Journal of Steroid Biochemistry & Molecular Biology 117 (2009) 139–145

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Epithelial cells are the major site of hydroxysteroid (17β) dehydrogenase 2 and androgen receptor expression in fetal mouse lungs during the period overlapping the surge of surfactant

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ARTICLE INFO

Article history:
Received 8 July 2009
Received in revised form 24 August 2009
Accepted 28 August 2009

Keywords:
Fetal lung maturation
Steroidogenesis
Testosterone
Hydroxysteroid (17beta) dehydrogenase
Immunohistochemistry
In situ hybridization

ABSTRACT

Many genes involved in the peripheral metabolism of androgens, including hydroxysteroid (17β) dehydrogenases (HSD17B) 2 and 5, steroid 5α reductase 1, and 3α-HSD, are expressed in the developing lung. Because lung development is delayed by androgens and pathologies related to lung immaturity are major concerns for preterm neonates, we are interested in the elucidation of the androgen metabolism in developing lung. In the present report we have identified the cell types expressing HSD17B2 (testosterone into androstenedione) and androgen receptor in normal male and female mouse developing lung between the gestation days 15.5 and 17.5. *In situ* hybridization and immunohistochemistry revealed that HSD17B2 is expressed in epithelial cells of respiratory and conducting zones, and in mesenchymal cells. The androgen receptor protein was observed in the same cell types that HSD17B2, and in α-smooth muscle actin-positive cells surrounding arteries. No difference was observed for the location of HSD17B2 and androgen receptor expression at any time points studied, or according to sex. Taken together, our results are in concordance with the hypothesis that in mouse fetal lungs the level of androgen receptor occupancy is finely tuned by local HSD17B2 expression.

1. Introduction

Peripheral androgen metabolism exists in the lung as demonstrated in cell culture models and *in vivo* in developing lung tissues. In the human pulmonary epithelial-like A549 cells, hydroxysteroid (17β) dehydrogenase 5 (*Hsd17b5*; *Akr1c6*) mRNA is expressed, along with the activity of the corresponding enzyme consisting in formation of testosterone from androstenedione [1]. *Hsd17b5* is also expressed in the mouse developing lung in late gestation with one major peak of expression on gestation day (GD) 17.5 [2]. Androgen synthesis within the developing lung is interesting since lung maturation is delayed by these steroids [3–7], and a sex difference has been observed in lung development and maturation [8–13]. As a consequence, sex is a determining factor in lung maturation with the risk to develop respiratory distress syndrome being higher in male than female premature infants [14–16]. However, whether the expression of *Hsd17b5* in the developing lung is somewhat involved in the sex difference in lung development remains an open question.

Three steroid metabolizing activities have been observed for HSD17B2 on steroids: inactivation of testosterone to androstenedione (the reverse reaction of HSD17B5 described above), inactivation of 17β-estradiol to estrone, and formation of progesterone from 20α-dihydroprogesterone. *Hsd17b2* mRNA has been detected in the mouse developing lung at all gestation times (GD 15.5–18.5) analyzed [2], and in cultured human lung fibroblasts [17]. In these cells, the androgen inactivating activity of HSD17B2 (testosterone → androstenedione) was also observed [17], and was considered to be important for lung development. Temporal and spatial regulation of *Hsd17b2* and androgen receptor was also demonstrated during neonatal lung development [18]. However mRNA and protein expression sites remained to be characterized in whole fetal lung tissues during the period overlapping the surge of surfactant.

Other enzymes involved in peripheral sex steroid metabolism are also expressed in the developing lung and/or in lung cell cul-

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doi:10.1016/j.jsbmb.2009.08.006

tures. Steroid 5α-reductase 1 is expressed at constant levels from GD 15.5 to 17.5 with a statistically significant decrease on GD 18.5 in the mouse [19]. The 5α-reductase activity has been also detected in human lung fibroblast cultures [17] and in A549 epithelial-like cells [1]. There was no formation of DHT from testosterone in human lung fibroblast cultures where the 5α-reductase activity appeared to be restricted to the conversion of androstenedione to androstanedione [17]. In contrast, accumulation of tritiated 3α,17β-diol, a metabolite of 5α-DHT, in A549 cell cultures in the presence of tritiated androstenedione strongly suggests that 5α-DHT was formed from the intermediate tritiated testosterone by 5α-reductase activity, while inactivation of 5α-DHT into 3α,17β-diol was catalyzed by type 3 3α-HSD [1]. Mouse type 1 3α-HSD gene is expressed in the developing lung with a marked increase from GD 17.5 when the emergence of mature type II pneumonocytes occurs [19]. In addition, constant levels of androgen receptor gene expression were observed in the mouse developing lung from GD 15.5 to 18.5 [2].

