ORIGINAL RESEARCH ARTICLE

Lack of Dystrophin Affects Bronchial Epithelium in *mdx* Mice

GIUSEPPE MORICI,[1,2]* FRANCESCA RAPPA,[1,3,4] FRANCESCO CAPPELLO,[1,4]
ELISABETTA PACE,[2] ANDREA PACE,[4,5] GIUSEPPA MUDÒ,[1] GRAZIA CRESCIMANNO,[2]
NATALE BELLUARDO,[1] AND MARIA R. BONSIGNORE[2,6]

[1]Dipartimento di Biomedicina e Neuroscienze Cliniche (BioNeC), University of Palermo, Palermo, Sicilia, Italy

[2]Istituto di Biomedicina e Immunologia Molecolare (IBIM), Consiglio Nazionale delle Ricerche (CNR), Palermo, Sicilia, Italy

[3]Dipartimento di Scienze Giuridiche della Società e dello Sport, University of Palermo, Palermo, Sicilia, Italy

[4]Istituto Euro-Mediterraneo di Scienza e Tecnologia, Palermo, Sicilia, Italy

[5]Dipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche (STEBICEF), University of Palermo, Palermo, Sicilia, Italy

[6]Dipartimento Biomedico di Medicina Interna e Specialistica (DiBiMIS), University of Palermo, Palermo, Sicilia, Italy

Mild exercise training may positively affect the course of Duchenne Muscular Dystrophy (DMD). Training causes mild bronchial epithelial injury in both humans and mice, but no study assessed the effects of exercise in *mdx* mice, a well known model of DMD. The airway epithelium was examined in *mdx* (C57BL/10ScSn-Dmdmdx) mice, and in wild type (WT, C57BL/10ScSc) mice either under sedentary conditions (*mdx*-SD, WT-SD) or during mild exercise training (mdx-EX, WT-EX). At baseline, and after 30 and 45 days of training (5 d/wk for 6 weeks), epithelial morphology and markers of regeneration, apoptosis, and cellular stress were assessed. The number of goblet cells in bronchial epithelium was much lower in *mdx* than in WT mice under all conditions. At 30 days, epithelial regeneration (PCNA positive cells) was higher in EX than SD animals in both groups; however, at 45 days, epithelial regeneration decreased in *mdx* mice irrespective of training, and the percentage of apoptotic (TUNEL positive) cells was higher in *mdx*-EX than in WT-EX mice. Epithelial expression of HSP60 (marker of stress) progressively decreased, and inversely correlated with epithelial apoptosis ($r = -0.66$, $P = 0.01$) only in *mdx* mice. Lack of dystrophin in *mdx* mice appears associated with defective epithelial differentiation, and transient epithelial regeneration during mild exercise training. Hence, lack of dystrophin might impair repair in bronchial epithelium, with potential clinical consequences in DMD patients.
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Duchenne muscular dystrophy (DMD) is a lethal X-linked muscle disease affecting 1:3500 newborn boys (Van Putten et al., 2012). DMD is characterized by a defect in the sub-sarcolemmal protein dystrophin, which leads to membrane fragility, muscle necrosis, motor weakness, myofibre death, and replacement of skeletal muscle by fibrous and fatty connective tissue, due to failed regeneration (Matthews et al., 1995). In patients with DMD, chronic respiratory insufficiency is inevitable in the course of disease progression, due to primary loss of respiratory muscle strength. Mechanical ventilation (MV) is the only established clinical strategy to decrease respiratory morbidity and mortality in young adults with DMD (Biggar, 2006).

