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Androgen receptor and androgen-dependent gene expression in lung

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ABSTRACT

The androgen receptor (AR) mediates the effects of male sex steroids. There are major sex differences in lung development and pathologies, including lung cancer. In this report, we show that *Ar* is mainly expressed in type II pneumocytes and the bronchial epithelium of murine lung and that androgen treatment increases AR protein levels in lung cells. Androgen administration altered significantly murine lung gene expression profiles; for example, by up-regulating transcripts involved in oxygen transport and down-regulating those in DNA repair and DNA recombination. Androgen exposure also affected the gene expression profile in a human lung adenocarcinoma-derived cell line, A549, by up- or down-regulating significantly some 200 transcripts, including down-regulation of genes involved in cell respiration. Dexamethasone treatment of A549 cells augmented expression of transcript sets that overlapped in part with those up-regulated by androgen in these cells. Moreover, a human lung cancer tissue array revealed that different lung cancer types are all AR-positive. Our results indicate that adult lung is an AR target tissue and suggest that AR plays a role in lung cancer biology.

1. Introduction

Lung alveoli comprise two epithelial cell types, type I and type II pneumocytes (PTI and PTII, respectively) that enable gaseous exchange. PTI cells form the majority of the epithelium, and while PTII cells account for only about 15% of peripheral lung cells (Stone et al., 1992), they serve as stem cells that produce new PTI and PTII cells and have other specialized functions. For instance, PTII cells secrete surfactant to reduce the surface tension of the alveoli and decrease the work of breathing by preventing the alveoli from collapsing. Glucocorticoid receptor (GR) is essential in promoting differentiation of PTII cells during embryonic life (Ballard, 1989; Gonzales et al., 2001, 2002) and, antenatal glucocorticoid administration expedites lung maturation in infants at risk of preterm delivery (Roberts and Dalziel, 2006), largely through increased surfactant protein expression (Ballard, 1989).

Androgen receptor (AR) mediates the effects of male sex steroids in a variety of reproductive and non-reproductive tissues both in males and females under physiological and pathophysiological conditions (Dehm and Tindall, 2006; Heemers and Tindall, 2007; Heinlein and Chang, 2002, 2004). Lungs of male fetuses develop more slowly than those of females, and males are more prone to neonatal respiratory distress syndrome due to reduced number of PTII cells and lack of surfactant (Perelman et al., 1986). The AR has been studied in the developing human and murine lungs, and it has been shown to influence branching morphogenesis (Kimura et al., 2003) and to attenuate PTII cell maturation (Dammann et al., 2000; Provost et al., 2000). During development, there are several differentially expressed genes in the lungs of female and male mouse fetuses, including apolipoproteins that may be involved in local lipid metabolism and transport related to surfactant lipid synthesis (Simard et al., 2006). However, the effect of androgens on the gene expression profile in adult lung has not been assessed.

Lung cancer is a disease with a clear sex difference. Female patients show better survival rates than males at any stage of the disease (Fu et al., 2005). Histological subtypes of the disease in women include proportionally more adenocarcinoma and less squamous cell carcinoma than men (Chen et al., 2005; Fu et al., 2005). Overall, men appear to have a higher rate of fatal outcome of lung cancer, but tend to be less vulnerable to tobacco carcinogens than women (International Early Lung Cancer Action Program Investigators et al., 2006). It remains to be elucidated whether these differences result from men having lower circulating female sex steroid (estrogen and progesterone) or higher androgen levels than women, or from some as yet unknown confounding associations.

There is a previous report on the presence of AR in adult human lung (Wilson and McPhaul, 1996), but very little is known about functional importance of AR and androgen signaling in lung physiology after embryonal development. To address

Abbreviations: AR, androgen receptor; ARE, androgen response element; ChIP, chromatin immunoprecipitation; Dex, dexamethasone; FBS, fetal bovine serum; GO, gene ontology; GR, glucocorticoid receptor; PTI and PTII, type I and type II pneumocytes, respectively; T, testosterone.

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these issues, we have examined expression of the *Ar* gene and searched for androgen-dependent target genes in adult murine lung. Androgen-treated A549 cells, representing a transformed human PTII adenocarcinoma cell line, were used to compare androgen-dependent gene expression profiles in human cells with those in murine lung. Since androgens and glucocorticoids are known to regulate expression of the same genes, such as the *prostate-specific antigen* gene (Cleutjens et al., 1997; Thompson et al., 2006), the A549 cells were also employed to examine similarities and differences between gene expression profiles regulated by androgens and glucocorticoids through the AR and GR, respectively.

2. Materials and methods

2.1. Mice and hormone treatments

Wild-type FVB mice were used. One week after castration, one group of male mice received androgen for 5 days [1 mg testosterone (T)/mouse/day in 0.1 ml of mineral oil as sc injections, corresponding to 30 μg T/g of body weight], whereas the control group received only vehicle. The dose of the androgen was based on similar previous experiments with mice (Pajunen et al., 1982). Animals were sacrificed on day 6, and the lungs were collected for RNA and protein isolation as well as for immunohistochemical analysis. The same hormone treatment (30 μg T/g of body weight for 5 days) was given in some experiments to intact females. All results originate from a minimum of three biological replicate experiments. All the experiments were performed according to the guidelines for animal experiments at the University of Helsinki and under the license from appropriate Finnish Review Boards for Animal Experiments.

2.2. Cells

Lung adenocarcinoma-derived A549 cells were purchased from American Type Culture Collection (Manassas, VA) and maintained in F-12K Nutrient Mixture, Kaighn's Modification (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS). This cell line originates from explant culture of lung carcinomatous tissue from a Caucasian male. For hormone exposure experiments, cells were cultured in charcoal-stripped 10% FBS for 24 h and then treated either with 100 nM T or 1 μM dexamethasone (Dex) in charcoal-stripped 2% FBS for 24 h. The hormone concentrations used were similar to those used by other investigators (So et al., 2007b; Wang et al., 2007). All results are representative of at least two independent experiments.

2.3. Protein isolation and immunoblotting

Proteins were isolated from cells or homogenized tissues using a buffer containing 50 mM Tris–HCl (pH 7.8), 300 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 10 mM N-ethylmaleimide (Sigma–Aldrich, St. Louis, MO), and 1× complete protease inhibitor set (Roche Diagnostics, Indianapolis, IN). Lysates were centrifuged, and soluble protein concentration was quantified with the BioRad protein assay. Protein samples (50 μg or 30 μg) were resolved on a 10% SDS-PAGE gel and transferred onto ECL membrane (GE Healthcare, Buckinghamshire, England). Immunoblotting was performed with rabbit polyclonal IgG against AR (sc-816, Santa Cruz Biotechnology Inc., Santa Cruz, CA) or rabbit polyclonal IgG against GR (sc-1004, Santa Cruz Biotechnology) or mouse monoclonal α-tubulin antibody (sc-5286, Santa Cruz Biotechnology). Horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG was used as secondary antibody. ECL reagent (GE Healthcare) was used for visualization. Band intensities were quantified using the Kodak 1D Scientific Imaging Systems version 3.5.3.