Together, the above observations strongly suggest that there is an active metabolism of androgens in the developing lung, and that androgen receptor occupancy is modulated through activation and inactivation of androgens. In order to get important insights on the androgen metabolism in the developing lung, we have characterized HSD17B2 expression in male and female fetal mouse lungs by *in situ* hybridization and immunohistochemistry, and the expression has been correlated with androgen receptor (AR) expression analyzed by immunohistochemistry.

2. Methods

2.1. Animals and tissue preparation

Balb/C mice were mated during the night (mating window ±8 h), and the day of copulatory plug was considered as GD 0.5. Pregnant females were killed by exposure to a CO_2 atmosphere. Protocols were approved by the Animal Care and Use Committee and the Institutional Review Board of the Centre de Recherche du Centre Hospitalier Universitaire de Québec (protocol no. 2005-156). At least two male and two female fetuses per litter from three pregnant mice were studied for each time of gestation (GDs 15.5, 16.5, and 17.5). The fetal sex was identified by examination of the genital tract using a dissecting microscope. Confirmation of individual sex was done by PCR amplification of the *Sry* gene. Fetal lungs were fixed in 4% paraformaldehyde for 48 h and then paraffin-embedded for use in immunohistochemistry and *in situ* hydridization. For red oil staining, tissues were embedded in Tissue-Tek OCT compound (Miles, IN, USA) and then kept at −80 °C until use. Slices of 5 and 6 μm were prepared for paraffin-embedded and OCT-embedded tissue sections, respectively.

2.2. Immunodetection of mouse HSD17B2

The following antibodies were characterized and used to detect HSD17B2 in mouse fetal lung tissues: polyclonal rabbit anti-peptide antibodies produced against amino acids 279–292 (#AB84) [20] and 318–333 (#4839) of the human HSD17B2; commercial polyclonal rabbit anti-human HSD17B2 raised against the full-length recombinant protein (ProteinTech Group Inc., IL, USA). The anti-peptide antiserum preparations were purified by the use of a Sulfo-Link affinity column (Pierce Biotechnology Inc., IL, USA), to which the corresponding HSD17B2 peptide was coupled.

2.3. Expression of recombinant mouse HSD17B2

Mouse HSD17B2 sequence was amplified by PCR from mouse lung cDNA using the following primers: 5′-GGGGAATTCTGGAGAATGAGCCCGTTTGC-3′; 5′-GGGCTCGAGGTGCACTGTCCTTAAAGCTTC-3′. The resulting amplicon was subcloned into pcDNA3.1/*myc*-His A vector (Invitrogen). Flp-In T-Rex 293 (FT293) cells (Invitrogen) were cultured at 37 °C in 5% CO_2 atmosphere in DMEM medium containing 4.5 g/l glucose, L-glutamine, sodium pyruvate (Wisent), and penicillin/streptomycin (Wisent), supplemented with 10% inactivated fetal bovine serum (Wisent) and nonessential amino acids (Wisent). FT293 cells were plated in 60 mm dishes, grown at 50–75% confluency, and transient transfection of plasmid DNA was performed with Effectene reagent (Qiagen) using the following conditions/dish: 1.0 μg DNA, 8 μl enhancer, 25 μl effectene. Cells were harvested 48 h after transfection, and total proteins were extracted.

2.4. Immunohistochemistry

Tissue sections were deparaffinized, rehydrated, incubated 30 min in 3% hydrogen peroxide in methanol, and then washed in distilled water. Slides were subjected to an antigen retrieval step consisting of incubation in hot citrate buffer (100 mM citric acid, 100 mM sodium citrate). Tissues were pre-incubated 1 h at room temperature in 10% goat serum in PBS (1× PBS: 135 mM NaCl, 2.65 mM KCl, 4.22 mM Na_2HPO_4, 1.45 mM KH_2PO_4) containing 0.1% BSA and 0.4% Triton X-100. Slides were then incubated overnight with the primary antibody at 4 °C: anti-human HSD17B2 #AB84 [20] (1:200), anti-human HSD17B2 #4839 (1:200), anti-human HSD17B2 (ProteinTech Group Inc.) (1:100), rabbit anti-androgen receptor polyclonal antibody (Santa Cruz Biotechnology Inc., CA, USA) (1:100), mouse anti-cytokeratin 18 monoclonal antibody (Abcam Inc., MA, USA) (1:200), mouse anti-α-SMA monoclonal antibody (Calbiochem, VWR International Ltd., QC, Canada) (1:200), or rabbit anti-human von Willebrand factor polyclonal antibody (Sigma, MO, USA) (1:200). Note that control and experiment sections were treated and photographed using the same conditions. Biotinylated goat anti-rabbit IgG (DakoCytomation Inc., ON, Canada) or anti-mouse IgG (Chemicon International Inc., ON, Canada) was added for 1 h at room temperature. Immunostaining was revealed with the streptavidin–biotin peroxidase reaction method using an ABC Vectastain elite kit (Vector Laboratories Inc., CA, USA) with AEC (3-amino-9-ethylcarbazole) as chromagen. Tissue sections were then counterstained with Mayer's hematoxylin.

