyses, mice were serially killed at 1–2, 3–4, 6–7, 14 and 21 months of age, a minimum of five mice in each group. Dissected lungs were fixed in 4% buffered paraformaldehyde and embedded in paraffin. Immunohistochemistry (IHC) was performed using avidin-biotinylated peroxidase method (21) and polyclonal anti-murine CC10 (1:10 000; a gift from Dr Francesco DeMayo, Baylor School of Medicine, Houston, TX), human prosurfactant protein C (proSP-C; 1:100, Chemicon International, Temecula, CA), involucrin and loricrin (1:500 each, Covance, San Diego, CA), K14, K8 and K18 (1:100 each, The Binding Site) and monoclonal anti-p63 (Clone 4A4; 1:500, Santa Cruz Biotechnology, Santa Cruz, CA) and cytokeratin (34βE12 cocktail; 1:100, Dako, Carpinteria, CA), which recognizes cytokeratins 1, 5, 10 and 14. Staining was analyzed with a Nikon Eclipse 400® microscope and Metamorph® software (Molecular Devices Corporation, Downingtown, PA).

Statistical analysis

Statistical analysis of data was carried by using SigmaStat3.5 software (Aspire Software International, Ashburn, VA). Mann–Whitney rank-sum and chi-square tests were used for analysis and a *P* value of 0.05 or less was considered to be significant.

Results

Cloning of hK14 and generation of CC10-hK14 TG mice

The hK14 gene was first cloned into an expression vector containing cytomegalovirus (CMV) in pcDNA3.1. In order to direct the expres-sion of *hK14* to airway epithelial cells, we prepared two different size constructs (0.8 and 2.1 kb) of mCC10 promoter (Figure 1A).

To confirm the functional expression of the cloned gene, we performed *in vitro* transfections using COS-7 and the human lung adenocarcinoma H441 cells. Using two different liposomal agents and CMV in pcDNA3.1, the 50 kDa hK14 was expressed at similar intensity. No expression was seen in untransfected parent COS-7 cells. Both 0.8 and 2.1 kb mCC10 promoters and CMV in pcDNA3.1 also resulted in hK14 expression at similar intensities in H441 cell line, which originally was negative (data not shown). The immortalized normal human bronchial epithelial cells BEAS-2B were used as positive control because of their endogenous expression of hK14 protein.

TG mice were generated using *hK14* gene fused to 2.1 kb of mCC10 5′-flanking DNA by standard methods. Several independent positive founders were obtained. Figure 1B shows a representative Southern blot analysis of tail DNA from 11 mice. Total DNA was digested with *BamHI*. A diagnostic 2.1 kb band was detected in mice J7, E10, E9, F8 and F7 migrating at the same level as the purified *BamHI* CC10 DNA fragment, which was used as probe. The 5.3 kb band in the gel represents a partial digest. The positive control for Southern blot included a mixture of plasmid DNA (transgene) and wild-type (WT) tail DNA, digested with the same enzyme *BamHI* (lane C). The diagnostic fragment was not detected in other mice (lanes J8, F1, F9, F6, F5 and F4). Mice J7, E10, E9, F8 and F7 were used as founders, which were outbred to establish breeding lines. The founders and first generation mice were followed up till 21 months.

The PCR-based genotyping strategy is illustrated in Figure 1C. In order to ensure the contiguity of the transgene that was incorporated into the mouse genome, we used two sets of primers (Table I) covering a 750 bp segment of the mCC10 promoter (PCR-1) as well as a 650 bp segment of the *hK14* gene (PCR-2). A representative gel (Figure 1C) demonstrates that there was contiguity of the transgene in 12 mice with similar expression levels following each PCR, thus confirming the incorporation. In eight of them there was strong expression (lanes B, C, E, F, G, J, L and N), whereas four of the mice showed weaker bands (lanes K, M, O and R).