2.4. Immunohistochemistry

Lung and prostate tissue samples were fixed immediately after dissection in 4% paraformaldehyde at 4 °C overnight, dehydrated and embedded in paraffin. Five micrometers sections were mounted onto Superfrost Plus slides (Menzel GmbH, Braunschweig, Germany), dewaxed with xylene, rehydrated and the endogenous peroxidase activity blocked by incubation in 3% hydrogen peroxide for 15 min. Antigen retrieval was carried out by boiling the slides in 10 mM sodium citrate (pH 6.0). The slides were subsequently blocked with 1% bovine serum albumin (Sigma–Aldrich) and normal goat serum (Vector Laboratories, Burlingame, CA), and incubated with antibodies against AR (1:1000 dilution, sc-816, Santa Cruz Biotechnology), GR (1:2000 dilution, sc-1004, Santa Cruz Biotechnology) or hemoglobin β/γ/δ (1:100 dilution, sc-21006, Santa Cruz Biotechnology) overnight at 4 °C. The slides were then incubated with biotinylated anti-rabbit IgG secondary antibody as a 1:200 dilution (Vector Laboratories). Negative controls with normal rabbit IgG (sc-2027, Santa Cruz Biotechnology) and in the absence of the primary antibody were included in each experiment. The immune complexes were visualized using the Vectastain Elite ABC and DAB substrate kits (Vector Laboratories). A set of slides were counterstained using Mayer's hemalum solution (Merck, Darmstadt, Germany). The slides were dehydrated and mounted using Permount (Fisher Chemicals, Fair Lawn, NJ).

2.5. Tissue array

A206 (Lung Cancer Tissues) AccuMax Array containing total 84 spots including different types of lung carcinoma was purchased from Petagen (Seoul, Korea). The array was immunostained with anti-AR-antibody (sc-816, Santa Cruz Biotechnology) as described above. Another polyclonal AR antibody raised against full-length human receptor (AR3, Thompson et al., 2006) was used to confirm the results.

2.6. RNA isolation and blotting

Total RNA was isolated from cells or homogenized tissues using TRIzol reagent (Invitrogen) or Nucleospin RNA II kit (Macherey-Nagel GmbH, Düren, Germany) according to the manufacturers' instructions. Ten micrograms of total RNA from different mouse tissues were resolved on a 1.2% denaturing agarose gel and transferred onto a Hybond XL nylon membrane (GE Healthcare). The blot was hybridized with a ^{32}P-labeled anti-sense RNA probe corresponding to nucleotides 1301–1655 of *Ar* cDNA (NM_013476), generated by using Riboprobe Synthesis II kit (Promega, Madison, WI). Hybridization was carried out at 68 °C in Ultrahyb buffer (Ambion, Austin, TX) overnight. The membrane was washed in 2× SSC, 0.1% SDS and 0.1× SSC, 0.1% SDS and exposed to Fuji X-ray film at −70 °C.

2.7. Microarray analysis

The quality of RNA was assessed by Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) prior to being subjected to microarray experiments, and the RNA integrity number was >8 for each sample. Five micrograms of total RNA samples from lungs of T-treated castrated male mice ($n = 3$), vehicle-treated castrated male mice ($n = 3$), intact male mice ($n = 3$), intact female mice ($n = 3$), non-treated A549 cells ($n = 2$), T-treated A549 cells ($n = 2$) or Dex-treated A549 cells ($n = 2$) were amplified and labeled to produce cRNA using the Low RNA Input Linear Amp Kit PLUS, Two-Color (Agilent). cRNA was hybridized onto G4122F Whole Mouse Genome chips or G4112F Whole Human Genome chips (Agilent) comprising over 40 000 mouse or human genes and transcripts, respectively, according to the manufacturer's instructions. The chips were scanned with Agilent DNA microarray scanner and Feature Extraction Software using Linear and Lowess normalizations. Each chip compared the relative abundance of the transcripts (*i.e.*, abundance differences in the sample pairs) in either T- and vehicle-treated lungs (G4122F chips), intact male and female lungs (G4122F chips), T- and vehicle-treated A549 cells (G4112F chips) or Dex- and vehicle-treated A549 cells (G4112F chips).

Microarray results were analyzed with Genespring GX 7.3.1 (Agilent). Data were normalized per spot and per chip as recommended by the manufacturer, and spots flagged as absent in more than half of the samples were removed. Transcripts with fold-change >1.8 and Student's *t*-test *p*-value <0.05 were considered significantly differentially expressed. Genes that were reported by several spots have been kept separate and may appear more than once in the lists. Moreover, the transcripts that did not have a current annotation were removed from the final lists. The resulting lists were analyzed further with Webgestalt (http://bioinfo.vanderbilt.edu/webgestalt) by organizing the lists into Gene Ontology (GO) categories and comparing the categories with those expected by random selection from the whole genome. Original microarray data sets are available at www.ebi.ac.uk with accession numbers E-MEXP-2001, E-MEXP-2002, E-MEXP-2003, and E-MEXP-2004.

2.8. Quantitative reverse transcription PCR (qRT-PCR)

cDNA was synthesized from 3 μg of total lung RNA samples using random hexamer primers and Superscript III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. qRT-PCR was performed with LightCycler® 480 Real-Time PCR System (Roche Diagnostics) in 20-μl reactions containing LightCycler® 480 SYBR Green I Master (Roche Diagnostics) and 1 μM forward and reverse primers (see Supplemental data, Table S1). PCR reactions included a 5-min denaturation step 95 °C followed by 40 cycles of 10-s denaturation at 95 °C, 5-s annealing at 57–60 °C, 20-s extension at 72 °C and 5-s SYBR Green signal measurement. Melting curve analysis was performed for each product to detect possible non-specific products. The results were analyzed with LightCycler analysis software (Roche Diagnostics). The results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels.

2.9. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed essentially as previously described (Kang et al., 2004; Thompson et al., 2006). Briefly, 10^6 A549 cells were seeded on 10-cm dishes and maintained for 4 days in 10% charcoal-stripped FBS, F-12K Nutrient Mixture medium. The cells were exposed to either 100 nM T or vehicle for 16 h and then cross-linked in 1% formaldehyde. The cells were harvested into lysis buffer and pelleted by centrifugation. Chromatin was sonicated to an average DNA length of 500–800 nt. Sonicated samples were precleared with normal rabbit

IgG and GammaBind G Sepharose (GE Healthcare) and immunoprecipitated with AR antiserum (sc-816, Santa Cruz Biotechnology). Antibody-bound complexes were adsorbed to GammaBind G Sepharose beads that were sequentially washed with TSE I, TSE II, TSE III, and TE buffers (Kang et al., 2004). DNA was eluted from the matrix with 1% SDS in 0.1 M $NaHCO_3$, cross-linking was reverted at 65 °C overnight, and DNA isolated using QIAquick PCR purification system (Qiagen GmbH, Hilden, Germany). Input samples were treated the same way except that no immunoprecipitation was performed. qRT-PCR was performed with LightCycler 480 System in 20-μl reactions containing LightCycler 480 SYBR Green I Master and 1 μM forward and reverse primers for the genomic regions examined (Supplemental data, Table S1). PCR reactions included a 5-min denaturation step at 95 °C, followed by 40 cycles of 10-s denaturation at 95 °C, 5-s annealing at 60 °C, 5-s extension at 72 °C and 5-s SYBR Green signal measurement. The immunoprecipitated samples were normalized to input values.