2.5. In situ hybridization

Two *Hsd17b2* cDNA fragments (nucleotides 374–672 and 653–927 of the mRNA sequence, NM_008290) were amplified by PCR from mouse lung cDNA using respectively the two following primer pairs: 5′-GGG-**GAA-TTC**-CAC-AGT-GTT-TGC-TGG-AGT-GC-3′, 5′-GGG-**AAG-CTT**-GTA-GAG-GCA-GAA-ACG-CCT-TG-3′ and 5′-GGG-**GAA-TTC**-CAA-GGC-GTT-TCT-GCC-TCT-AC-3′, 5′-GGG-**AAG-CTT**-TCC-TGG-CCA-TAG-TTC-TCC-TG-3′. The resulting amplicons were sub-cloned into pSV-SPORT-1 (Invitrogen Life Technologies Inc., CA, USA) and pGEM-4Z (Promega Corp., WI, USA), respectively. RNA probes were synthesized using DIG-UTP substrate (Roche, IN, USA) with 1 μg DNA, 2 μl of 10× transcription buffer (Roche), 2 μl NTP labeling mix (Roche), 20 U RNAse inhibitor (Promega), 40 U SP6 (sense) or T7 (antisense) polymerase (Roche) in 20 μl. Probes were purified by ethanol precipitation with 20 μg tRNA and quantified by UV band densitometry following agarose gel electrophoresis. The specificity of the first antisense probe was confirmed by Northern blot analysis (data not shown). The two resulting antisense probes gave identical *in situ* hybridization results (data not shown). Tissues were deparaffinized, rehydrated, and fixed in 4% paraformaldehyde (Sigma, MO, USA) in PBS for 5 min. Slides were incubated in ethanol:glacial acetic acid (95:5) for 10 min at −20 °C, and rehydrated. Tissues were equilibrated in

PBS, and incubated in preheated proteinase K (Roche) (10 μg/ml in PBS) 15 min at 37 °C. Protease action was stopped with glycine (2 mg/ml). Tissues were fixed 5 min in 4% paraformaldehyde in PBS, then acetylated in 0.25% acetic anhydride in 100 mM triethanolamine pH 8.0 for 10 min, and washed in PBS. Slides were prehybridized 2 h at 42 °C in a humid chamber with 200 μl of hybridization solution/slide (0.3 M NaCl, 10 mM Tris–HCl pH 7.5, 1 mM EDTA, 1× Denhardt's (100× = 2% BSA, 2% polyvinylpyrrolidone, and 2% Ficoll), 250 μg/ml tRNA, 5% dextran sulfate, 0.02% SDS, 50% formamide, 250 μg/ml herring sperm). DIG-cRNA probes were denaturated and diluted at 2.5 ng/μl. Hybridization was performed overnight at 42 °C in a humid chamber. Slides were washed 2× 5 min in 2× SSC, followed by RNAse A treatment for 10 min in 2× SSC, washed 10 min in 2× SSC, 2× 10 min in 1× SSC at 42 °C, and 2× 10 min in 0.2× SSC at 42 °C. Slides were equilibrated 2× 10 min in detection buffer #1 (200 mM Tris–HCl pH 7.5,