Characterization of CC10-hK14 TG mice

The CC10-hK14 TG mice were breeding without difficulty. Grossly, lungs of TG mice appeared normal. Microscopic and ultrastructural (data not shown) examination failed to reveal significant abnormalities in younger animals. Clara cells in TG mice retained their hobnail appearance. However, the airway epithelium in older mice (6–21 months) showed multifocal hyperplasia and occasional squamous metaplasia (Figure 2A–D). By IHC, the lungs of WT littermates were consistently negative for K14, whereas the expression in older TG mice revealed several patterns including basal cell hyperplasia and positive cuboidal airway lining cells (Figure 2E and F). The immu-

noreactivity of K14 was highly variable, and it was not uncommon to see low levels or no expression in TG airways. A delicate, granular pattern was seen in CC10-hK14 TG mice using an antibody cocktail clone 34βE12 against human high-molecular weight cytokeratins (supplementary Figure 3s is available at *Carcinogenesis* Online). In contrast, most airway lining cells in WT and TG lungs contained CC10 (Figure 2G and H). Interestingly, there was a 20% reduction in body weight of TG mice compared with their WT littermates at 14 months ($P = 0.014$) (Figure 2I).

In order to measure the expression of the *hK14* transgene, lung tissues derived from CC10-hK14 TG mice were analyzed by
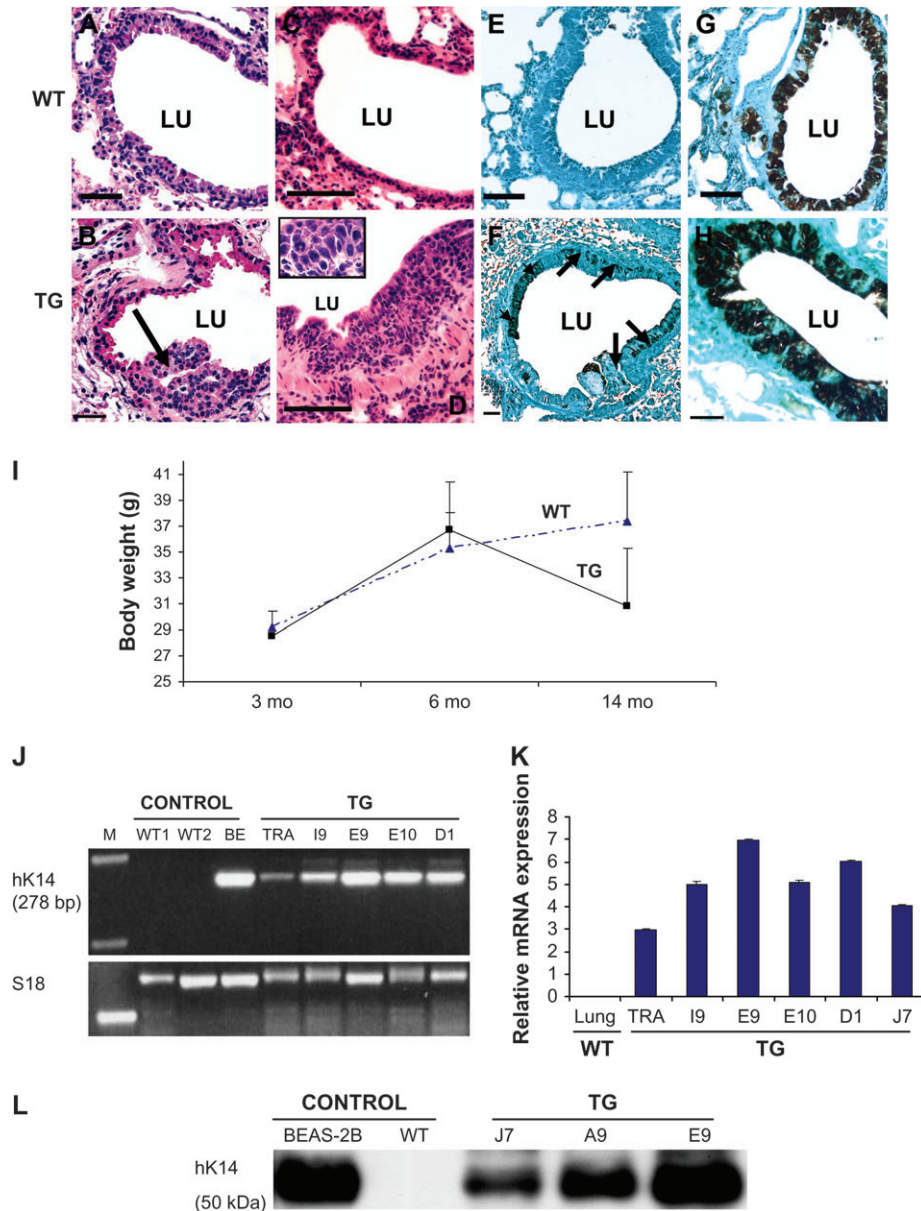

