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From Cell Biology to Tissue Engineering

Adrenomedullin regulates club cell recovery following lung epithelial injury

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Summary. The equilibrium between lung epithelium damage and recovery in the context of chronic injury is at the basis of numerous lung diseases, including lung cancer and COPD. Understanding the contribution of growth factors and other molecular intermediates to this crosstalk may help in devising new therapeutic approaches. To better understand the contribution of adrenomedullin (AM) to lung homeostasis, we built club cell-specific conditional knockout (KO) mice for AM and subjected them to naphthalene injury. Untreated KO mice had lower levels of club cell 10 KDa protein (CC10) immunoreactivity than their wild type (WT) littermates in both terminal and regular bronchioles. Naphthalene injury resulted in a rapid necrosis of club cells followed by a progressive recovery of the epithelium. Club cells proliferated at higher rates in the KO mice and at 21 days post-injury the club cell coverage of the main bronchioles was higher and more homogeneous than in the WT animals. In conclusion, the paracrine/autocrine influence of AM in club cells subtly modulates their proliferation and spreading kinetics during lung epithelium recovery.

Key words: Adrenomedullin, Club cells, Naphthalene, Lung injury, BrdU

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Introduction

Cigarette smoke is connected to an array of chronic lung diseases and is a major source of morbidity and mortality. In the United States over 400,000 deaths per year are attributed to smoking (Bhalla et al., 2009). Active smoking is responsible for approximately 90% of lung cancers (Alberg and Samet, 2003). In addition, cigarette smoke is associated with other chronic pulmonary diseases such as edema, chronic bronchitis, and emphysema, the last two also termed chronic obstructive pulmonary disease (COPD). It has been known for a long time that lung cancer incidence increases in patients with COPD (Tockman et al., 1987), apparently through an increase in inflammation, oxidative stress, matrix destruction, and release of growth factors (Goldkorn and Filosto, 2010). This results in a chronic loop of epithelial injury followed by subsequent regeneration of the lung epithelium. Understanding the molecular mechanisms that control lung epithelium repair is critical to design interventions for patients with high risk of developing lung cancer.

Out of all the cell types lining the airway epithelium, club cells are in charge of xenotoxic detoxification through the action of cytochrome P450 and related enzymes (Buckpitt et al., 1995). As a consequence, club cells are very sensitive to chemically-induced toxicity. Many laboratories have used naphthalene treatment in experimental animals as a model to study the molecular and cellular mechanisms of airway epithelium regeneration. Naphthalene is an abundant polycyclic aromatic hydrocarbon in tobacco smoke (Sutherland et al., 2012). After treatment with naphthalene, club cells

become necrotic and are replaced by FoxJ1-positive ciliated cells, which undergo dynamic changes involving cell migration, proliferation, and differentiation (Rawlins and Hogan, 2006; Gorissen et al., 2013). However, the role of specific growth factors in regulating the process of airway epithelial regeneration requires further investigation.

Adrenomedullin (AM) is a 52 amino acid regulatory peptide, which together with proadrenomedullin N-terminal 20 peptide (PAMP), is coded by the *adm* gene. These peptides have a widespread distribution and many physiological functions where they usually act as autocrine or paracrine growth factors (Miller et al., 1996; Lopez and Martinez, 2002). The highest expression of this gene occurs in the lung (Kitamura et al. 1993) and this organ is also the major clearing site for circulating AM (Sabates et al., 1996; Lewis et al., 1997). By immunohistochemical and *in situ* hybridization studies of the lung, AM has been shown to be expressed by the airway epithelium, macrophages, chondrocytes, and smooth muscle covering both the airways and the blood vessels (Martinez et al., 1995; Montuenga et al., 1997). Many specific AM binding sites are located in the lung, to the point that labeled AM may be used as an imaging tool (Letourneau et al., 2013). In addition, circulating AM levels are elevated in a number of pulmonary diseases, including COPD (Cheung and Leung, 1997), pulmonary hypertension (Yoshibayashi et al., 1997), septic shock (Nishio et al., 1997), and lung cancer (Portal-Nunez et al., 2012), among others.

The relevance of the AM system and its receptors has been emphasized by several knockout models which result in 100% embryonic lethality (Caron and Smithies, 2001; Shindo et al., 2001; Dackor et al., 2006), but these animals do not permit physiological studies of the lung or other adult organs. To circumvent this problem, we generated a "floxed" conditional mouse model which allows cell type-specific targeting of the AM deletion (Fernandez et al., 2008; Koyama et al., 2013). Here we present the results of deleting AM expression from club cells in the airways and their modified response to lung epithelium injury.