3. Results

3.1. Lung cell expression of the Ar gene

Ar mRNA was detected in hybridization blots of total murine lung RNA as a distinct ~10-kb band. The presence of *Ar* mRNA size heterogeneity has been reported previously (Tilley et al., 1990; Shan et al., 1990), with two mRNA species of ~10 kb and ~8 kb in size. This latter *Ar* mRNA species was barely detectable in murine lung but corresponded to the principal *Ar* mRNA from in brain (Fig. 1A). The abundance of *Ar* mRNA in lung was comparable to that in brain, but less than in kidney or prostate. This result was confirmed with RT-PCR (data not shown). Five-day T treatment increased AR protein levels in lungs of both castrated males and intact females, as revealed by immunoblotting of tissue extracts (Fig. 1B), and the amount of AR in the castrated male lung appeared to be higher than that in female lung. Likewise, lung AR protein level was lower in castrated than in intact male mice (Fig. 1C and D). Up-regulation of AR levels either by physiological or pharmacological amounts of androgen is likely to be due to ligand-dependent stabilization of AR, as has been found in other AR-containing tissues or cells (Shan et al., 1990; Kemppainen et al., 1992; Zhou et al., 1995; Mora et al., 1996). In support to this notion, 5-day T treatment failed to alter significantly *Ar* mRNA levels in murine lung (results not shown). As reported previously (Wollmer et al., 1996), we detected AR protein in a human cancer cell line derived from PTII cells (the A549 cell line), and upon androgen exposure, A549 cell AR protein level was increased in a fashion similar to that in murine lung (Fig. 1E).

AR antigen was detected by immunohistochemistry in nuclei of murine PTII and bronchial epithelial cells of intact male mice (Fig. 2A). In these experiments, a murine prostate sample served as a positive control, and showed clear staining of the epithelial cell nuclei (Supplemental Fig. S1). In comparison, GR immunoreactivity exhibited a more universal distribution than that of AR in

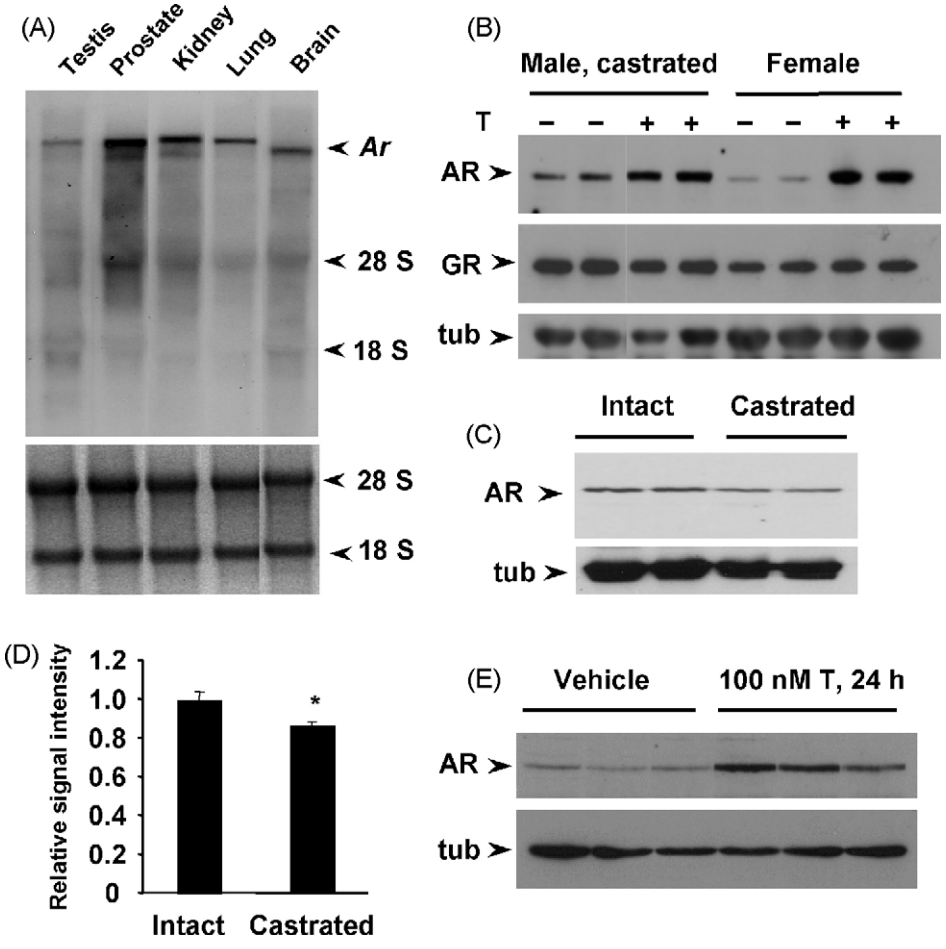

Fig. 1. Androgen receptor mRNA and protein expression in the lung. (A) Ten micrograms of total RNA from different mouse tissues were resolved on a 1.2% formaldehyde denaturing agarose gel, transferred onto a nylon membrane and hybridized with a ^{32}P-labeled *Ar* cRNA probe as described in Section 2. (B) Immunoblot analysis of AR protein in murine lung. Female or castrated male mice were treated testosterone (T) for 5 days; control animals received vehicle only. Soluble lung proteins (50 μg) were resolved by SDS-PAGE gel electrophoresis and immunoblotted using antibodies against AR or GR. (C) AR protein levels in the lung of intact and castrated male mice. Soluble protein extracts (50 μg protein) were resolved by SDS-PAGE and immunoblotted using antibodies against AR. (D) The bands of the immunoblot shown in panel C were quantified. The columns represent AR protein values normalized to α-tubulin levels, and the asterisk indicates a p-value < 0.05. (E) A549 cells were treated with 100 nM testosterone (T) for 24 h. Total protein extracts (50 μg) were resolved on an SDS-PAGE and immunoblotted with an antibody against AR. α-Tubulin level was used to control for equal protein loading.