Fig. 2. Characterization of CC10-hK14 TG mice. Photomicrographs of normal bronchiolar epithelium from a WT mouse (**A**) (hematoxylin and eosin stain; bar = 200 μm) and focal epithelial hyperplasia (arrow) from TG mouse (**B**) (hematoxylin and eosin stain; bar = 100 μm). Normal bronchiolar epithelium from a young TG mouse (**C**) and squamous metaplasia in a TG mouse (**D**) (hematoxylin and eosin stain; bar = 400 μm). Inset shows a mitotic figure and intercellular bridges. Bronchiolar epithelium from a WT mouse (**E**) negative for K14 (immunoperoxidase stain; bar = 200 μm) and K14 immunoreactivity in basal cells (long arrows) and focal squamous metaplasia (short arrows) in TG bronchiolar epithelium (**F**) (immunoperoxidase stain; bar = 100 μm). CC10 immunorectivity in bronchiolar epithelium from a WT mouse (**G**) and in hyperplastic, thickened epithelium from a TG mouse (**H**) (immunoperoxidase stain; bar = 200 μm). LU = lumen. (**I**) A graph of body weights. Hatched line = WT mice; solid line = TG mice; mo = months. (**J**) Expression of *hK14* mRNA expression by Q-RT-PCR with ribosomal S18 as housekeeping gene. Controls included lungs from two lines of WT mice (WT1 and WT2) and BEAS-2B cells (BE). TG tissues included trachea (TRA) and lungs from independent lines I9, E9, E10 and D1 of mice. M = DNA markers. The primers used in this experiment detected only *hK14* gene with a 278 bp product. (**K**) A bar graph of relative *hK14* mRNA expression by Q-RT–PCR in a trachea and lungs from TG lines I9, E9, E10, D1, J7 and a WT mouse. (**L**) Immunoprecipitation analysis of *hK14* protein in human BEAS-2B cells and WT lung (control) and in lungs from TG mouse lines J7, A9 and E9.

semiquantitative and Q-RT–PCR (Figure 2J and K). As expected, no *hK14* messenger RNA (mRNA) was detected in lungs from WT littermates using human-specific primers (Table I). In lungs from TG mice, the expression level of *hK14* mRNA was comparable with that found in the positive control of normal human bronchial epithelial cells BEAS-2B, generating the specific *hK14* band of 278 bp (Figure 2J). By Q-RT–PCR, there was a consistent 4- to 7-fold increase in the relative expression of *hK14* mRNA in lungs of several independent lines of TG mice (Figure 2K). The *hK14* mRNA was also detected in the trachea of TG mice.

Moreover, we analyzed the expression of hK14 protein in the CC10-hK14 TG mouse lungs by immunoprecipitation (Figure 2L). Strong but variable expression of *hK14* was present in the lungs of several independent lines of TG animals, comparable with the level seen in the positive control of normal human bronchial epithelial cells BEAS-2B. As expected, WT lung showed no expression. These experiments confirmed that the transgene *hK14* was expressed both at mRNA and protein level in the lungs of CC10-hK14 mice.