In the last decade, new strategies have been explored in order to reduce the muscle wasting associated to DMD (Odom et al., 2007; Strimpakos et al., 2014; De Arcangelis et al., 2016), including the implementation of exercise training, but the effects of exercise in patients with DMD have not yet been adequately studied (Grange and Call, 2007). In this context, the *mdx* mouse model is a useful intermediate step to assess the effects of training on dystrophic muscle (Grounds et al., 2008). Lack of dystrophin in *mdx* mice leads to cycles of muscle degeneration and regeneration (Radley-Crabb et al., 2014). While excessive or otherwise inappropriate activity may induce muscle damage and increase pathology in *mdx* mice (Brussee et al., 1997; De Luca et al., 2003; Okano et al., 2005) some protective effects of low-intensity or voluntary exercise (Hayes and Williams, 1996; Call et al., 2010; Baltgalvis et al., 2012; Gordon et al., 2014) have been reported. Exercise training may also affect airway cells in *mdx* mice. We previously documented increased epithelial thickness, and increased epithelial turnover in small airways of Swiss mice undergoing low-intensity endurance training (Chimenti et al., 2007). Overall, data obtained in mice (Chimenti et al., 2007) and human endurance athletes (Chimenti et al., 2010) suggest that habitual exercise might cause low-level inflammation in the airways secondary to mild epithelial injury. Whether low-intensity endurance training affects the airways of *mdx* mice is yet unknown.

Aims of this study were to analyze: (a) the characteristics of airway epithelium in *mdx* compared to WT mice and (b) the effects of low-intensity endurance exercise training on the airways of *mdx* mice. In more detail, we assessed whether training induced epithelial remodelling, apoptosis, and changes in the stress marker HSP60 at bronchial and bronchiolar levels

Materials and Methods

We analyzed lung tissue from the animals previously analyzed for skeletal muscle changes (Frinchi et al., 2014). Briefly, 8-week-old male *mdx* mice (C57BL/10ScSn-Dmdmdx/J Jackson Laboratories, Bar Harbor, ME), and C57/BL wild type (WT) mice (C57BL/10ScSn, Harlan, Udine, Italy) were studied. Both *mdx* and WT mice were randomly assigned to sedentary (S) (MDX-S n = 17; WT-S n = 19) or trained (EX) (MDX-EX n = 14; WT-EX n = 16) groups. Training was performed using a motorized rotating treadmill (Rota-Rod; Ugo Basile, Biological Research Apparatus, Comerio Varese, Italy), in a protected environment and in the same room where the mice were housed, as previously reported (Chimenti et al., 2007). After 2 weeks of acclimatization, mice ran 5 days/week for 6 weeks at progressively increasing loads (Table 1).

Mice undergoing exercise training and sedentary controls in each group (WT and *mdx*) were sacrificed by an overdose of chloral hydrate anesthesia at time 0 and after 30 and 45 days. The Human Physiology Laboratory of the Department of Experimental Biomedicine and Clinical Neurosciences (BioNeC) of the University of Palermo has been formally authorized by the Italian Ministry of Health to conduct animal studies and gave authorization for this protocol. Procedures involving animals and their care were conducted in conformity with the Italian institutional guidelines (D.L. 116, G.U., suppl. 40, February 18, 1992).

Tissue preparation, histochemistry, and morphological analysis

Samples of lungs were processed for light microscopy examination. Each sample was routinely fixed in formalin and embedded in paraffin. Five μm-thick sections of lung parenchyma were obtained from paraffin blocks, deparaffinized with xylene for 10 min at 60°C and hydrated with a decreasing ethanol gradient. They were stained with haematoxylin-eosin (Hematoxylin, DAKO, Glostrup, Denmark, Cat. No. CS 700; Eosin, DAKO, Denmark, Cat. No. CS701). The sections were dehydrated and mounted with a coverslip for histological examination using an automated Leica DM5000 B microscope connected to a high-resolution camera, Leica DC300 F (Leica, Milan, Italy). All the histological evaluations were carried out by two different readers (FC and FR).

Peripheral airways, that is, conducting airways that are less than 2 mm in their internal diameter, were analyzed, and we will refer to them as either bronchi (with a diameter greater than 1 mm) or bronchioles (with a diameter lower than 1 mm).

In particular, we evaluated epithelial morphology and inflammatory infiltrate, as previously described (Chimenti et al., 2007). We also performed Periodic acid Schiff (PAS) staining (Sigma–Aldrich, Milan, Italy) to highlight goblet cells in the mucosa. The sections were examined using an automated Leica DM5000 B

microscope (Leica, Milan, Italy) connected to a high-resolution camera, Leica DC300 F. Morphological examination was performed on 10 random fields at 400× magnification. The goblet cells were quantified in the bronchi of all samples, and expressed as the percentage of goblet/total cells.