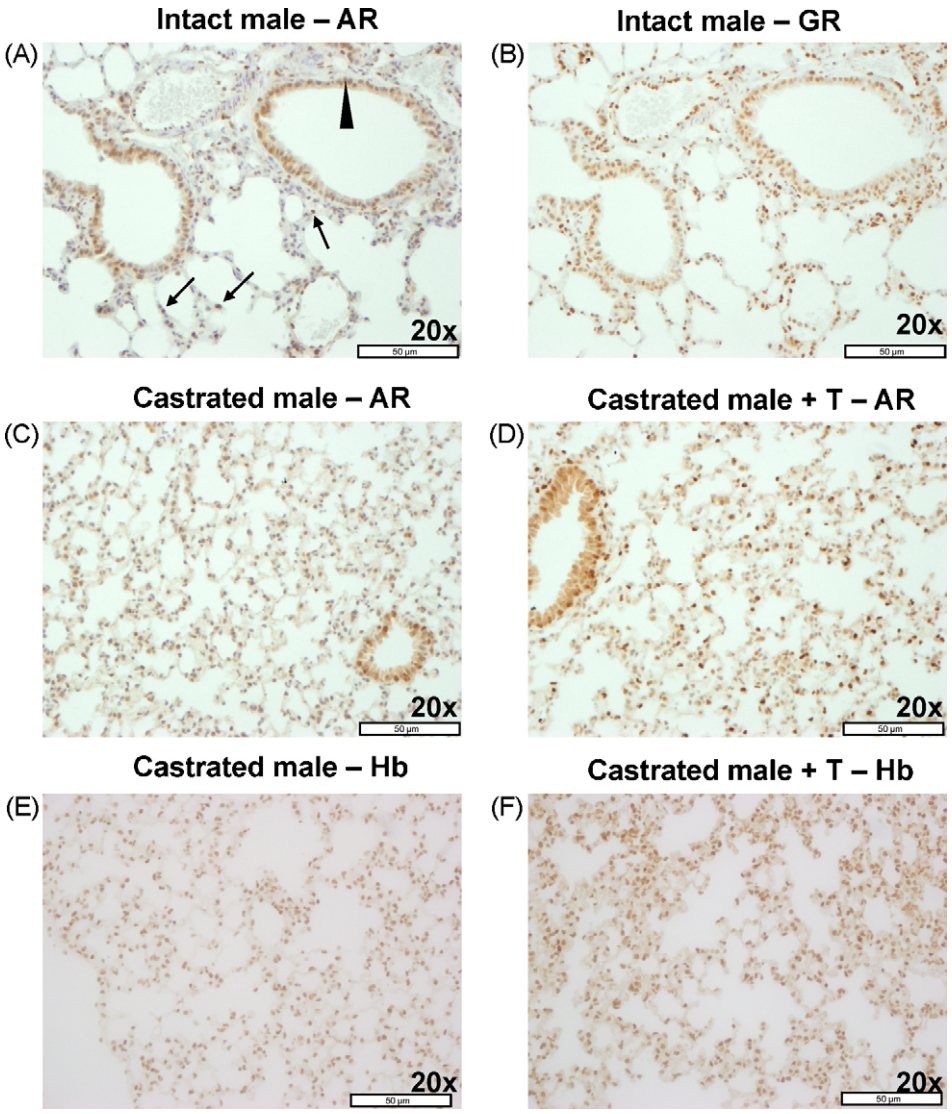

Fig. 2. Localization of AR, GR and hemoglobin in lung cells. (A and B) Serial lung sections of male mice were immunostained with an antibody against AR (A) or GR (B) to show localization of AR to type II pneumocytes and bronchial epithelium, and the ubiquitous presence of GR. (C and D) Influence of androgen treatment on AR localization. Castrated mice were given vehicle (C) or testosterone for 5 days (D). (E and F) Influence of androgen treatment on hemoglobin immunostaining. Tissue sections from lungs of castrated (E) or androgen-treated (F) male mice were immunostained with antibody against hemoglobin β/γ/δ. Panels (A) and (B) were counterstained with hematoxylin. Scale bar = 50 μm.

the same tissue sections, and all lung cell types expressed GR antigen (Fig. 2B). Upon androgen treatment of castrated male mice, AR immunostaining of the lung became more intense, but the subcellular localization of AR antigen did not change significantly (Fig. 2C and D), whereas the intensity of GR immunostaining was independent of androgen exposure (data not shown).

3.2. Androgen treatment affects gene expression profile in murine lung

In search of androgen-regulated genes in murine lung, we performed gene expression profiling using the Agilent Whole Mouse Genome platform. By using the cut-off values of fold-change >1.8 and *p*-value <0.05, we identified 46 up-regulated and 134 down-regulated transcripts in lung of castrated male mice after T treatment (Supplemental data, Table S2). To infer the biological processes potentially regulated by androgens, the genes were grouped into gene ontology (GO) categories. These categories were compared with the expected ones by using the Webgestalt mouse as a reference gene set, and the hypergeometric test was used to calculate the *p* values. Among the enriched categories in the up-regulated genes were those involved in oxygen transport (4 genes, $p = 3.59e{-8}$), negative regulation of apoptosis (3 genes, $p = 3.79e{-3}$), hemopoiesis (3 genes, $p = 8.02e{-3}$) and heme biosynthesis (2 genes, $p = 2.14e{-4}$). The down-regulated genes belonged to categories such as DNA repair (5 genes, $p = 6.65e{-3}$), DNA recombination (3 genes, $p = 9.19e{-3}$), and double-strand break repair (2 genes $p = 4.20e{-3}$) (Table 1). Quantitative RT-PCR was used to confirm expression levels of a few selected transcripts, and all transcript levels quantified by qPCR were in good agreement with the microarray results (Supplemental data, Table S3).

To examine sexual dimorphism in lung gene expression and to search for genes potentially regulated by physiological androgen concentrations, gene expression profiling was performed on male and female lungs. With the same cut-off values as above (fold-change >1.8 and *p*-value <0.05), we identified 28 up-regulated and 99 down-regulated transcripts in male lungs as compared to female lungs (Supplemental data, Table S4). Some of the up-regulated genes in males belonged to muscle development (3 genes, $p = 2.28e{-4}$) and muscle contraction (6 genes, $p = 3.78e{-11}$). The

Table 1
Enriched gene ontology (GO) categories in murine lung upon T treatment.

GO category	*p*-Value (GO)	Gene symbol	Common name	Entrez ID	Fold change	*p*-Value
Up-regulated genes						
Oxygen transport	3.59e−08	Hbb-b1	Hemoglobin, beta adult major chain	15129	3.20	0.0276
		Hbb-y	Hemoglobin Y, beta-like embryonic chain	15135	2.00	0.0330
		Hbq1	Hemoglobin, theta 1	216635	1.86	0.0028
		Mb	Myoglobin	17189	1.86	0.0059
Negative regulation of apoptosis	3.79e−03	Angptl4	Angiopoietin-like 4	57875	2.02	0.0075
		Tsc22d3	TSC22 domain family 3	14605	1.86	0.0088
		Fgf8	Fibroblast growth factor 8	14179	1.84	0.0182
Iron ion binding	5.93e−06	Hbb-b1	Hemoglobin, beta adult major chain	15129	3.20	0.0276
		Rsad2	Radical S-adenosyl methionine domain containing 2	58185	2.40	0.0007
		Fech	Ferrochelatase	14151	2.38	0.0013
		Slc25a37	Solute carrier family 25, member 37	67712	2.18	0.0034
		Hbb-y	Hemoglobin Y, beta-like embryonic chain	15135	2.00	0.0330
		Hbq1	Hemoglobin, theta 1	216635	1.86	0.0028
		Mb	Myoglobin	17189	1.86	0.0059
Heme biosynthesis	2.14e−04	Alas2	Aminolevulinic acid synthase 2, erythroid	11656	2.50	0.0023
		Fech	Ferrochelatase	14151	2.38	0.0013
Down-regulated genes						
DNA repair	6.65e−03	Brca2	Breast cancer 2	12190	−2.08	0.0049
		Msh6	mutS homolog 6 (*E. coli*)	17688	−1.90	0.0269
		Pold3	Polymerase (DNA-directed), delta 3, accessory subunit	67967	−1.90	0.0277
		H2afx	H2A histone family, member X	15270	−1.82	0.0219
		Rev3l	REV3-like, catalytic subunit of DNA polymerase zeta RAD54 like (*S. cerevisiae*)	19714	−1.81	0.0095
DNA recombination	9.19e−03	Brca2	Breast cancer 2	12190	−2.08	0.0049
		Msh6	mutS homolog 6 (*E. coli*)	17688	−1.90	0.0269
		H2afx	H2A histone family, member X	15270	−1.82	0.0219
Double-strand break repair	4.20e−03	Brca2	Breast cancer 2	12190	−2.08	0.0049
		H2afx	H2A histone family, member X	15270	−1.82	0.0219
Response to stress	1.73e−02	Brca2	Breast cancer 2	12190	−2.08	0.0049
		C1s	Complement component 1, s subcomponent	50908	−2.02	0.0044
		Hsp90ab1	Heat shock protein 90 kDa alpha (cytosolic), class B member 1	15516	−1.90	0.0227
		Msh6	mutS homolog 6 (*E. coli*)	17688	−1.90	0.0269
		Pold3	Polymerase (DNA-directed), delta 3, accessory subunit	67967	−1.90	0.0277
		Fn1	Fibronectin 1	14268	−1.86	0.0171
		Txnip	Thioredoxin interacting protein	56338	−1.85	0.0061
		Lta4h	Leukotriene A4 hydrolase	16993	−1.85	0.0158
		H2afx	H2A histone family, member X	15270	−1.82	0.0219
		Rev3l	REV3-like, catalytic subunit of DNA polymerase zeta RAD54 like (*S. cerevisiae*)	19714	−1.81	0.0095