In order to extend our observation that *hK14* was expressed in the trachea of TG animals (Figure 2J and K), we assessed the expression of K14 in tracheas from several lines of WT and TG mice by semiquantitative and Q-RT–PCR and IHC (Figure 3). Using common primers that have the ability to amplify both human and mouse *K14*, we observed a specific band of *K14* with 202 bp size in tracheas (supplementary Figure 4s is available at *Carcinogenesis* Online). Whereas WT mouse tracheas revealed low levels of K14 mRNA expression, the tracheas from TG mice showed 20–100% increase in the relative expression of *K14* mRNA compared with the ones obtained from WT mice (Figure 3A). No expression was detected in WT lung. Immunohistochemical analysis demonstrated multifocal basal cell hyperplasia as evidenced by continuous rows of K14-immunoreactive cells and occasional foci of squamous metaplasia occupying the entire thickness of the epithelial layer (Figure 3B-I). In contrast, the WT trachea typically revealed only a few, mostly scattered positive cells (Figure 3B-II).

Evidence for squamous differentiation and increased incidence of lung tumors in CC10-hK14 mice

A specific indicator of squamous differentiation is the formation of CCE (11,12,22). In order to examine whether increased expression of *hK14* was able to induce squamous differentiation in the respiratory tract of TG mice, we used Q-RT–PCR to measure CCE precursor proteins involucrin, loricrin, small proline-rich protein 1A and the enzymes cholesterol sulfotransferase 2B1 as well as transglutaminase 1 that mediates the CCE formation (23–27) (Figure 4). Although the levels were variable, five of five TG mice revealed up to 5-fold increase in involucrin, loricrin and small proline-rich protein 1A mRNAs in their lungs, and four of five TG mice up to 2- to 2.5-fold increase in cholesterol sulfotransferase 2B1 and transglutaminase 1 mRNAs. WT mouse lungs remained negative. The results indicated that squamous differentiation program was initiated.

In order to investigate whether the constitutive expression of *hK14* would induce lung tumors, groups of mice were serially killed and their lungs were examined. As in many mouse strains, older mice tend to develop more lung tumors (28), we followed the TG mice until 21 months of age (supplementary Table Is is available at *Carcinogenesis* Online). At 6–8 months, <10% of CC10-hK14 TG mice developed lung tumors, which was no different than in WT mice. Twenty and 67% (8 of 12) of TG mice had lung tumors at 14 and 21 months, respectively. In contrast, 27% and only 46% (11 of 24) WT mice had lung tumors at 14 and 21 months, respectively. Furthermore, detailed necropsies of four TG mice at the age of 21 months revealed incidental other tumors characteristic of older FVB/N mice including an adrenal pheochromocytoma and a mammary carcinoma (data not shown). No metastases were seen. The results suggested increased lung tumorigenesis in older TG animals.

Histologically, the pulmonary tumors ranged from adenomas to poorly differentiated carcinomas of the alveolar compartment and lacked overt squamous maturation (Figure 5A–D). However, by IHC focal positivity for the squamous markers p63 and K14 was detected in the airways and 2 of 10 tumors from TG mice,
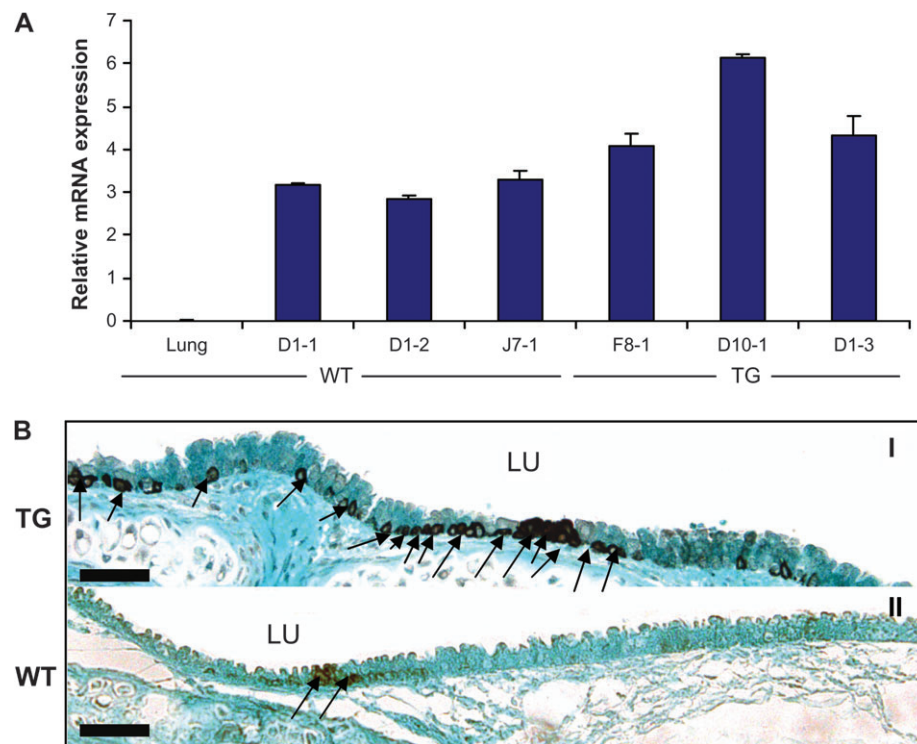