Immunohistochemistry

From all paraffin blocks, 5-μm thick sections were obtained with a cutting microtome. These sections were dewaxed in xylene for 10 min and rehydrated by sequential immersion in a descending scale of alcohols and transition in water for 5 min. Subsequently, the sections were immersed for 8 min in Sodium Citrate Buffer (pH 6) at 95°C for antigen retrieval and then immersed for 8 min in acetone at −20°C. All subsequent reactions were conducted at room temperature. The reactions were performed by a streptavidin–biotin complex method using Histostain®-Plus 3rd Gen IHC Detection Kit (Invitrogen Corporation, Waltham, MA, 85–8943). The used primary antibodies were anti-HSP60 (HSP 60 (K-19)-R Antibody rabbit polyclonal sc-1722-R diluted 1:100) and anti-PCNA (PCNA (F-2) Antibody mouse monoclonal sc-25280 diluted 1:100). Appropriate positive and negative (isotype) controls, were run concurrently. Nuclear counterstaining was carried out using hematoxylin (Hematoxylin aqueosus formula, S2020, DAKO). Finally, the slides were prepared for observation with coverslips with an aqueous mounting solution. All the observations were made at a magnification of 400× and the means of triplicate counts were used for statistical analyses. The observation of the sections was performed with an optical microscope (Leica DM 5000 B) connected to a digital camera (Leica DC 300F). Results were expressed as percentage of HSP60- or PCNA-positive cells/total cells.

Detection of apoptosis

Detection of apoptosis was performed using "In Situ Cell Death Detection Kit, AP" (Roche, Basel, Switzerland, 11684809910) on samples of paraffin embedded lung tissue. Five-micrometer thick sections were deparafinized in a 60 °C water bath, in two xylene baths and rehydrated in decreasing alcohol baths (absolute, 95, 80, and 50%). Positive and negative controls were run concurrently. Then the slides were incubated with proteinase K at 37°C for 30 min. Later, the labeling protocol of TUNEL reaction mixture addition for 60 min at 37°C was performed. The slides were incubated with Converter AP for 30 min at 37°C, and, with NBT/BCIP Substrate Solution (BCIP/NBT Color Development Substrate, Promega, S3771) for 10 min at room temperature in the dark. After rinsing with PBS, slides were mounted with glass coverslips and observed using light automated Leica DM5000 B microscope connected to a high-resolution camera, Leica DC300 F (Leica, Milan, Italy). The results were evaluated separately by two different observers (FC, FR) who calculated the percentage of positive nuclei of epithelial cells in the mucosa on 10 random fields at 400× magnification. The mean of the two independent observations was considered for evaluation. Results were expressed as percentage of TUNEL-positive epithelial cells/total epithelial cells.

Statistics

Data are expressed as mean ± SD. Unpaired *t*-test was used to compare *mdx* and control mice at different time points according to sedentary or trained status. Time trends in the examined variables were assessed by simple linear regression in each group according to the sedentary or trained status. The relationship between apoptosis and HSP60 expression was assessed by linear regression separately in WT and *mdx* groups. Unpaired *t*-test was used to compare *mdx* and control mice at similar time points according to sedentary or trained status. Significance was at

TABLE 1. Acclimatization (A) and training (T) protocol in WT and *mdx* mice

Week	Session duration (min)	Rotations min^{-1}	Distance (m)
1 (A)	15	16	48
2 (A)	30	16	96
3 (T)	30	20	120
4 (T)	45	20	180
5 (T)	60	20	240
6 (T)	60	24	288

$P < 0.05$ for all tests. The software used for all analyses was Statview 5.0 for Windows.