Agilent Whole Mouse Genome expression microarray was used to compare gene expression in lungs of castrated male mice treated with testosterone for 5 days to those of vehicle-treated animals. The GO categories are shown for transcripts that were differentially expressed (fold-change >1.8, *t*-test *p*-value <0.05).

down-regulated genes included genes involved in acute inflammatory response (3 genes, $p = 6.05e{-}3$) and regulation of translation (2 genes, $p = 4.58e{-}2$) (Table 2). As could have been expected, Y chromosome-encoded transcripts were highly overrepresented in the male samples. These included genes such as *Eif2s3y*, *Ddx3y* and *Uty*. *Xist*, the X inactive specific transcript in females, was at the top of the list of down-regulated genes in males.

The gene lists covering the two data sets – T-treated lungs *vs*. non-treated lungs of castrated male mice, and intact male *vs*. intact female lungs – exhibited very limited overlap. There were 7 overlapping down-regulated transcripts that included 6 genes: *Kcnj8*, *Txk*, *D0H4S114*, *Hsd17b12*, *BC002199*, and *Tmem46*. The up-regulated lists did not overlap when the cut-off was set at fold change of 1.8. However, when the cut-off value was relaxed to fold change of 1.5, the lists of up-regulated genes overlapped by 9 transcripts (7 genes: *Myl7*, *Hrc*, *Tnnt2*, *Mb*, *Fgl1*, *Cdh2* and *Junb*).

Gene expression profiling experiments revealed androgen-dependent up-regulation of several hemoglobin-related transcripts (*Hbb-b1*, *Hbb-y*, *Hbq1*, *Mb*) in the murine lung. To examine whether up-regulation was due to increased expression of these genes by lung cells or increased blood cell retention in lung as a consequence of T administration, we performed immunohistochemical analyses with an antibody recognizing several hemoglobin variants (β, γ and δ) on murine lung tissue sections. This antibody stained primarily the nuclei of all lung cell types, and T treatment appeared to bring about an increase in signal intensity within these cells (Fig. 2E and F).

3.3. AR is present in normal and malignant human lung cells

Lung carcinoma tumors and normal adjacent tissue showed AR immunoreactivity in normal lung, and all lung cancer types within the array included at least a few samples that were clearly AR-positive (Fig. 3). AR staining was mainly nuclear, although diffuse cytoplasmic staining was also present in many samples. The tissue array comprised samples from adenocarcinoma (7 AR-positive of 15 samples), squamous cell carcinoma (11/15), bronchioloalveolar carcinoma (2/3), large cell neuroendocrine carcinoma (2/2), large

Table 2
Enriched gene ontology (GO) categories between intact male and female mouse lung.

GO category	*p*-Value (GO)	Gene symbol	Common name	Entrez ID	Fold change	*p*-Value
Up-regulated genes						
Muscle contraction	3.78e−11	Ttn	Titin	22138	2.41	0.0497
		Ttn	Titin	22138	2.171	0.0014
		Atp2a2	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	11938	2.059	0.0021
		Myh8	Myosin, heavy polypeptide 8, skeletal muscle, perinatal	17885	2.038	0.0041
		Myl7	Myosin, light polypeptide 7, regulatory	17898	2.017	0.0020
		Myh6	Myosin, heavy polypeptide 6, cardiac muscle, alpha	17888	1.978	0.0027
		Tnnt2	Troponin T2, cardiac	21956	1.878	0.0039
Muscle development	2.28e−04	Myh6	Myosin, heavy polypeptide 6, cardiac muscle, alpha	17888	1.978	0.0027
		Ttn	Titin	22138	2.41	0.0497
		Ttn	Titin	22138	2.171	0.0014
		Tcap	Titin-cap	21393	1.957	0.0034
Down-regulated genes						
Acute inflammatory response	6.05e−03	Saa3	Serum amyloid A 3	20210	−3.12	0.0011
		Ptger3	Prostaglandin E receptor 3 (subtype EP3)	19218	−1.85	0.0082
		C1qa	Complement component 1, q subcomponent, alpha polypeptide	12259	−1.81	0.0041
Regulation of translation	4.58e−02	Eif2s3x	Eukaryotic translation initiation factor 2, subunit 3, structural gene X-linked	26905	−2.06	0.0043
		Ireb2	Iron responsive element binding protein 2	64602	−1.82	0.0139
Enzyme linked receptor protein signaling pathway	3.11e−02	Plat	Plasminogen activator, tissue	18791	−2.05	0.0041
		Angpt1	Angiopoietin 1	11600	−1.93	0.0073
		D0H4S114	DNA segment, human D4S114	27528	−1.93	0.0054
		Bmp5	Bone morphogenetic protein 5	12160	−1.86	0.0121
Inorganic anion transport	4.25e−02	Fcna	Ficolin A	14133	−2.06	0.0033
		Ptger3	Prostaglandin E receptor 3 (subtype EP3)	19218	−1.85	0.0082
		C1qa	Complement component 1, q subcomponent, alpha polypeptide	12259	−1.81	0.0041

Agilent Whole Mouse Genome expression microarray was used to compare gene expression in the lung tissue of intact male and female mice. The GO categories are shown for transcripts that were differentially expressed (fold-change >1.8, *t*-test *p*-value <0.05).

cell carcinoma (2/3), and small cell carcinoma (2/2). The staining of the tumor was not dependent on the sex of the patient. All normal lung tissue specimens in the array exhibited clear AR immunoreactivity, but the signal intensity varied among the samples. An other AR antibody (Thompson et al., 2006) was used to confirm the immunostaining results.

3.4. Androgen exposure affects gene expression of A549 cells

To study the effects of T on cultured human lung cancer cells, we profiled gene expression in A549 cells with or without T treatment. Androgen exposure for 24 h resulted in up-regulation of 126 transcripts, but only 62 of these were annotated as transcripts encoded by known genes (Supplemental data, Table S5). Of the known AR target genes, the list included *TMPRSS2* and *EPB41L4B* (Bolton et al., 2007; Wang et al., 2007). The genes belonged to the following GO categories: phospholipid metabolism (2 genes, $p = 0.03$), iron ion binding (3 genes, $p = 0.046$), oxygen binding (2 genes, $p = 0.0057$), heme binding (3 genes, $p = 0.0028$) and clathrin-coated vesicle (2 genes, $p = 0.03$) (Supplemental data, Table S6). T elicited down-regulation of 111 transcripts, 98 of which corresponded to currently known genes (Supplemental data, Table S5), and these included the following GO categories: cellular respiration (3 genes, $p = 0.0006$), metabolism (44 genes, $p = 0.009$), mitochondrion (8 genes, $p = 0.008$) and clathrin coat of trans-Golgi network vesicle (3 genes, $p = 0.00004$). Among the enriched categories of down-regulated genes was also regulation of apoptosis (6 genes, $p = 0.008$); these genes included both negative regulation (3 genes, $p = 0.02$) and positive regulation of apoptosis (4 genes, $p = 0.009$) (Supplemental data, Table S6). Unlike mouse lung *in vivo*, expression of hemoglobin genes was not significant up-regulated by androgen in A549 cells. This result may relate to the fact that expression levels of all hemoglobin genes were extremely low in A549 cells.