Fig. 3. Increased expression of K14 and basal cell hyperplasia in TG trachea. (**A**) Relative expression of *K14* mRNA by Q-RT–PCR in the lung and tracheas from WT littermates of mouse lines D1-1, D1-2 and J7-1 and from TG animals of mouse lines F8-1, D10-1 and D1-3. The primers used in this experiment detected both mouse and human *K14* mRNA. Ribosomal S15 and S18 were used as housekeeping genes. Photomicrographs of (**B**) basal cell hyperplasia (arrows) in tracheal epithelium from a TG mouse (I) and scattered basal cells (arrows) in the trachea of a WT mouse (K14 immunoperoxidase stain; bar = 200 μm). Lu = lumen.
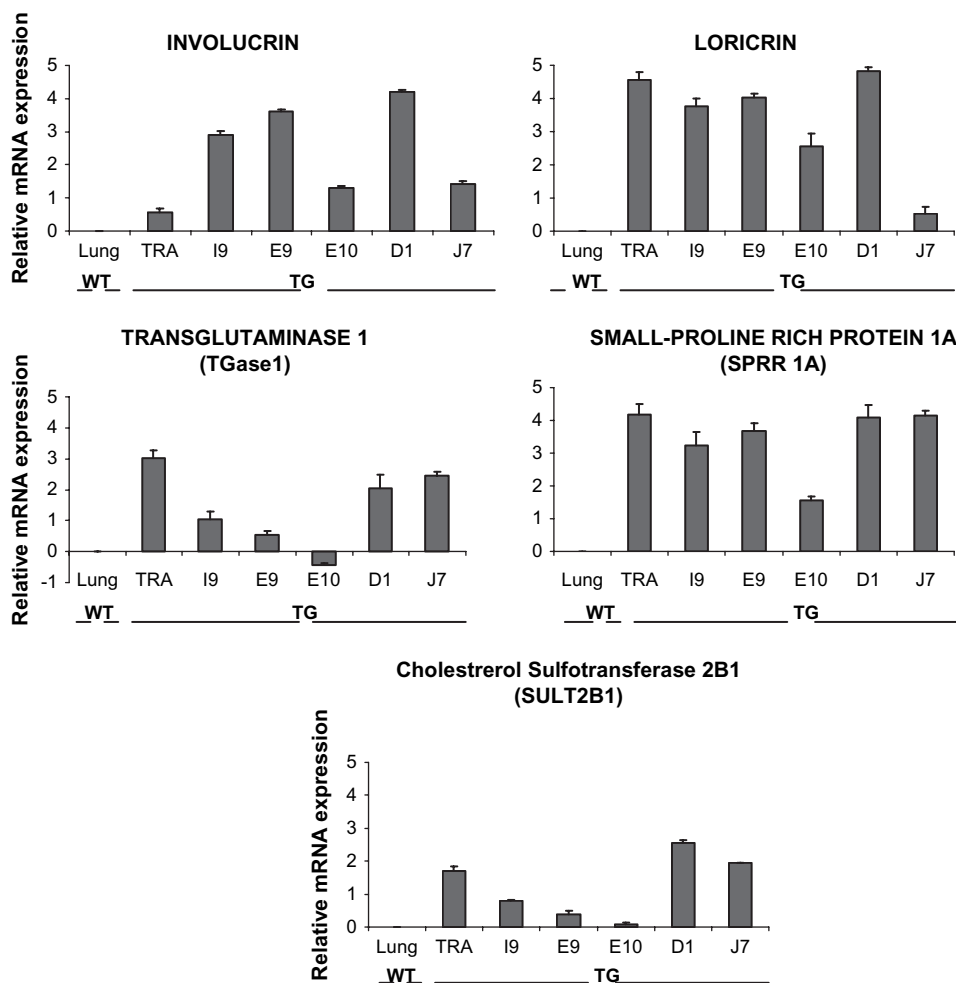
Fig. 4. Expression of CCE precursor mRNAs in the lungs and trachea of TG mice. Relative expression of involucrin, loricrin, transglutaminase 1 (TGase 1), small-proline rich protein 1A (SPRR 1A) and cholesterol sulfotransferase 2B1 (SULT2B1) mRNAs was analyzed by Q-RT–PCR in the lung tissue of WT mouse and in the trachea (TRA) and lungs from CC10-hK14 TG mouse lines I9, E9, E10, D1 and J7. Ribosomal S15 and S18 were used as housekeeping genes.