Materials and methods

Conditional knockout for AM in club cells

Mice were obtained by crossing animals whose *adm* gene was flanked by LoxP sequences (Fernandez et al., 2008) with mice expressing Cre recombinase under the CCSP promoter (gL4-CCSP-Cre, European Mouse Mutant Archive, München, Germany). In these animals Cre expression is restricted to club cells (Bertin et al., 2005). Both mouse strains had been previously backcrossed into a homogeneous C57BL/6 genetic background. All experiments were performed with female littermates whose genotypes where either f/f Cre- (named here as wild type, WT), or f/f Cre+ (conditional knockouts or KO). All procedures involving animals were carried out in accordance with the European Communities Council Directive (2010/63/UE) and Spanish legislation (RD53/2013) on animal experiments and with approval from the ethical committee on animal welfare of our institution (Órgano Encargado del Bienestar Animal del Centro de Investigación Biomédica de La Rioja, OEBA-CIBIR).

Naphthalene and BrdU treatment

Drug administration was carried out as described (Oliver et al., 2011). Control animals received the same amount of corn oil as a vehicle control. Groups of mice (n=5) were killed at different time points (0, 2, 5, 14, and 21 days). Two hours before sacrifice, mice were injected with 0.1 mg/g body weight BrdU (Roche, Basel, Switzerland).

Gene expression

Immediately after euthanasia, the left lung of each specimen was ligated, excised, and used to study gene expression. Gene expression was quantified as described (Sackett et al., 2008). Primers are summarized in Table 1.

Histopathological techniques

Animals were perfused with 4% buffered formalin through the right ventricle to clear and fix the pulmonary circulation. The remaining right lung lobes were gingerly inflated with the fixative through a tracheal canula, and then immersed in fixative for 8 h at room temperature, before dehydratation and paraffin embedding. Sections (3 μm-thick) were stained with hematoxylin and eosin or with multiple immuno-

Table 1. Primers used for diagnostic (first 4 pairs) and quantitative real time PCR.

Target	Sequence	Expected band size
AMKOD (F)	AAGGGAAGTCCTGCTCCAGT	2500/600 bp
AMKOD (R)	GCCTTAGCTCAGGTCCAGTG	
AMKO (F)	TGATTATCGCCTGTGGATGA	500/600 bp
AMKO (R)	GCCTTAGCTCAGGTCCAGTG	
WT (F)	CTAGGCCACAGAATTGAAAGATCT	324 bp
WT (R)	GTAGGTGGAAATTCTAGCATCATCC	
Cre (F)	CGACCAAGTGACAGCAATGCTGTTTCA	625 bp
Cre (R)	CACCAGCTTGCATGATCTCCGGTATT	
Mouse CCSP (F)	ATGAAGATCGCCATCACAATCAC	135 bp
Mouse CCSP (R)	GGATGCCACATAACCAGACTCT	
Mouse FoxJ1 (F)	CCCTGACGACGTGGACTATG	114 bp
Mouse FoxJ1 (R)	GCCGACAGAGTGATCTTGGT	
Mouse GAPDH (F)	CATGTTCCAGTATGACTCCACTC	136 bp
Mouse GAPDH (R)	GGCCTCACCCCATTTGATGT	
18S (F)	ATGCTCTTAGCTGAGTGTCCCG	101 bp
18S (R)	ATTCCTAGCTGCGGTATCCAGG	

Annealing temperature for all primers was 60°C

fluorescence using primary and secondary antibodies as shown in Table 2. To ensure simultaneous detection of all markers, antigen retrieval had to be optimized and was accomplished by exposure to 0.1 M citrate buffer, pH 6.0, in a Coplin jar immersed in boiling water for 40 min. A confocal microscope (Leica TCS SP5, Leica, Badalona, Spain) was used to visualize the slides. Quantification of immunostaining was achieved by image analysis (ImageJ, NIH) and expressed as mean positive pixels per area unit or the number of BrdU-labeled club cells per area unit.

Statistical analysis

Differences between WT and KO mice along experimental times were calculated by two way ANOVA followed by post-hoc Bonferroni tests, and were considered statistically significant when $p<0.05$.

Results

Club cell-specific deletion of the *adm* gene was achieved by targeted expression of Cre recombinase under the CCSP promoter in the context of homozygote mice for "floxed" *adm*. To ensure proper deletion of the *adm* gene in the airways, PCR analysis of genomic DNA was performed with primers external to the *LoxP* sequences. The lower band, corresponding to the excised allele, was only seen in the lung of KO animals whereas all other organs were unaffected, independently of the genotype (Fig. 1). Since *adm* is only deleted from the club cells, and not from the other cells of the lung, the higher band is also present in the lung of KO animals.

KO mice did not present any overt phenotypic disadvantage as assessed by weight loss, longevity, reproductive fitness, respiratory dysfunction, or discomfort behavior. Conventional histological analysis of the lung and trachea of the KO animals did not find any morphological difference with their WT counterparts at any age. However, quantification of the