Results

Morphology of lung parenchyma and small airways

Figure 1 and supplemental Figures S1–S4 summarize histochemical and immunohistochemical data in sedentary and trained WT and *mdx* groups, respectively, at the different time points tested. In bronchi, epithelial thickness progressively decreased in WT mice, irrespective of training (linear regression for time trends: WT-SD: $R^2 = 0.43$, $r = -0.65$, $P < 0.0005$; WT-EX: $R^2 = 0.68$, $r = -0.82$, $P < 0.0001$). No change over time was evident in *mdx* mice (*mdx*-SD: $R^2 = 0.02$, NS; *mdx*-EX: $R^2 = 0.09$, NS), and epithelial thickness was significantly higher in *mdx* compared to WT mice at 45 days in both the sedentary and exercised groups (Fig. 1, part A; Fig. S1). A very modest number of inflammatory cells was found, with a non significant increase in WT-EX mice at 30 days (data not shown).

Few PAS-positive (goblet) cells were found in *mdx* compared to WT mice at all time points, irrespective of sedentary or trained conditions (Fig. 1, part B; Fig. S2). Moreover, WT mice showed a significant linear increase of PAS-positive cells over time irrespective of sedentary or trained conditions (linear regression for time trends: WT-SD: $R^2 = 0.23$, $r = 0.48$, $P = 0.015$; WT-EX: $R^2 = 0.36$, $r = 0.60$, $P = 0.0015$). No similar trend was found in *mdx* mice (not shown).

In summary, the main result was that mdx mice showed a paucity of goblet cells in airway epithelium, scarce progressive thinning of airway epithelium over time, and little evidence of airway inflammation after training.

Apoptosis and regeneration of airway epithelium

Both at bronchial and bronchiolar level, frequency of apoptosis of airway epithelial cells at baseline was higher in WT than in *mdx* mice, but there was a trend at 30 and 45 days for higher apoptosis in *mdx* than WT mice irrespective of sedentary or trained status (Fig. 1, part C, and Fig. S3). In mice trained for 45 days, the percentage of TUNEL-positive epithelial cells was significantly higher in *mdx* than in WT mice. In particular, in bronchi linear regression revealed opposite trends over time in WT (WT-SD: $R^2 = 0.51$, $r = -0.71$, $P < 0.0001$; WT-EX: $R^2 = 0.51$, $r = -0.72$, $P < 0.0001$) and *mdx* mice (*mdx*-SD: $R^2 = 0.29$, $r = 0.54$, $P = 0.006$; *mdx*-EX: $R^2 = 0.38$, $r = 0.62$, $P = 0.00$). Similar trends were found in bronchioli (WT-SD: $R^2 = 0.49$, $r = -0.70$, $P = 0.04$; WT-EX: $R^2 = 0.45$, $r = -0.67$, $P < 0.0001$; *mdx*-SD: $R^2 = 0.58$, $r = 0.76$, $P = 0.03$; *mdx*-EX: $R^2 = 0.38$, $r = 0.62$, $P < 0.0001$).