AR regulates gene expression through canonical androgen response elements (AREs) and non-canonical AREs (Wang et al., 2007), and class I nuclear receptors, such as AR, GR, progesterone receptor, and mineralocorticoid receptor, may recognize the same canonical response elements (for a review, see Verrijdt et al., 2003). It was, therefore, of interest to expose A549 cells to Dex and compare AR and GR target genes in the context of a single cell line. As expected, more A549 transcripts were regulated by Dex than T exposure. Likewise, Dex responses were more robust than those with T. With the cut-off value of 2-fold change, Dex up-regulated the abundance of 1510 transcripts (compared to 74 with T at the same fold change), and 1285 transcripts were down-regulated by Dex (compared to 56 with T). Nevertheless, there was some overlap in transcript regulation, in that 40 transcripts were common for T and Dex among the up-regulated genes, whereas only 11 common ones were among the down-regulated genes (Fig. 4). Among the most Dex-responsive genes [>3-fold up-regulation (510 transcripts corresponding to 416 known genes); Supplemental data, Table S7] were those involved in lipid metabolism (23 genes, $p = 9.8e{-}5$), suggesting a possible increase in surfactant production (Supplemental data, Table S8). A few of the surfactant-related genes were up-regulated in Dex-treated cells, such as *SFTPD* (10.4-fold), *SFTPG* (3.7-fold), and *PGC* (12.2-fold). Some other genes encoding surfactant-associated proteins were expressed at very low

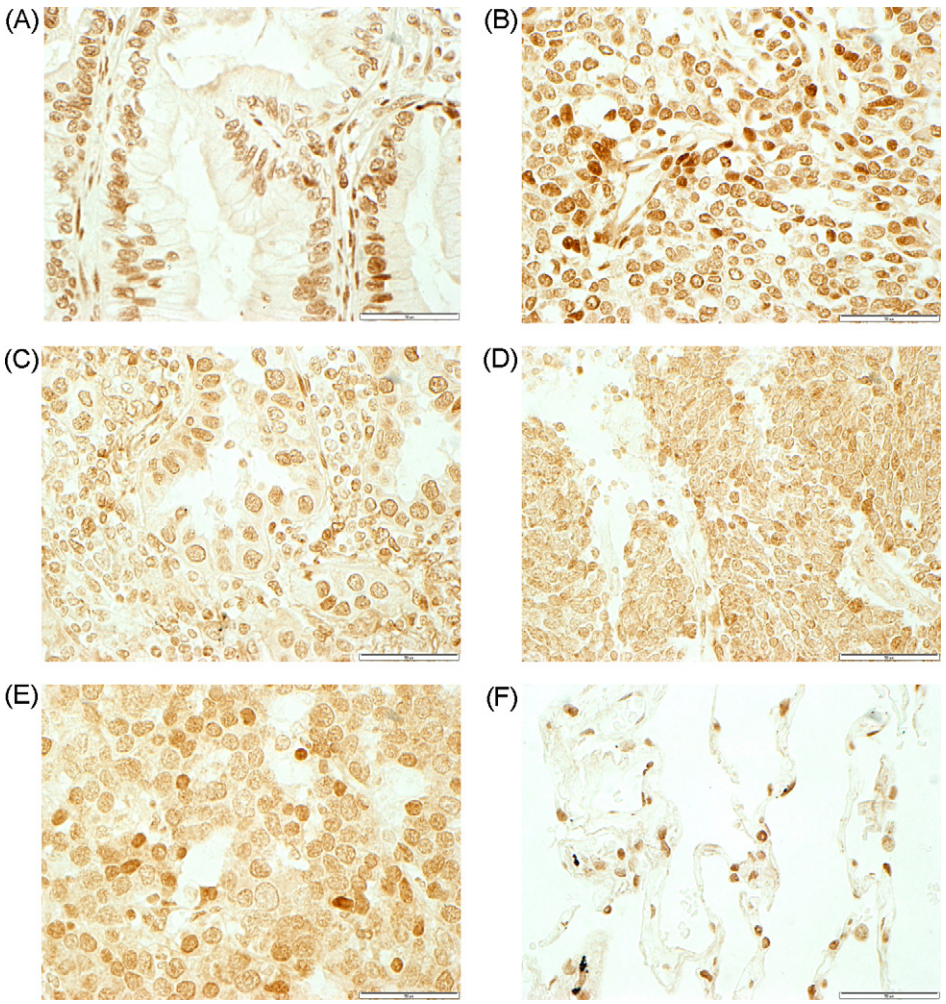

Fig. 3. Immunoreactive AR in lung cancer specimens. Shown are examples of AR-positive sections from a human lung cancer tissue array comprising 80 spots from 40 lung cancer specimens and 4 non-neoplastic spots. The array was immunostained with AR antibody. The pictures show examples of immunoreactive spots from adenocarcinoma (A), squamous cell carcinoma (B), bronchioloalveolar carcinoma (C), small cell carcinoma (D), large cell carcinoma (E), and normal human lung tissue (F). Scale bar = 50 μm.

abundance and were, therefore, flagged in the analysis as being absent. Genes down-regulated by Dex (225 transcripts, 209 known genes) included those in stress response (15 genes, $p = 7e{-}3$), DNA metabolism (19 genes, $p = 5e{-}5$), and mitosis (12 genes, $p = 8e{-}8$) (Supplemental data, Table S8).

The transcripts that were up-regulated at least 2-fold by both T and Dex included several interesting genes that are listed in Table 3. One of the genes listed in this table, *TMPRSS2*, is known to harbor an androgen-responsive enhancer at ~14 kb of transcription start site (Wang et al., 2007). The *TMPRSS2* locus plays also an important role in prostate cancer biology, as it is a common place for translocation of genes encoding members of the ETS transcription factor family (Tomlins et al., 2005). In view of this, it was of interest to observe that, in A549 cells, there was androgen-dependent loading of AR onto the *TMPRSS2* enhancer, as revealed by ChIP assays (Fig. 5). There is no other information on potential AR-binding sites in the regulatory regions of androgen-regulated genes in A549 cells. However, our ongoing experiments on LNCaP-1F5 prostate cancer cell line have employed ChIP with AR antibody followed by ultrahigh-throughput parallel sequencing (ChIP-seq) to delineate genome-wide AR-binding sites on chromatin (our unpublished observations). Even though this AR-binding site information originates from a different cell line, it was still pertinent to examine their occurrence in androgen-regulated genes of the A549 cells. We found that 39% of androgen up-regulated and 36% of down-regulated genes contained putative AR-dinding sites within 200 kb of transcription start sites. On the basis of this analysis, additional ChIP assays were performed on two other loci, an AR-binding site at 21 kb upstream of the *TAS2R50* gene encoding a G-protein coupled taste receptor and a negative control site at 57 kb down-stream of the *PDGFRA* gene. Upon androgen exposure for 16 h, there was significantly increased loading of AR on the former but not onto the latter site (Fig. 5). Up-regulation of *TMPRSS2* and *TAS2R50* mRNA levels upon T treatment was confirmed by qPCR (Supplemental data, Table S3).