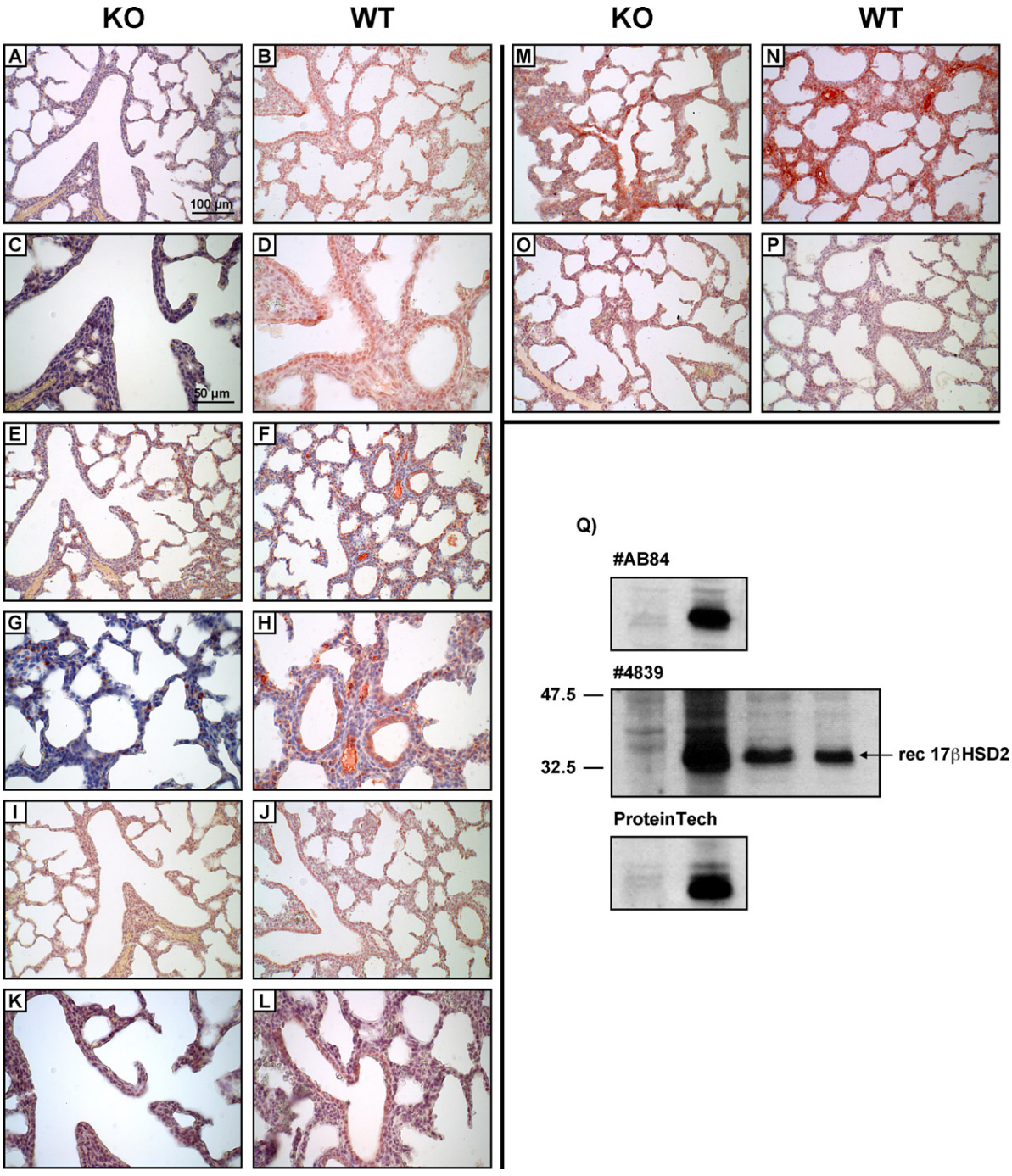

Fig. 1. Immunohistochemistry on GD 18.5 HSD17B2-knockout (KO) and wild-type (WT) fetal lung tissues using anti-HSD17B2 antibodies; and Western blot on protein extracts of FT293 cells expressing a recombinant mouse HSD17B2. Similar positive signals were observed on WT fetal lung tissues using the anti-human HSD17B2 #AB84 [20] (panels B and D), the anti-HSD17B2 #4839 (panels F and H), and the commercial anti-human HSD17B2 (ProteinTech) (panels J and L). No specific signal was observed on KO fetal lung tissues using these antibody preparations (panels A, C, E, G, I, K). Immunodetection of α-smooth muscle actin was done on KO (panel M) and WT (panel N) fetal lungs to confirm protein integrity. Rabbit IgG was used as negative control for KO (panel O) and WT (panel P) lung tissues. WT and KO mice were from the mixed (SV129/C57BL/6N 1:1) strain [24]. Positive signal is red. Magnifications: A, B, E, F, I, J, M–P, 200×; C, D, G, H, K, L, 400×. One specific band was detected on total protein extracts of FT293 cells transfected with a plasmid encoding for recombinant mouse HSD17B2 using antibodies #AB84 (panel Q, lane 2), #4839 (panel Q, lanes 2–4, three different preparations of transfected cells), and anti-HSD17B2 from ProteinTech (panel Q, lane 2). Non-transfected FT293 cells were used as negative control (panel Q, lane 1).