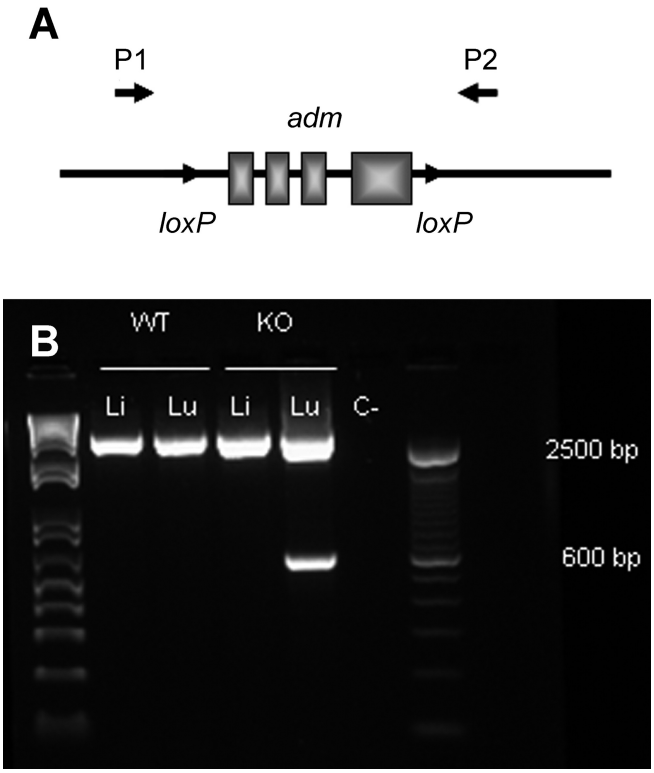

Fig. 1. PCR strategy **(A)** and analysis **(B)** to check for proper targeting of the conditional construct. Primers P1 and P2 anneal outside the LoxP sequences (triangles) surrounding the whole *adm* gene (which has 4 exons). Amplification of the unmodified allele renders a ~2,500 bp product whereas the recombined allele produces a ~600 bp amplicon. Genomic DNA was purified from liver (Li) and lung (Lu) of WT and KO mice and subjected to PCR with primers P1 and P2. The lower band (recombined allele) was found only in the lung of KO animals. A negative control **(C-)** containing no DNA was added to ensure reaction specificity. Since the adm gene is only deleted in the club cells, the lungs of KO animals still have a higher band corresponding to the intact *adm* gene in all other cell types.

Table 2. Antibodies used for multiple immunofluorescence.

Primary antibodies				
Target	Species	Dilution	Source	Reference
CC10	Goat polyclonal	1:250	Santa Cruz Biotech.	sc-9772
FoxJ1	Mouse monoclonal	1:100	Santa Cruz Biotech.	sc-53139
BrdU	Rat monoclonal	1:200	Abcam	ab6326
Secondary antibodies				
Specificity	Fluorochrome	Dilution	Source	Reference
Donkey anti goat	Alexa Fluor® 633	1:600	Life Technologies	A-21082
Donkey anti mouse	Alexa Fluor® 488	1:800	Life Technologies	A21202
Donkey anti rat	Cy3	1:400	Jackson Immunores.	712-165-153

To achieve 4-color immunofluorescence (3 antibodies + DAPI), antigen retrieval had to be optimized and was accomplished by exposure to 0.1 M citrate buffer, pH 6.0, in a Coplin jar immersed in boiling water for 40 min.