4. Discussion

4.1. Localization of AR expression to specific lung cell types

In addition to fetal lung (Dammann et al., 2000; Kimura et al., 2003; Provost et al., 2004), we have now shown that adult lung is an androgen-responsive tissue in both mice and humans, and the AR is expressed predominantly in the bronchial epithelium and PTII cells. The latter cells are important in the maintenance of alveolar epithelium by (i) providing new epithelial cells and (ii) secreting surfactant. Since T delays maturation of PTII cells during embryonic development (Provost et al., 2004), it is not surprising that *Ar* is also expressed in these cells in adult mice. In view of the specific role of PTII cells in surfactant production, AR might

Table 3
Genes differentially expressed by both T and Dex in A549 cells.

Gene symbol	Common name	Entrez gene
Up-regulated genes		
AQP10	Aquaporin 10	89872
CILP2	Cartilage intermediate layer protein 2	148113
CNGB1	Cyclic nucleotide gated channel beta 1	1258
CXorf36	Chromosome X open reading frame 36	79742
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1	1543
CYP2E1	Cytochrome P450, family 2, subfamily E, polypeptide 1	1571
CYP7A1	Cytochrome P450, family 7, subfamily A, polypeptide 1	1581
DKKL1	Dickkopf-like 1 (soggy)	27120
DMRTC1	DMRT-like family C1	63947
DPCR1	Diffuse panbronchiolitis critical region 1	135656
FLJ21438	Hypothetical protein FLJ21438	64926
FOLH1	Folate hydrolase (prostate-specific membrane antigen) 1	2346
FOLR2	Folate receptor 2 (fetal)	2350
GIPR	Gastric inhibitory polypeptide receptor	2696
GYPC	Glycophorin C (Gerbich blood group) (GYPC)	2995
HEMK1	HemK methyltransferase family member 1	51409
HIF3A	Hypoxia inducible factor 3, alpha subunit	64344
KIAA1881	KIAA1881	114782
LOC387761	Hypothetical LOC387761	387761
PLA2G3	Phospholipase A2, group III	50487
PPP1R14C	Protein phosphatase 1, regulatory (inhibitor) subunit 14C	81706
PSORS1C2	Psoriasis susceptibility 1 candidate 2	170680
SEMA6A	Sema domain, transmembrane domain (TM), and cytoplasmic domain (semaphorin) 6A	57556
SERINC2	Serine incorporator 2	347735
SPRR2D	Small proline-rich protein 2D	6703
TAS2R50	Taste receptor, type 2, member 50	259296
TCL1A	T-cell leukemia/lymphoma 1A	8115
TMPRSS2	Transmembrane protease, serine 2	7113
TTC21B	Tetratricopeptide repeat domain 21B	79809
Down-regulated genes		
AK097130	cDNA FLJ39811 fis, clone SPLEN2009581	
FLJ20464	cDNA FLJ20464 fis, clone KAT06158	54944
GINS4	GINS complex subunit 4 (Sld5 homolog)	84296
GRLF1	Glucocorticoid receptor DNA binding factor 1	2909
IGSF11	Immunoglobulin superfamily, member 11	152404
PNMA6A	Paraneoplastic antigen like 6A	84968
SMOC1	SPARC related modular calcium binding 1	64093
TMEM142B	Transmembrane protein 142B	80228
UBXN7	UBX domain protein 7	26043

Agilent Whole Human Genome expression microarray was used to compare gene expression in A549 cells. Genes included in the lists were differentially expressed >2-fold compared to vehicle (*t*-test *p*-value <0.05).

also influence surfactant biosynthesis, but there are no reports to indicate that androgen insensitive *Tfm*/*Y* mice or human androgen insensitive patients have a lung phenotype. Moreover, although lung surfactant composition of *Tfm*/*Y* mice differs from that of wild-type mice during development (Nielsen, 1985), we failed to observe significant androgen-dependent differences in transcripts encoding surfactant proteins in adult lung, between either T-treated and castrated male mice or intact male and female mice.

4.2. Androgen-regulated gene expression in mouse lung and A549 cells

Androgen-dependent genes in the murine lung include those in iron binding and oxygen transport and, in particular, many hemoglobin-related gene transcripts. Hemoglobin genes are expressed, not only in blood cells, but also in cultured PTII and Clara cells (Newton et al., 2006), and cellular hemoglobin is proposed to serve as a protective agent against oxidative and nitrosative stress (Liu et al., 2000; Minning et al., 1999). Androgen treatment not only increased expression of genes involved in oxygen transport, such as those encoding hemoglobins, but it also decreased expression of genes involved in DNA damage repair. It is tempting to speculate that cells with increased protection against oxidative stress would require less DNA repair proteins. Case–control studies have shown that women have an increased risk of lung cancer over that in men with the same consumption of tobacco (Risch et al., 1993; Zang and Wynder, 1996). Interestingly, one of the genes that was most down-regulated in male *vs*. female murine lung was *Cyp1a1* that encodes an enzyme involved in metabolism of polycyclic aromatic hydrocarbons. Increased expression of this enzyme in women has been proposed to play a role in their elevated lung cancer risk (Mollerup et al., 1999).

Several androgen-regulated genes did not respond in lung in the same ways as in some other androgen target tissues. Tissue-specific differences are not surprising, since gene expression depends on chromatin modifications, coactivators and corepressors typical of a given cellular environment. For example, *Igf1* was significantly down-regulated in lung, whereas it is up-regulated in other androgen target tissues (Bolton et al., 2007). In addition, *Angptl4*, a gene that is linked to lung cancer metastasis seeding *via* the TGF-β signaling pathway (Padua et al., 2008), is down-regulated by androgen in prostate cancer cells (Bolton et al., 2007) but up-regulated in murine lung. Up-regulation of the *ANGPTL4* gene has also been linked to hypoxic conditions in human fetal cardiac cells and neonatal rat cardiomyocytes (Belanger et al., 2002). This, together with up-regulated hemoglobin genes, could relate to the ways by which the lung reacts to changing oxygen levels. Another interesting gene that was one of the most androgen down-regulated genes in murine lung is D0H4S114, also known as *P311*, which has a role in distal lung morphogenesis during development,