200 mM NaCl). Unspecific binding was blocked with blocking solution (10 ml = 9.3 ml 0.3% Triton X-100 in detection buffer #1, 500 μl goat serum, 200 μl 100 mM levamisole in detection buffer #1) for 1 h in a humid chamber. Slides were incubated with anti-DIG-AP fab fragment (1/1000 in blocking solution) for 2 h, washed 2× 10 min in detection buffer #1, and then incubated 10 min in detection buffer #2 (100 mM Tris–HCl pH 9.5, 100 mM NaCl, 10 mM $MgCl_2$). Tissue sections were incubated overnight at room temperature in substrate solution (10 ml = 0.2 ml of 100 mg/ml NBT–50 mg/ml BCIP, 20 μl of 100 mM levamisole in buffer #1, 9.78 ml buffer #2). Slides were then immersed in stop solution (1 mM EDTA, 10 mM Tris–HCl pH 7.5), washed 5 min in water, and counterstained 2 min with 0.25% neutral red. Tissue sections were then washed, dehydrated, cleared in xylene, and mounted with Permount (Fisher Scientific, ON, Canada).

2.6. Red oil staining

Red oil (Fluka/Chemica, ON, Canada) was prepared at 0.7% (w/v) in propylene glycol. OCT-embedded tissue sections were thawed, fixed in 10% formalin, and washed in water. Slides were then incubated 10 min in 100% propylene glycol, 1 min 20 s in the red oil solution, 3 min in 85% propylene glycol, and then washed in water. Tissue sections were counterstained in hematoxylin (Gill's Formulation #2, Fisher Scientific) and washed in water. Slides were then dipped in 0.3% ammonia solution (Fisher Scientific) and washed in water.

3. Results

Endometrium [21,22], placenta, and liver [23] were included as positive controls for the detection of HSD17B2. Immunohistochemistry with the three anti-HSD17B2 antibodies on mouse endometrial tissues showed a strong positive signal specifically on epithelial cells, as expected (data not shown). Western blots using mouse lung, placental, and liver protein extracts confirmed that antibody #4839, as well as the anti-HSD17B2 from ProteinTech, detect mouse HSD17B2 (data not shown). Protein extracts from FT293 cells expressing a recombinant mouse HSD17B2 were also analyzed by Western blot with antibodies #AB84 and #4839, and the anti-HSD17B2 from ProteinTech (Fig. 1Q). One specific band was observed in transfected cells, but not in non-transfected cells.