Frequency of PCNA-positive cells, that is, cells under active proliferation, at baseline was higher in WT compared to *mdx* mice in both the bronchial and bronchiolar epithelium. Both strains were similar at 30 days for PCNA positivity, that is, higher proliferation in exercised than sedentary animals in both WT and *mdx* groups. At 45 days, PCNA positivity was
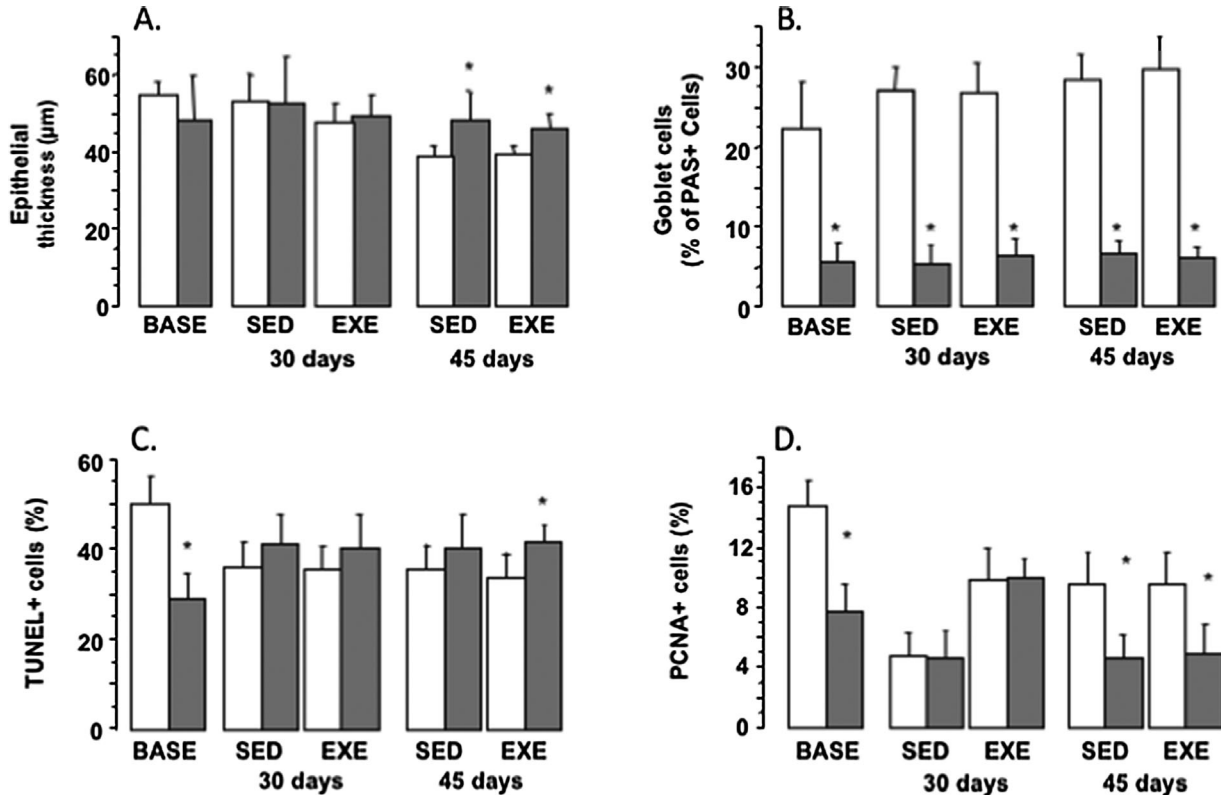

Fig. 1. Summary data on morphological and immunohistochemical analyses of lung sections from **WT** (empty bars) and *mdx* (full bars) mice under sedentary (SED) or trained (EXE) conditions at baseline and at 30 and 45 days. Part A: Mean bronchial epithelial thickness (expressed in μm, error bars indicate SD); part B: Mean percentage of goblet cells; part C: mean percentage of TUNEL-positive cells; part D: mean percentage of PCNA positive cells. *Significant difference between WT and *mdx* groups.

unchanged in WT mice, but sharply decreased in *mdx* mice irrespective of training (Fig. 1, part D, and Fig. S4).

In summary, epithelial apoptosis progressively increased in *mdx* mice over time. Epithelial regeneration sharply decreased in *mdx* mice at 45 days.

Chaperonin HSP60

The level of HSP60, an oxidative stress marker, was higher at baseline in *mdx* compared to WT mice in both the bronchial (Fig. 2, part A) and bronchiolar (data not shown) epithelium. In particular, HSP60 levels were higher in WT than *mdx* mice, with the highest values recorded in the WT-EX mice at 30 days. No significant time trend was evident in WT mice (data not shown), while linear regression revealed a progressive decrease in HSP60 levels in *mdx* mice over time in both bronchial (*mdx*-SD: $R^2 = 0.81$, $r = -0.90$, $P < 0.002$; *mdx*-EX: $R^2 = 0.78$, $r = -0.88$, $P < 0.0015$) and bronchiolar epithelium (*mdx*-SD: $R^2 = 0.88$, $r = -0.94$, $P = 0.0006$; *mdx*-EX: $R^2 = 0.67$, $r = -0.82$, $P < 0.0005$). The percentage of apoptotic cells in bronchial epithelium was inversely related to HSP60 expression in *mdx* but not in WT mice (Fig. 2, part B), and a similar relationship was found in bronchioles ($r = -0.59$, $P = 0.03$).

In summary, HSP60 levels progressively decreased over time, and were inversely correlated to apoptosis, in *mdx* mice.