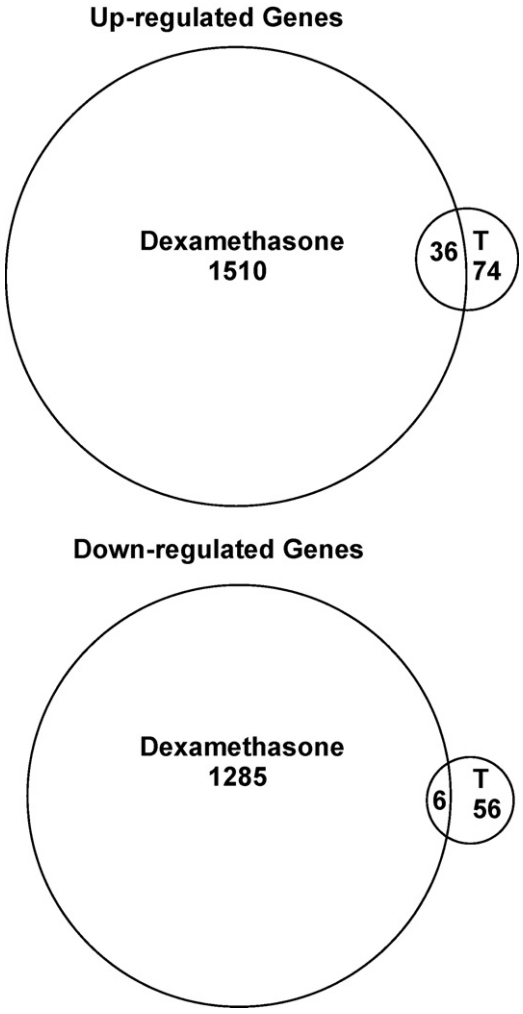

Fig. 4. Comparison of transcript numbers regulated by androgen and glucocorticoid in A549 cells. The cells were treated with 100 nM T, 1 μM Dex or vehicle for 24 h. Gene expression profiling was conducted as described in Section 2. The figures show the overlap of the transcripts that were significantly regulated by either T or Dex.

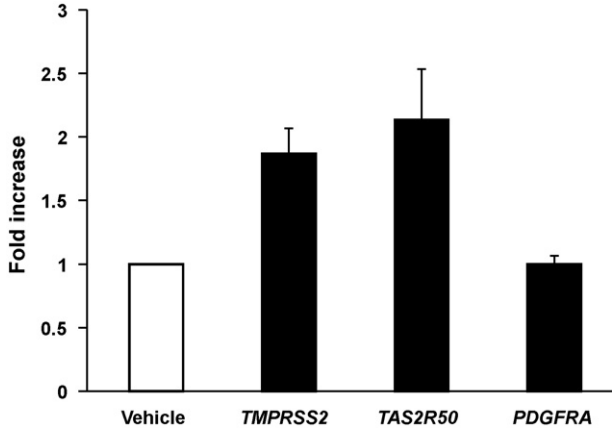

Fig. 5. Loading of AR onto the *TMPRSS2* and *TAS2R50* enhancers in A549 cells upon androgen exposure. The cells were exposed to 100 nM T or vehicle for 16 h. Pre-cleared samples were immunoprecipitated with anti-AR-antibody. qRT-PCR was performed using primers for the *TMPRSS2* and *TAS2R50* enhancers and the bars represent relative fold-changes (+SEM) for T treatment in comparison to vehicle-treated samples (C) set at 1.0. *PDGFRA* refers to a control chromosomal site located at 57 kb down-stream of the *PDGFRA* gene.

and its absence in adults may result in emphysema (Zhao et al., 2006).

Many genes that were androgen-regulated in A549 cells are known AR targets in other tissues. These genes include *TMPRSS2* (up-regulated by androgens in prostate cancer, Tomlins et al., 2005; Wang et al., 2007) and *EPB41L4B* (Bolton et al., 2007). Similar to mouse lung, the list of up-regulated genes in A549 cells included significant enrichment of GO categories dealing with oxygen binding, heme binding and iron ion binding, even though the genes whose expression was modulated by androgens were not the same. Collectively, our results imply that androgens play a role in transport and utilization of oxygen in lung.

Although the enriched GO categories showed similarity between murine lung and human A549 lung cancer cells, differentially expressed single genes were not the same. However, A549 cells represent a malignant human cell line and may not reflect all functions of intact PTII cells. In addition, PTII cells are not the only lung cells expressing AR. As the lung tissue represents the first-line defense between the body and its surroundings, environmental conditions ought to play a more significant role in modulating gene expression *in vivo* than in cultured cells. Therefore, an intact organism (murine lung) is likely to express different gene sets than cells (A549 cells) under culture conditions.

4.3. Comparison of T and Dex regulation of A549 cell gene expression profiles

Glucocorticoid receptor (*Gr*) null mice die during the first few hours after birth due to respiratory failure (Cole et al., 1995), and GR protein is expressed in all cell types of the lung. To examine a potential overlap between androgen- and glucocorticoid-dependent genes in lung cells, we compared gene expression profiles of A549 cells exposed to either T or Dex. Most of the genes identified previously by chromatin immunoprecipitation-microarray (ChIP-on-chip) studies in Dex-treated A549 cells (So et al., 2007a) were included in our list of differentially expressed (>2-fold) genes in Dex-treated cells. The majority of genes regulated by T or Dex in A549 cells were distinct, indicating that AR possesses functions that are independent of GR signaling in lung cells. Nevertheless, gene expression profiles from T- and Dex-treated A549 cells also showed the presence of overlapping sets of transcripts, and it is therefore likely that some of the androgen-responsive genes are also regulated by GR signaling. Interestingly, the majority of these genes were up-regulated by both T and Dex. Whether or not this is due to binding of ligand-occupied AR and GR to the same genomic elements remains to be elucidated.

4.4. AR and lung cancer

There are several reports implicating the estrogen signaling pathway in lung cancer (Marquez-Garban et al., 2007), and clear sex differences exist in lung cancer type and survival (Belani et al., 2007; Chen et al., 2005; Fu et al., 2005; Patel, 2005; Thomas et al., 2005). In particular, women who do not smoke are more susceptible to developing adenocarcinoma, a cancer type that derives from PTII cells expressing AR. Since epidermal growth factor receptor (EGFR) plays a major role in adenocarcinoma (Johnson and Jänne, 2005), it is interesting that an inhibitor of EGFR which is widely used to treat lung adenocarcinoma, gefitinib, has been shown to lower androgen levels especially in patients responding to treatment (Nishio et al., 2005). Our results show that some human lung cancers express AR, and a human alveolar carcinoma-derived cell line with PTII-like properties, A549, not only contains AR but also exhibits androgen-dependent gene expression. Some of the androgen-responsive genes in these cells belong to interesting categories with regard to survival of malignant cells, such as oxygen utilization and

apoptosis. This result combined to androgen-responsive genes in murine lung, involving iron ion binding, DNA repair and angiogenesis, makes the role of androgens in lung cancer biology worthy of further inquiry. In this regard, *TMPRSS2* was one of the genes that was up-regulated upon androgen exposure and a direct AR target in A549 cells, as judged by ChIP experiments. This gene is also an AR target in prostate (Wang et al., 2007), and a locus for translocation of ETS transcription factors in about 50% of prostate cancers (Kumar-Sinha et al., 2008; Tomlins et al., 2005). It is currently not known whether a similar translocation takes place in a subset of lung cancers. With regard to lung cancer development and metastasis seeding, it was of particular interest to find androgen-dependent up-regulation of *Angptl4* in murine lung, as this TGF-β regulated gene has been implicated in lung cancer progression and metastatic potential (Padua et al., 2008). This gene is also a PPARγ target (Yoon et al., 2000), and PPARγ agonists reduce the growth of non-small cell lung cancer derived cells – including adenocarcinoma (Chang and Szabo, 2000) – *in vitro*, and patients with diabetes using PPARγ agonists have a reduced risk of lung cancer (Govindarajan et al., 2007). In conclusion, our data suggest that AR, along with other nuclear receptors, plays a role in lung cancer biology.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mce.2009.12.022.

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