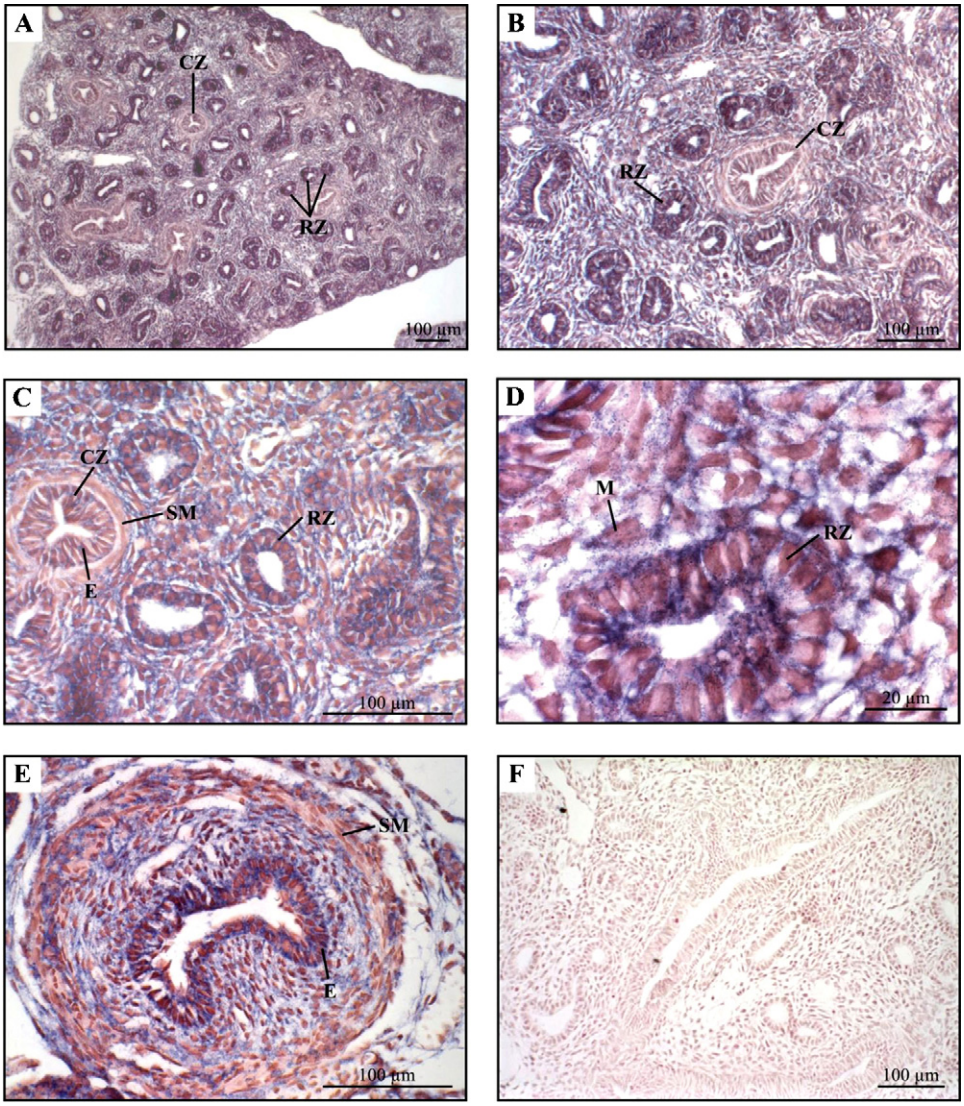

Fig. 2. *Hsd17b2* mRNA in Balb/C mouse fetal lungs. *In situ* hybridization was performed on mouse fetal lung tissues, isolated on GD 15.5 (panels A–D, F) or GD 17.5 (panel E), with an antisense (A–E) or a sense (F, negative control) *Hsd17b2* RNA probe. Panels A–E are adjacent slices of panel F. Positive signal is blue. Identical staining patterns were obtained on GD 16.5 (data not shown). Results were reproduced using another set of probes specific to another segment of *Hsd17b2*. Magnifications: A, 100×; B and F, 200×; C and E, 400×; D, 1000×. CZ, conducting zone; RZ, respiratory zone; M, mesenchyme; SM, smooth muscle; E, epithelium.

Noteworthily, immunohistochemistry was performed on GD 18.5 fetal lung tissues obtained from HSD17B2 deficient (knockout) mice and their wild-type counterparts [24] to further address the specificity of the antibody preparations. In wild-type tissues, specific staining was observed with the three antibody preparations, while no signal was obtained with KO tissues. This confirms the specificity of the positive signal obtained with the anti-HSD17B2 antibodies (Fig. 1A–L).

Expression of HSD17B2 was detected by *in situ* hybridization (Fig. 2 and data not shown) and immunohistochemistry (Fig. 3 and