whereas the lungs of WT mice were negative (Figure 5E–J, O and P). Not surprisingly, all tumors were positive for alveolar type II cells (proSP-C), whereas only selective tumors of TG mice were immuno-reactive for CC10 (Figure 5K–N). Involucrin was positive in most WT and TG tumors, whereas loricrin immunoreactivity was limited to tumor cells of TG mice (Figure 5Q and R).

Discussion

We hypothesized that mice rarely get pulmonary SQCAs because they do not express K14 and have rare if any K14-containing cells in the airways. We showed that expression of *hK14* in the airway epithelium initiates squamous differentiation program and induces a number of airway abnormalities that greatly mimic premalignant changes of human lung carcinogenesis.

We used a mCC10 promoter in order to direct the expression of *hK14* to Clara cells that are the progenitor cells of normal and neo-plastic pulmonary epithelium (29–31) because mouse promoters allow the gene expression in a specific, single cell type in mouse lungs (32–35). Multiple lines of the CC10-hK14 TG mice revealed a 4- to 7-fold relative increase in the mRNA of *hK14*. Moreover, the protein levels of K14 were comparable with the levels observed in human bronchial epithelial cells, which were used as a positive control.

The constitutive expression of hK14 under the mCC10 promoter was well tolerated in the airways that normally express K8 and K18 of simple epithelium. The type II keratin K5 mRNA, which is normally the partner for the type I keratin K14, was slightly downregulated (data

not shown). However, the expression of K5 was strong in the basal cells and squamous metaplasias of TG mice that also expressed high levels of K14 providing the preferred and conceivably stabilizing part-ner (data not shown) (36,37). Our results are in accordance with pre-vious studies in which simple epithelial cells transfected with either an ectopic type I (K14) or type II (K6) epidermal keratin. The foreign proteins were merely incorporated into the existing endogenous ker-atin network without induction of partner or host keratins (38).

The strongest functional evidence that constitutive expression of *hK14* in the lung may have an impact on squamous pathway of dif-ferentiation came from the induction of involucrin, loricrin and other markers of CCE in the CC10-hK14 TG mice. In the epidermis, the plasma membrane of terminally differentiated, dead keratinocytes is replaced by insoluble cross-linked proteins and lipids called the CCE (11,12), whereas in the human lung cancer cells CCE formation can be induced *in vitro* by calcium ions serving as evidence for squamous differentiation pathway (22).