Discussion

Subtle epithelial abnormalities were found in *mdx* mice, that is, a low number of goblet cells, and lower PCNA expression at

45 days in *mdx* compared to WT animals. Moreover, in *mdx* mice there was a significant trend for increasing apoptosis to be associated with low epithelial expression of HSP60 in airway epithelium. These results suggest that dystrophin might be involved not only at the muscle level, but also in the preservation of bronchial epithelial integrity. In response to low-intensity endurance training, no significant inflammatory infiltrate was observed in the airways of trained WT or *mdx* mice, at variance with the mild airway epithelial damage and leucocyte influx into and around airways previously documented in a different mouse strain (Chimenti et al., 2007).

The number of goblet cells in the airways of *mdx* mice was very low in both sedentary and trained animals. To our knowledge, this is a new and intriguing finding. We can only speculate on the potential pathway(s) involved. Recent work has shown that the Notch pathway is central in epithelial homeostasis and remodelling. The Notch pathway is involved not only in airway cell distribution during development (Mori et al., 2015) but also in homeostatic transdifferentiation in the adult lung (Marcet et al., 2011; Tsao et al., 2011; Gomi et al., 2015; Lafkas et al., 2015; Mori et al., 2015). Notch or Jagged inhibition caused an almost complete loss of goblet/club cells in favour of ciliated cells (Marcet et al., 2011; Firth et al., 2014; Lafkas et al., 2015). Our data strongly suggest that low activity of the Notch pathway might be involved in the decreased number of goblet cells found in the airways of *mdx* mice, as schematically depicted in Figure 3. No data are currently available on the expression and role of dystrophin in airway epithelium.

In patients with Duchenne muscular dystrophy (DMD), mucus elimination from the airways is a major clinical problem
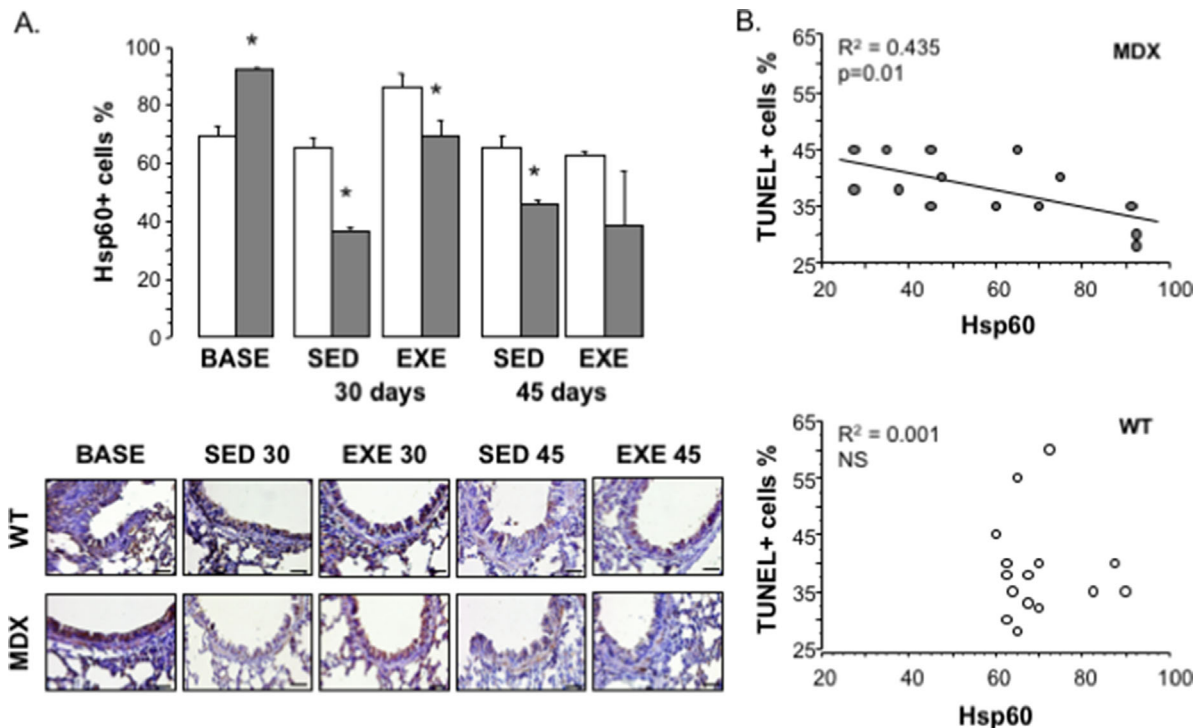
Fig. 2. Analysis of HSP60. Part A, above: mean percentage of HSP60-positive cells in bronchial epithelium of WT (empty bars) and *mdx* (full bars) mice under sedentary (SED) or trained (EXE) conditions at baseline and at 30 and 45 days; below: representative pictures of HSP60 assay in lung sections. Error bars in bar graphs: SD; horizontal bars in sections: 100 μm. Part B: relationship between HSP60 and TUNEL positivity in bronchial epithelium of *mdx* (upper graph) and WT (lower graph) mice. Pooled data from sedentary and trained conditions at baseline and at 30 and 45 days are shown. *Significant difference between WT and *mdx* groups.