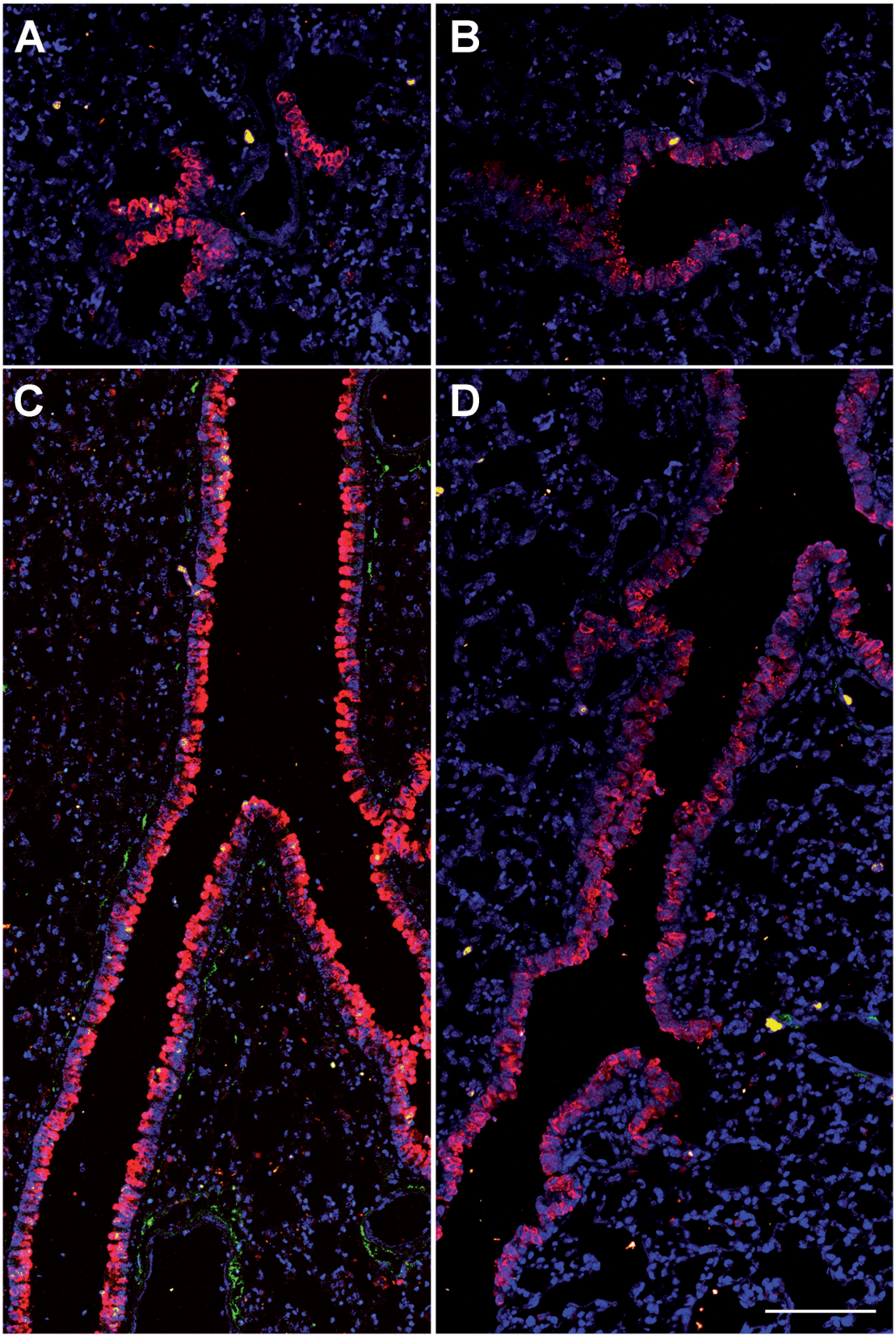

Fig. 2. Representative confocal microscopy images of terminal **(A, B)** and main **(C, D)** bronchioles in the lung of WT **(A, C)** and KO **(B, D)** mice treated with vehicle (corn oil), without naphthalene. Red labels CC10 protein, green labels FoxJ1-positive ciliated cells, and yellow labels BrdU-positive cells. Nuclei are counterstained in blue (DAPI). Scale bar: 100 μm.

staining for the different cell lineages of the lung found significant differences in the immunoreactivity in KO animals for club cell 10 KDa protein (CC10) in both terminal and main bronchioles (Figs. 2, 3A,C).

Distribution of CC10-positive cells during recovery

Intraperitoneal injection of naphthalene resulted in mild breathing difficulty for the first 3 days, concomitant with a significant body weight loss in both genotypes. After the 4th day, both genotypes quickly recovered. Consistent with previous reports (Oliver et al., 2011; Gorissen et al., 2013), at day 2 after naphthalene injection we observed a massive necrosis and shedding of club cells in the bronchioles of both genotypes (arrows in Fig. 4A) and a time-dependent recovery achieved by the proliferation and migration of these cells from their stem cell niches (Figs. 3A, 4C-H). Interestingly, the speed of recovery was not the same for both genotypes. At days 2 and 14 after naphthalene injury, there was a significantly higher CC10 immunoreactivity in WT animals in the terminal