The molecular changes in CC10-hK14 TG mice were accompanied by histological changes that were distributed from trachea to periph-eral airways. This is consistent with the fact that unlike in humans, where Clara cells are restricted to the peripheral lung, mice have abundant CC10-containing cells also in trachea (39). One of the alter-ations was multifocal Clara cell hyperplasia, suggesting that *hK14* elicited a proliferative response. Other abnormalities were basal cell hyperplasia and squamous metaplasia/dyplasia, which are common early lesions in human squamous cell carcinogenesis (9) but rare in WT mouse lungs. The origin of basal cells in the murine lung is
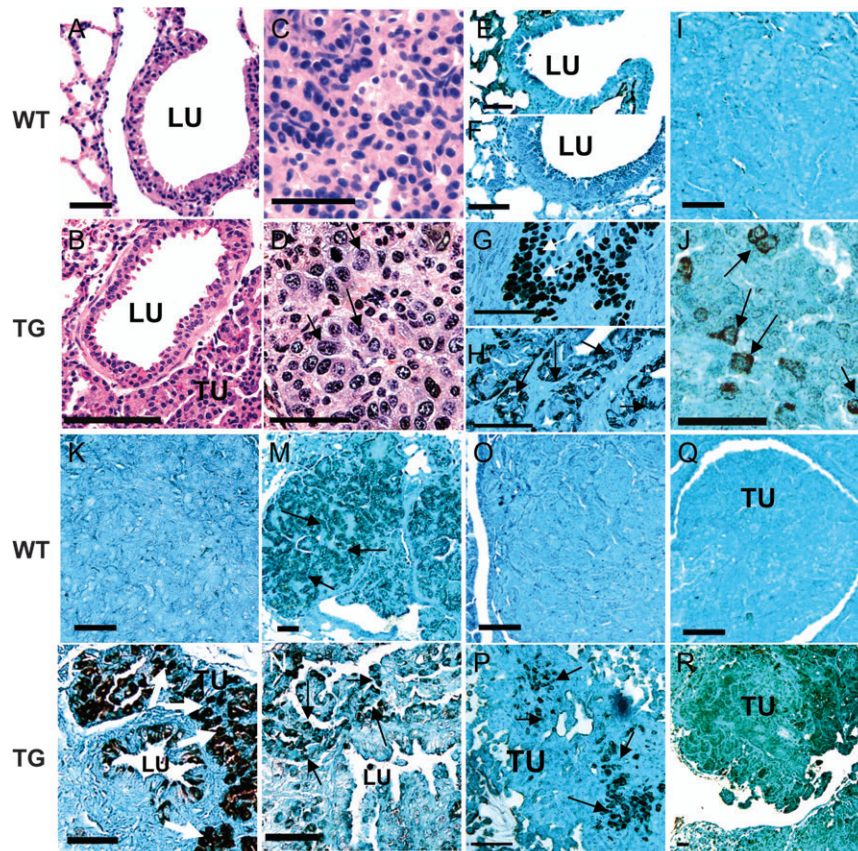
Fig. 5. Tumor histology and immunohistochemical analysis of differentiation in the lungs from CC10-hK14 TG mice. (**A**) Photomicrograph of a normal airway from a WT mice (hematoxylin and eosin stain; bar = 200 μm). (**B**) An adenoma adjacent to a bronchiolus with mild hyperplasia and atypia from a TG mouse (hematoxylin and eosin stain; bar = 400 μm). (**C**) Histology of a spontaneous adenoma in the lung of a 48-week-old WT mouse (hematoxylin and eosin stain; bar = 400 μm). (**D**) A poorly differentiated spontaneous carcinoma with marked atypia, increased nuclear:cytoplasmic ratio (arrows) and prominent nucleoli in tumor cells from a TG mouse (hematoxylin and eosin stain; bar = 400 μm). Panels (**E–R**) show immunoperoxidase stain as follows: bronchiolar epithelium of a WT mouse negative for p63 (E) and K14 (F) (bar = 200 μm). Bronchiolar epithelium from a TG mouse positive for p63 (G) and K14 (H) in basal cells (arrows; bar = 200 μm). (I) A spontaneous tumor from a WT mouse negative for K14 (bar = 200 μm). (J) Scattered K14-positive tumor cells in a TG mouse (arrows; bar = 400 μm). (K) A tumor from WT lung negative for CC10 (bar = 200 μm). (L) A tumor with intense CC10 immunoreactivity (white arrows) from a TG mouse. The epithelial cells lining an airway lumen (LU) are also positive (bar = 200 μm). (M) Characteristic immunoreactivity for proSP-C (arrows) in a pulmonary adenoma from a WT mouse (bar = 200 μm). (N) The same tumor as in (L) from a TG mouse is also positive for proSP-C (arrows), whereas the cells lining an airway lumen (LU) are negative (stain; bar = 200 μm). (O) A tumor from a WT mouse negative for p63 (bar = 200 μm). (P) Focal immunoreactivity for p63 (arrows) in a tumor from a TG mouse (bar = 200 μm). (Q) A tumor (TU) from a WT mouse negative for loricrin (bar = 200 μm). (R) A tumor from a TG mouse with strong immunoreactivity for loricrin (bar = 200 μm).