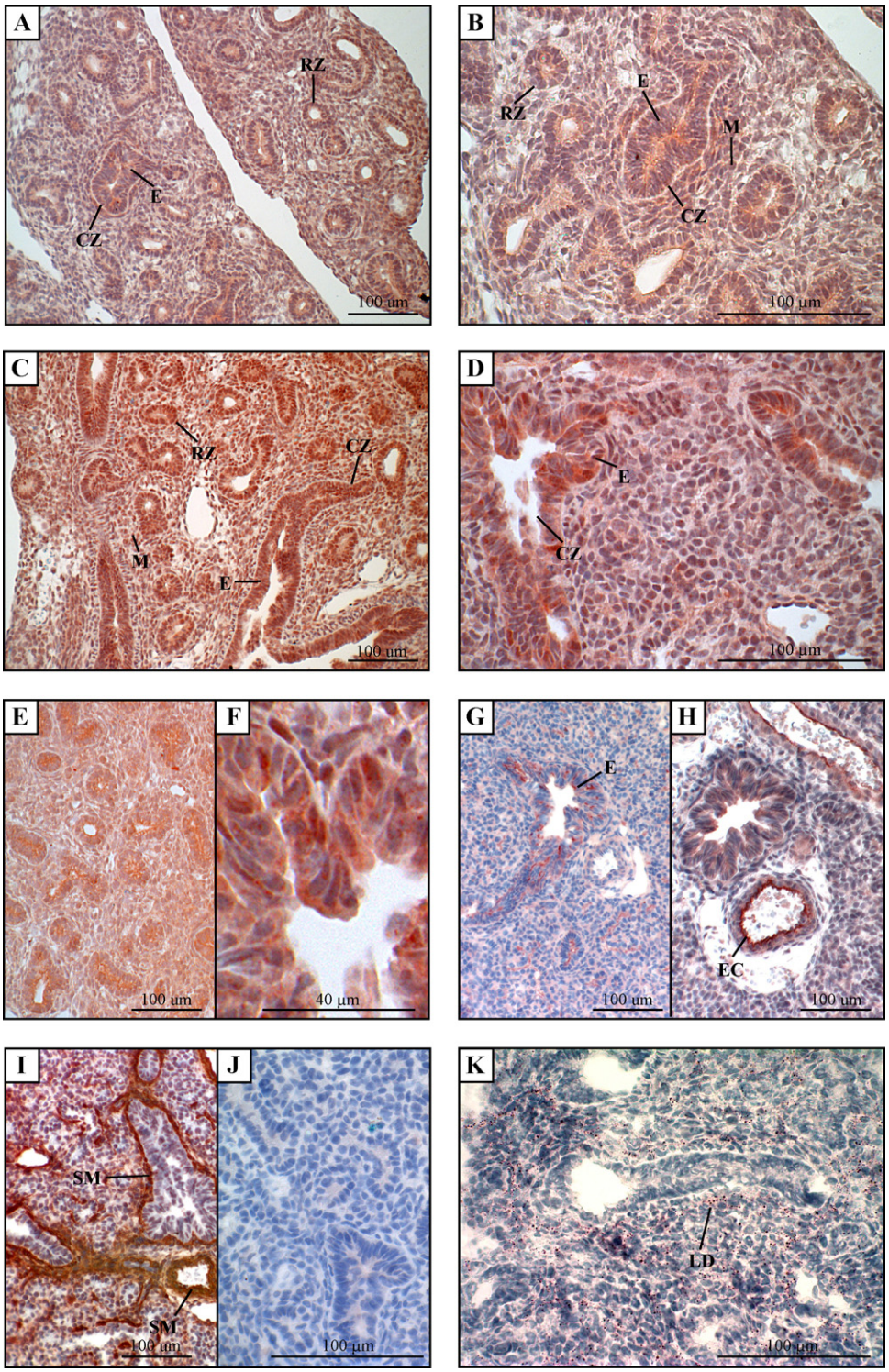

Fig. 3. Detection of HSD17B2 and androgen receptor proteins in the Balb/C mouse fetal lung by immunohistochemistry. Anti-HSD17B2 #AB84 [20] (panels A and B); anti-HSD17B2 #4839 (panel E); negative control (anti-HSD17B2 #AB84-depleted antiserum after adsorption on an affinity column) (panel J). Results were reproduced using the anti-HSD17B2 antibody from ProteinTech (data not shown). Anti-androgen receptor (panels C, D, and F); anti-cytokeratin 18 (panel G); anti-von Willebrand factor (panel H); anti-α-smooth muscle actin (panel I); red oil staining (panel K). Immunohistochemistry positive signals (panels A–J) are red. All the selected photographs show lung tissues isolated on GD 17.5, but identical staining patterns were obtained on GD 15.5 and 16.5 (data not shown). Magnifications: A, C, E, G–I, 200×; B, D, J, K, 400×; F, 1000× CZ, conducting zone; RZ, respiratory zone; M, mesenchyme; SM, smooth muscle; E, epithelium; EC, endothelial cell; LD, lipid droplets.

data not shown) in male and female fetal mouse lungs harvested on GD 15.5, 16.5, and 17.5. Several cell types were positively stained with both approaches, showing that *Hsd17b2* was not only transcribed but also translated in these cells. By *in situ* hybridization, all epithelial cells of the respiratory zone, comprising lower airways from bronchioles to alveolar ducts, were positive (Fig. 2). There is no alveolar sac in the developing lung from GD 15.5 to 17.5. Most epithelial cells of the conducting zone, comprising upper airways from trachea to bronchus, were positive (Fig. 2), while some negative cells were also observed (data not shown). All the epithelial cells showed a positive signal with each of the three anti-HSD17B2 antibodies (Fig. 3A, B, E, and data not shown). Positive signals of the respiratory zones were oriented within the lumina as for the signal obtained with an anti-cytokeratin 18 antibody (Fig. 3G), which is a marker of epithelial cells [25]. Most mesenchymal cells were positive by *in situ* hybridization (Fig. 2) including cells with lipid droplets (Fig. 3K) detected by red oil staining [26]. The mesenchyme was also positive by immunohistochemistry (Fig. 3A, B, and E), although it was impossible to determine whether all the cells were positive. Tissue sections of mouse developing lung were also immunostained with the anti-α-smooth muscle actin antibody, which is a marker of smooth muscle cells, myofibroblasts, and microvascular pericytes [27]. Anti-α-smooth muscle actin-positive cells surrounding conducting zones (Fig. 3I) were negative for HSD17B2 as analyzed by *in situ* hybridization (Fig. 2C) and immunohistochemistry (Fig. 3B). Most of endothelial cells gave positive signals close to the background with both techniques while some vessels were clearly negative (data not shown). No difference was observed in sites of HSD17B2 expression according to sex and time of gestation within the gestational period analyzed using three different litters per time point.