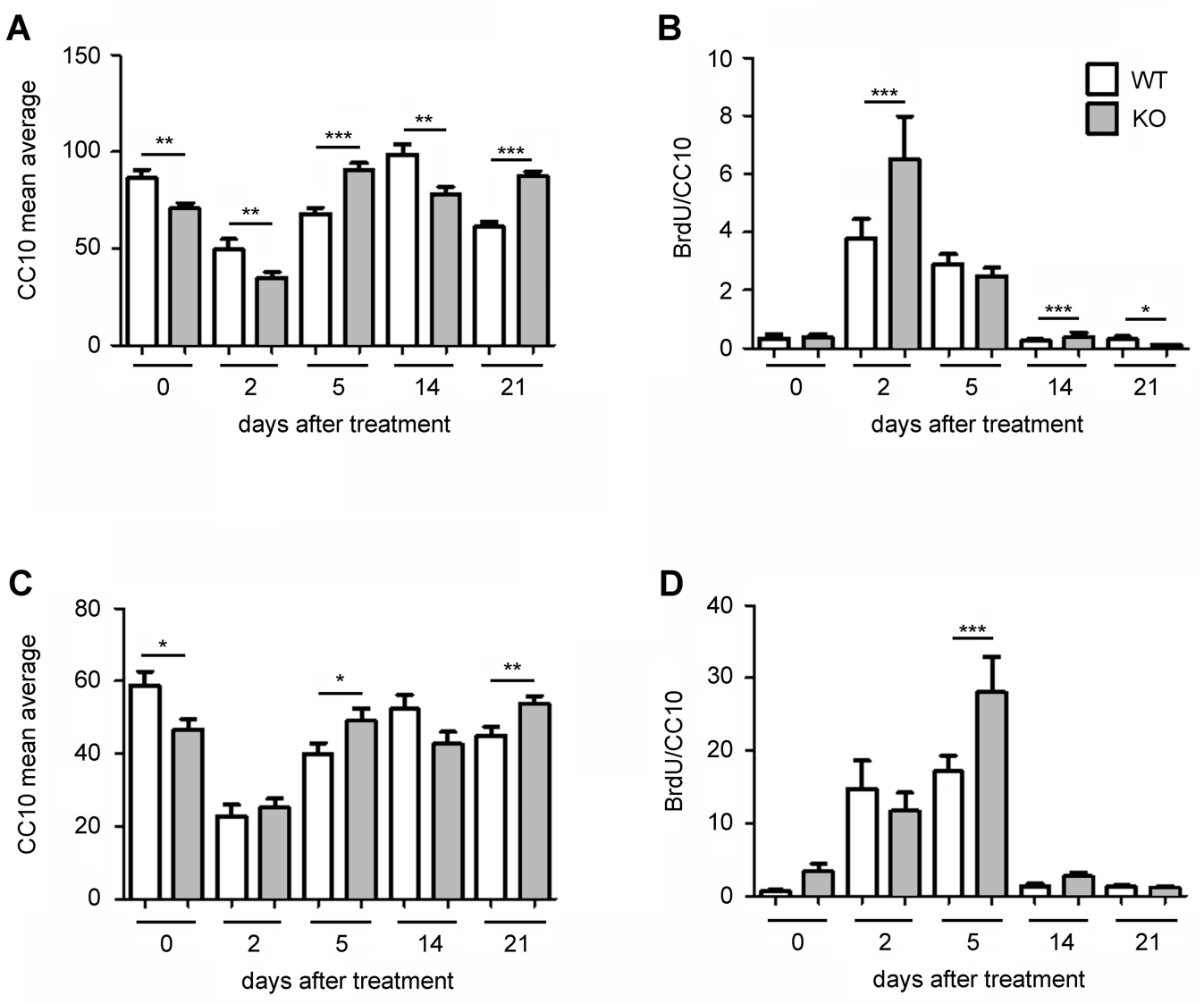

Fig. 3. Quantification of CC10 staining intensity **(A, C)** and the number of BrdU-positive CC10 cells **(B, D)** in terminal **(A, B)** and main **(C, D)** bronchioles in wild type (WT, open bars) and knockout (KO, closed bars) mice at different times after naphthalene injury. Each bar represents the mean and standard deviation for all bronchioles in that particular time frame (n> 30 in all cases). Two way ANOVA analysis showed a high significance for time ($p<0.001$) in all graphs and genotype differences ($p<0.01$) in terminal bronchioles **(A, B)**. Asterisks represent statistically significant differences between genotypes in the post-hoc analysis. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