uncertain. Like Clara cells, they mature late during embryogenesis. Studies on injury and vitamin A deficiency have suggested that secretory or Clara cells are pivotal for the regeneration of basal cells of tracheobronchial epithelium (40–42). We speculate that the expression of *hK14* under the mCC10 promoter might have targeted a common embryonal precursor cell, which then gave rise both to presecretory (pre-Clara cells) and prebasal cells by the end of the pseudoglandular period in the embryonic lung (43,44). In addition to Clara cells, *hK14* transgene may have triggered a proliferative response also in basal cells.

Despite the proliferative phenotype in the airways of TG mice, the expression of *hK14* had limited impact on tumorigenesis, which requires the accumulation of multiple genetic and epigenetic changes probably to occur with the increasing age (28). While the number of animals was too small to reach a definite conclusion, 67% of the CC10-hK14 mice had lung tumors at 21 months of age in comparison with 46% of WT mice. The tumor incidence cited in the literature for old (24 months) FVB mice is even lower (38%). Taken together, this data may warrant further examination of the role of *hK14* in tumorigenesis (28). Histologically, the tumors ranged from adenomas to poorly differentiated carcinomas that might have been expected as the expression of K14 in the TG lungs was not associated with overt keratinization. On the other hand, the impact of *hK14* manifested in the simultaneous expression of diverse markers such as the alveolar type II cell marker proSP-C, airway secretory (Clara) cell marker CC10, epidermal K14 and CCE proteins, suggesting multidirectional differentiation.

There are few previously reported mouse models of lung SQCAs. In one study, eight different inbred mouse strains were exposed to *N*-nitroso-tris-chloroethylurea by skin painting and five developed both SQCAs and characteristic precursor lesions that were positive for the epidermal keratins K5/6 (45). Wang *et al.* also conducted linkage analyses and found specific loci that were associated with susceptibility to SQCA. There were marked differences between the strains in the ability to develop SQCAs. Interestingly, as with the tendency to form pulmonary adenomas, FVB mice were in the middle of the spectrum and developed low multiplicity of tumors that correlated with less frequent precursor lesions as well. It would be interesting to see if the constitutive expression of *hK14* in a more susceptible strain would result in more pronounced squamous metaplasias than the ones observed in the present study utilizing FVB mice. In another study, a somatically activatable mutant Kras-driven model of mouse lung cancer was used to evaluate the role of LKB1 and other tumor suppressors in lung carcinogenesis (46). Inactivation of LKB1 resulted in aggressive and highly metastatic tumors, 60% of

which revealed squamous or mixed squamous histology although there was no evidence of squamous precursor lesions in the airways. In contrast, our results suggest that the constitutive expression of *hK14* is only weakly oncogenic.

In conclusion, constitutive expression of *hK14* in the lung resulted in increased expression of K14 and squamous differentiation program. The mice demonstrated multifocal alterations in the airways ranging from hyperplasias to squamous metaplasia that were reminiscent of precursor lesions of human pulmonary SQCA.

Supplementary material

Supplementary Figures 1s–4s and Table Is can be found at http://carcin.oxfordjournals.org/

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