The presence of AR was also studied by immunohistochemistry in mouse developing lung on GD 15.5, 16.5, and 17.5 (Fig. 3C, D, F, and data not shown). Based on the structural organization of the developing lung, the distribution of the protein was very similar to that of HSD17B2. Epithelial cells of conducting and respiratory zones and mesenchymal cells were positive, while anti-α-smooth muscle actin-positive cells surrounding conducting zone were negative. Sub-localization of the positive signal within the nucleus, indicating that the androgen receptor is activated, was observed for a few epithelial cells of the conducting zone and, to a lesser extent, of the respiratory zone (Fig. 3C, D, and F). Positive nuclei were also observed on some mesenchymal cells (Fig. 3D) and on a few smooth muscle cells surrounding arteries (data not shown). Endothelial cells were negative as evidenced by comparison with the signal obtained with an anti-von Willebrand factor antibody (Fig. 3H), used as endothelial cell marker [28]. No difference was observed in sites of AR expression according to sex and time of gestation within the gestational period analyzed.

4. Discussion

HSD17B2 has the potential to negatively regulate both androgen and estrogen receptor occupancy, and to positively regulate progesterone receptor occupancy. Accordingly, its ability to convert testosterone to androstenedione is considered to be of first importance in developing lung due to the reported deleterious effects of androgens on lung development. This activity has been observed in cultured human lung fibroblasts along with the presence of *Hsd17b2* mRNA [17]. Expression of *Hsd17b2* in the developing lung was also observed in RNA extracts prepared from mice between GD 15.5 and 18.5 [2]. So far cultured human lung fibroblasts cells are the only pulmonary cell type identified to express HSD17B2 [17]. The present report shows that HSD17B2 is more widely expressed in the developing lung than previously expected, and that in addition to mesenchymal cells, the majority of epithelial cells also express HSD17B2.

In fetal lung, the expression pattern of HSD17B2 is very close to that of AR. Co-expression of these two proteins in the same structures suggests a metabolic relationship. Thus, HSD17B2 could be involved in fine tuning of AR occupancy through the control of active androgen levels available for intracrine and/or paracrine action(s). Androgens in turn are already known to control developmental pulmonary events, particularly during lung maturation [7].

Type II pneumonocyte maturation, leading to surfactant production, is regulated by cell-cell communication and delayed by androgens in males [4] through their primary effects on lung fibroblasts [3,29,30]. Besides, a positive role for androgens in lung development was proposed on the basis that *Hsd17b5* (androstenedione → testosterone) is expressed in male and female mouse lungs with a peak of expression on GD 17.5 [2]. In the mouse, the surge of surfactant synthesis occurs on GD 17.5 as indicated by the appearance of lamellar bodies [31], an increase in surface activity in the mouse lung homogenate [31], and by increases in the activity of some enzymes involved in pulmonary lipid metabolism [32,33]. Therefore, we have proposed that a peak of androgen production occurs in the developing lung during the surge of surfactant synthesis to play a role in cell reprogramming [2]. The present study suggests that HSD17B2 may also participate in these endocrine functions by regulating the intracellular levels of AR ligands in the developing lung of both sexes. This assumption is favored by co-expression of AR and HSD17B2 in both epithelial and mesenchymal cells of the fetal lung.

In the human placenta, endothelial cells are the exclusive site of HSD17B2 expression [34–36]. This is compatible with a specialized function. It is not yet determined whether HSD17B2 has specialized function(s) in the developing lung. In addition to type II pneumonocyte maturation, other mechanisms would be influenced by HSD17B2 activity. It was reported that androgens stimulate cell proliferation and programmed cell death during branching morphogenesis [37]. Therefore, HSD17B2 could be expressed in late gestation to control these processes.

Our results support the hypothesis that fine tuning of AR occupancy is regulated by HSD17B2. The wide variety of cell types expressing HSD17B2 suggests that androgens would influence not only lung maturation but also other developmental events in late gestation.

Disclosure statement

The authors of this manuscript have nothing to disclose.

Acknowledgments

The authors thank Dr. Pei Min Rong for excellent technical assistance, Marc Cloutier for red oil staining experiment, Stéphanie Cloutier for manuscript edition, and Dr. Tommy Seaborn for critical reading of the manuscript. This work was supported by grants from the Canadian Institutes of Health Research (CIHR) and Natural Sciences and Engineering Research Council of Canada (NSERC) to YT. MS was supported by a Ph.D. studentship award from the FRSQ-RSR.

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