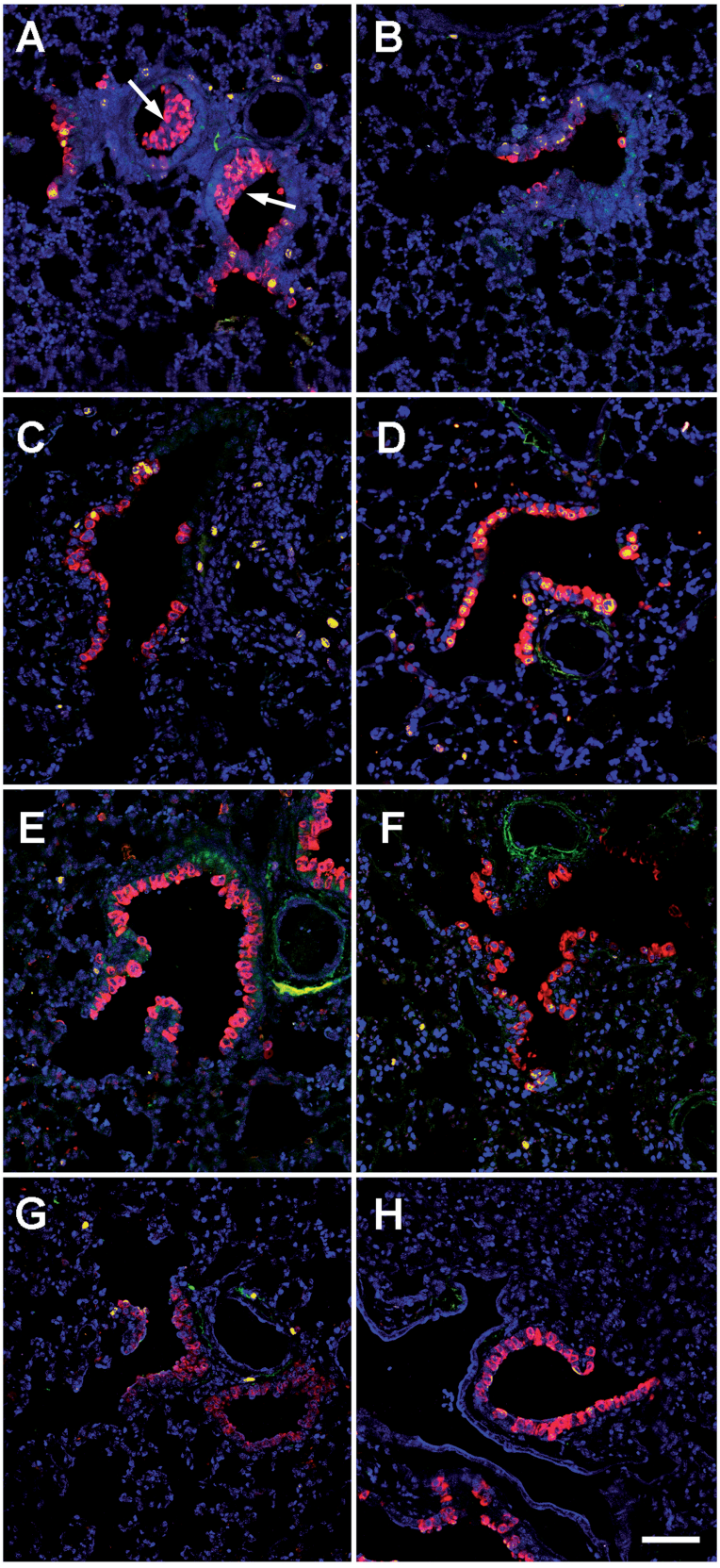

Fig. 4. Representative confocal images of terminal bronchioles in wild type **(A, C, E, G)** and knockout **(B, D, F, H)** mouse lungs taken at days 2 **(A, B)**, 5 **(C, D)**, 14 **(E, F)**, and 21 **(G, H)** following naphthalene injection. At day 2 remnants of the shedding epithelium can be seen in the bronchiolar lumen (arrows in **A**). Colors label CC10 (red), FoxJ1 (green), BrdU (yellow), and nuclear DNA (blue). Scale bar: 50 μm.