due to inefficient cough (LoMauro et al., 2014). A reduced number of goblet cells in the airways might actually be favourable in the setting of reduced respiratory muscle function by limiting mucus production. Notch was shown to be downregulated in skeletal muscle of *mdx* mice, and this finding might be causally related to age-dependent depletion of satellite cells (Jiang et al., 2014). Positive modulation of Notch is a strategy currently explored to rescue the dystrophic muscle, even though increasing Notch expression in animal models of DMD did not improve muscle regeneration (Church et al., 2014). Our data suggest the possibility that rescuing Notch to counteract skeletal muscle damage might exert detrimental consequences at the respiratory level in DMD patients. Further studies are needed to clarify this point.

The reduction in goblet cells in *mdx* mice might be associated with an increase in ciliated cells, since the airways mucosa is mainly constituted by these two differentiated cytotypes. However, while goblet cells can be easily recognized by PAS staining, there is no histochemical stain available allowing the identification of ciliated cells. Further studies by electron microscopy are needed to confirm the hypothesis that in our model the decrease of goblet cells was associated with an increase in ciliated cells.
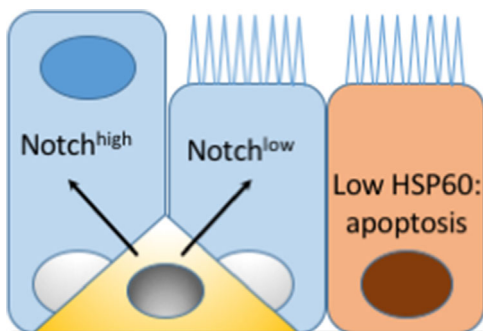
At variance with our previous results (Chimenti et al., 2007), we found little epithelial damage associated with exercise training in both *mdx* and WT mice, despite we used exactly the same training protocol as in our earlier study. Since inflammatory cell infiltration was low in both strains, the lack of an inflammatory response in the airways cannot be attributed to absence of dystrophin, but rather suggests a difference between strains. Such interpretation is supported by other differences between the two studies. For example, in Swiss mice, we found loss of cilia and increased epithelial thickness after training (Chimenti et al., 2007); conversely, no loss of cilia was found in the present study.

Analysis of epithelial apoptosis and regeneration provided some insight into the mechanisms involved in bronchial epithelial integrity in *mdx* mice. In our previous study, we documented a progressive increase in both PCNA- and TUNEL-positive cells during training, and interpreted this result as indicative of exercise-dependent increase in epithelial turnover (Chimenti et al., 2007). In the present study, the analysis of the PCNA time course showed that at 30 days

Fig. 3.   Schematic drawing summarizing the hypothetical changes occurring in *mdx* mice in the airway epithelium. Differentiation of basal epithelial cells (triangle) into goblet cells (left) or ciliated cells (middle) is modulated by Notch expression. Predominance of ciliated cells in mdx mice might be explained by low Notch expression. A high expression of HSP60 may protect epithelial cells against apoptosis (right) early in the course of the disease.