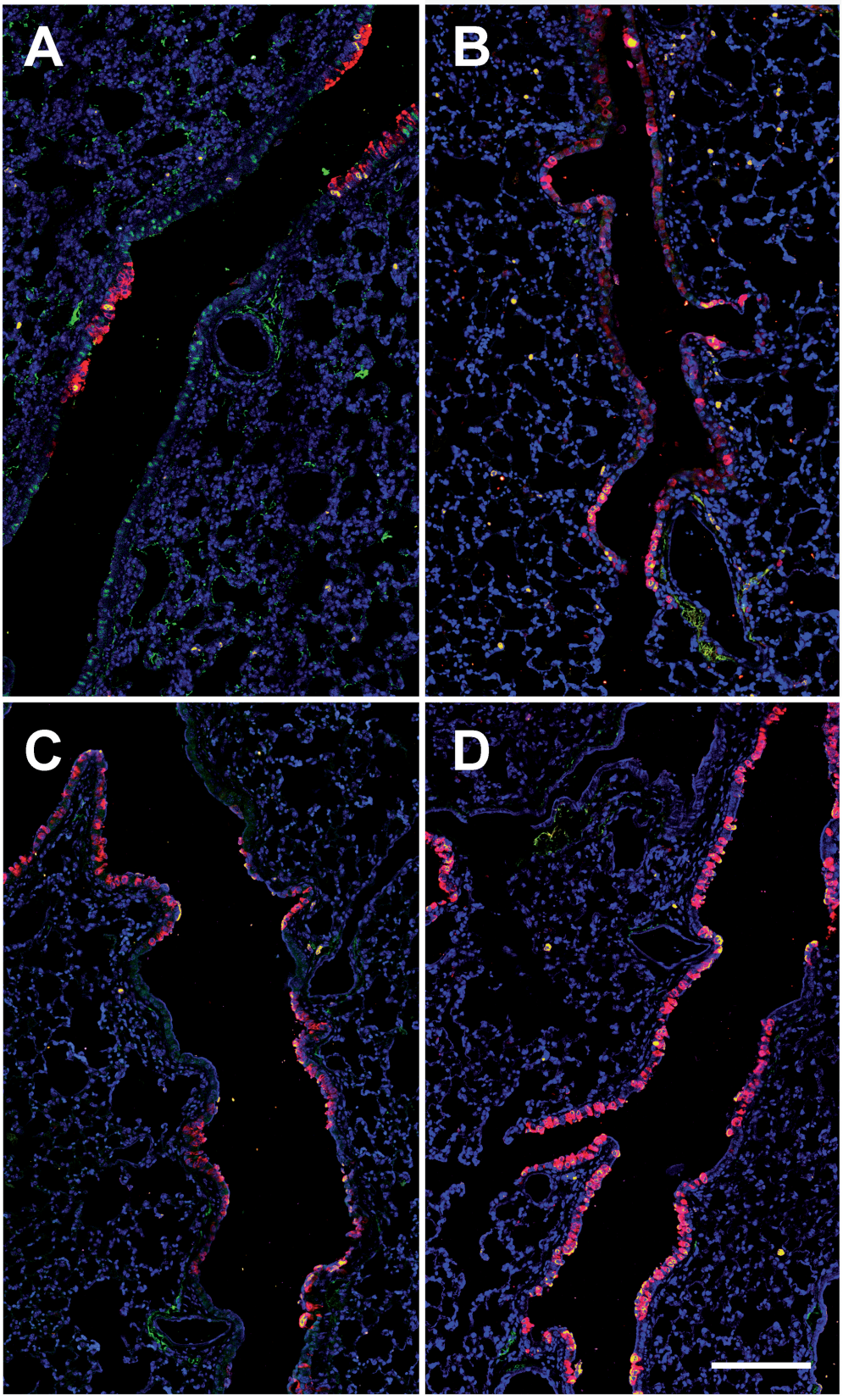

Fig. 5. Representative confocal images of main bronchioles in wild type **(A, C)** and knockout **(B, D)** mice taken at days 5 **(A, B)**, and 21 **(C, D)** following naphthalene injection. Colors label CC10 (red), FoxJ1 (green), BrdU (yellow), and nuclear DNA (blue). Scale bar: 100 μm.

bronchioles when compared to KO mice (Figs. 3A, 4A,B,E,F). This difference swapped on days 5 and 21, with the KO animals having higher CC10 immunoreactivity than their WT counterparts (Figs. 3A, 4C,D,G,H). In the main bronchioles a similar pattern was observed but significant differences were only achieved on days 5 and 21 (Figs. 3C, 5). Interestingly, the main bronchioles of the KO animals were more homogeneously covered by CC10-positive club cells than their WT counterparts, which presented broad areas of the epithelium lined by FoxJ1-positive ciliated cells (Fig. 5).

Proliferation dynamics

Analysis of proliferating club cells was performed by evaluating the number of CC10-positive cells that incorporated BrdU during the 2 h previous to sacrifice. In terminal bronchioles there was a clear increase in the number of proliferating club cells at days 2 and 5 after injury. Significant differences between genotypes were observed at days 2, 14, and 21. KO mice had more proliferating club cells at days 2 and 14 whereas WT mice had more BrdU-positive club cells at day 21 (Fig. 3B). In the main bronchioles, proliferation was higher at day 5 than at day 2, and the only significant differences between genotypes happened at day 5, with a clear advantage for the KO animals (Fig. 3D).

Molecular analysis

Quantitative real time-PCR (qRT-PCR) with mRNA extracted from the same lungs used for immunohistochemistry was performed (Fig. 6). CC10 expression (Fig. 6A) dropped significantly 2 days after naphthalene injection, coinciding with the massive death of club cells observed under the microscope (Fig. 4A). CC10 expression recovered slowly after this. Expression of FoxJ1 (Fig. 6B) followed a reverse pattern, increasing at day 2 and going back to normal after 21 days. No differences between genotypes were found at any time point.

Discussion

In this study we found that eliminating AM expression from the club cells of the mouse lung results in subtle changes in the number of these cells and in their capability to regenerate the airway epithelium following xenotoxic damage. We need to point out that the "floxed" construction targets the whole gene (Fernandez et al., 2008) so expression of both AM and PAMP is abrogated and either peptide or both of them may be responsible for the phenotype. The knockout model we are using deletes the *adm* gene from just the club cells, leaving all other sources of AM or PAMP undisturbed; therefore the physiological effects we see must be due to specific autocrine/paracrine signalling of club cell-produced AM or PAMP. In addition, recent reports point to potential intracrine functions for AM and PAMP acting on the cytoskeleton (Sackett et al., 2008; Larrayoz and Martinez, 2012), which may also be involved in the observed phenotype.

First of all, the number of proliferating club cells was higher in KO than in WT mice at days 2 and 14 in terminal bronchioles and at day 5 in main bronchioles.

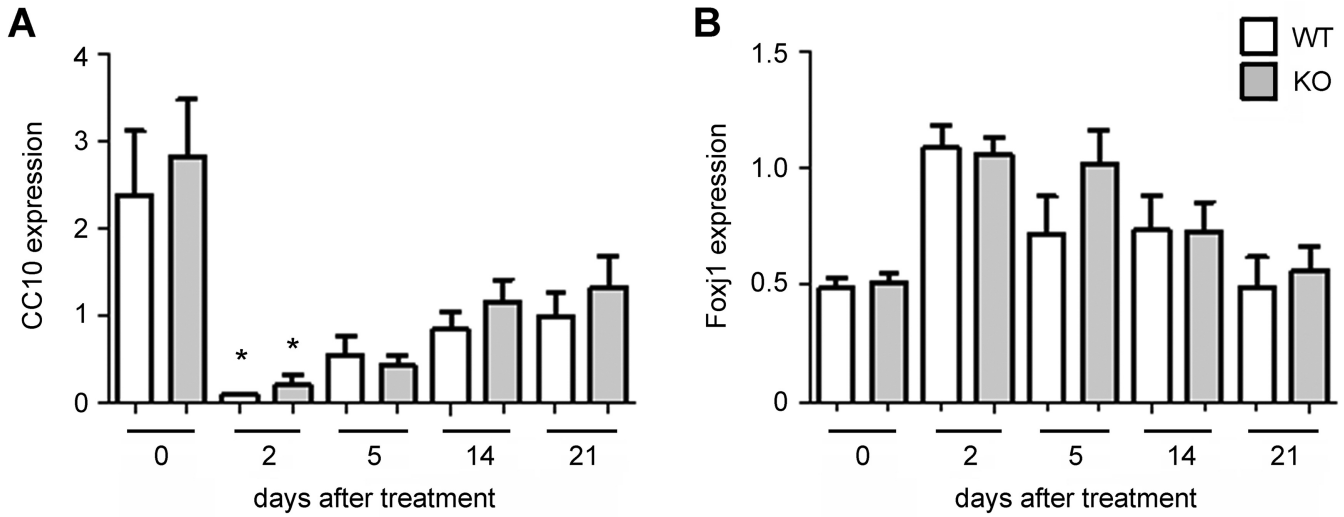

Fig. 6. Quantitative expression (qRT-PCR) of CC10 **(A)** and FoxJ1 **(B)** mRNA in the lung of wild type (WT, open bars) and knockout (KO, closed bars) mice through the recovery period after naphthalene treatment. Each bar represents the mean and standard deviation for all mice at that time point. All values were corrected by the house keeping gene 18S. Two way ANOVA analysis showed a high significance for time ($p<0.001$), but not for genotype, in both graphs. Asterisks represent statistically significant differences with day 0 in the post-hoc analysis. *: $p<0.05$.

This phenomenon resulted, by day 21 post injury, in a significantly higher number of club cells in the recovered KOs than in the WTs. In addition, the distribution pattern also changed and the club cells in KO animals were more homogenously distributed through the bronchioles, whereas in the WT mice there were broad areas not covered by club cells. These areas were covered by FoxJ1-positive ciliated cells.

In our study, qRT-PCR data for CC10 follows in general the expected pattern for this mRNA's expression and shows a significant difference at day 2 in favour of the KOs, in clear coincidence with the peak of club cell proliferation shown in the terminal bronchioles. Nevertheless this technique lacks the finesse of the immunofluorescent technique. With immunofluorescence we can distinguish between terminal and main bronchioles where the kinetic of club cell proliferation is different. In qRT-PCR we work with whole lung extracts thus losing the anatomical variants.

It has been shown that smoking and COPD reduce the number of club cells in the airways, adversely affecting small airway function (Lumsden et al., 1984; Shijubo et al., 1997; Liao et al., 2010). In our experiment, WT animals that had received naphthalene had less club cells after 21 days than the original number in untreated animals. On the other hand, KO mice had a higher number of club cells that was very close to the original number in untreated WT animals, indicating that lack of AM may prevent the loss of club cells associated with each cycle of xenotoxic injury. This was also supported by a higher number of proliferating club cells in the KOs, as assessed by BrdU staining. Further experiments are needed to check whether a repetitive exposure to naphthalene, as would be the case with a smoker, affects the protective effect of the lack of AM.

Current knowledge indicates that regeneration of club cells following xenotoxic damage emanates from a naphthalene-resistant population of club cell-like cells located in the bronchoalveolar duct junction, a well known niche of stem-like cells in the lung (Hogan et al., 2014; Kotton and Morrisey, 2014). From here, the proliferating club cells should spread through the bronchioles until they cover the whole epithelium. Our data seem to support this theory. We see an early wave of BrdU-positive club cells by day 2 in terminal bronchioles whereas the zenith is reached in main bronchioles at day 5.

It has been shown that AM is a mediator of tobacco-induced lung tumor progression (Portal-Nunez et al., 2012). At least in theory, KO animals should be partially protected from cigarette smoke. The better response to injury shown by these animals may be the basis for this behaviour.

AM expression is strongly regulated by hypoxia through a HIF-1-dependent mechanism (Garayoa et al., 2000). Hypoxia preconditioning is a proven method to protect lungs from injury (Zhang et al., 2009; Lin et al., 2011) and AM may be one of the survival mediators induced by hypoxia in this context. KO animals should have a protracted response to hypoxia preconditioning due to their lack of club cell-produced AM. In addition, both AM and PAMP have antimicrobial activity (Martinez et al., 2006) and we can predict that KO animals should have less protection against pulmonary infections than WT animals.

Previous studies have shown the beneficial effects of treating lung disorders with an external source of AM. These include, among others, septic shock (Temmesfeld-Wollbruck et al., 2007), pulmonary hypertension (Dani et al., 2007), emphysema (Murakami et al., 2005), bronchopulmonary dysplasia (Vadivel et al., 2010), hyperoxia-induced lung injury (Tao et al., 2012), ventilator-induced lung injury (Muller et al., 2010), and pulmonary fibrosis (Kach et al., 2013). Nevertheless, in the context of lung cancer promotion and progression, a reduction of AM levels should be recommended (Martinez et al., 1995, 1997; Portal-Nunez et al., 2012). A series of small molecules that could either enhance or reduce the physiological actions of AM have been described (Martinez et al., 2004; Garcia et al., 2005) and they may provide a new avenue to treat these pulmonary diseases.

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