trained animals showed twofold PCNA expression compared to sedentary animals in both WT and *mdx* groups. However, later on (i.e., 45 days) WT mice were able to maintain PCNA expression level, whereas *mdx* mice showed decreased PCNA expression. Moreover, TUNEL positivity at 30 and 45 days tended to be higher in *mdx* than WT mice, reaching significance in the exercised group at 45 days. A possible interpretation of this complex picture is that *mdx* mice can effectively increase epithelial regeneration only at an early stage, but such ability shows signs of exhaustion in later phases. Again, this time course is similar to the late depletion of satellite cells in *mdx* muscle (Jiang et al., 2014), in agreement with a possible role of Notch signalling. The late changes in epithelial thickness in the course of the experiment can be interpreted as an indirect proof of altered homeostasis of airway epithelium. From a morphological point of view, the increased epithelial thickness may indicate a modification in cell differentiation and, in turn, morphology: for example, cells could be taller or cilia longer, or the overall number of cells are increased so that the epithelium results crowded. Again, further studies by transmission electron microscopy are necessary to solve this conundrum. Extension of the training period in future experiments can also help to better understand the relationship between airway epithelial damage and increased epithelial thickness.

HSP60-positive cells progressively decreased over time in *mdx* mice. Rather than indicating low cellular stress, the low expression of HSP60 at 45 days in *mdx* mice suggests the progressive exhaustion of a protecting mechanism preserving epithelial integrity, as confirmed by the inverse correlation between HSP60 expression and the percentage of apoptotic cells, as schematically summarized in Figure 3. A similar time course of HSP60 was observed in the diaphragm of *mdx* mice (Cappello, personal communication). HSP60 is known to exert an anti-apoptotic activity in bronchial epithelial cells by blocking the activation of the caspase cascade (Campanella et al., 2008). Our data extend to the lung previous findings indicating a role of HSP60 in epithelial preservation in the kidney of *mdx* mice (Loh et al., 2000). The role of HSP60 in bronchial mucosa homeostasis is still under investigation. Low HSP60 levels were found during carcinogenesis of bronchial mucosa in patients with chronic obstructive pulmonary disease (COPD) (Cappello et al., 2005). Moreover, the normal epithelium close to the tumor showed reduced immunopositivity for HSP60 compared to healthy subjects (Cappello et al., 2006). Viceversa, high HSP60 levels were documented in severe/very severe COPD compared to smokers and nonsmoker controls (Cappello et al., 2011). Interestingly, oxidative stress increased the expression and secretion of HSP60 in a human bronchial epithelial cell line (Cappello et al., 2011) while cigarette smoke extracts did not (Corrao et al., 2014). Secreted HSP60 may also act in a paracrine fashion on epithelial cells by downregulating inflammation (Cappello F, personal communication). Therefore, HSP60 appears to exert protective effects in the bronchial epithelium in disease models like cancer or COPD (Loh et al., 2000; Cappello et al., 2005, 2006, 2011; Campanella et al., 2008; Corrao et al., 2014), and the current data extend these observation to the *mdx* model.

The more relevant implication of our results is that lack of dystrophin affects airway epithelium in addition to the known effects on skeletal muscle. This result is in line with a generalized perturbation of cell metabolism in DMD, which has not been given much consideration until now. Instead, DMD should be considered a systemic disease with major, but not exclusive, involvement of skeletal muscle. From a clinical point of view, it is unknown whether the epithelial changes found in *mdx* mice also occur in human DMD, and further studies are necessary to test this hypothesis and assess potential pathological effects of exercise training or test specific pharmacologic interventions. In patients with DMD, positive

effects of inspiratory muscle training have been reported, but data on airway epithelium have not been collected (Wanke et al., 1994).

In conclusion, to our knowledge, this is the first report on airway cells in *mdx* mice. *mdx* mice showed a low number of goblet cells, limited epithelial regeneration, and decreased expression of HSP60 in airway epithelium irrespective of sedentary or training conditions. No major inflammatory changes related to mild exercise training were found in WT or *mdx* mice. Further studies are needed to assess the potential clinical implications of our findings in patients with DMD.

Acknowledgments

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