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Molecular and Cellular Endocrinology xxx (2016) 1–9

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

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

DNA methylation is not involved in specific down-regulation of *HSD3B2*, *NR4A1* and *RARB* genes in androgen-secreting cells of human adrenal cortex

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A R T I C L E I N F O

Article history:
Received 16 June 2016
Received in revised form
18 September 2016
Accepted 21 September 2016
Available online xxx

Keywords:
Adrenal zonation
DNA methylation
HSD3B2
NR4A1
RARB

A B S T R A C T

We hypothesized that DNA methylation is involved in human adrenal functional zonation. mRNAs expression and methylation pattern of RARB, NR4A1 and HSD3B2 genes in human adrenal tissues (HAT) and in pediatric virilizing adrenocortical tumors (VAT) were analyzed. For analysis of the results samples were divided into 3 age groups according to FeZ involution, pre and post-adrenarche ages. In all HAT, similar RARB mRNA was found including microdissected zona reticularis (ZR) and zona fasciculata, but HSD3B2 and NR4A1 mRNAs were lower in ZR ($p < 0.05$). NR4A1 and RARB promoters remained unmethylated in HAT and VAT. No adrenal zone-specific differences in NR4A1 methylation were observed.

In summary, RARB was not associated with ZR-specific downregulation of HSD3B2 in postnatal human adrenocotical zonation. DNA methylation would not be involved in NR4A1 adrenocortical cell-type specific downregulation. Lack of CpG islands in HSD3B2 suggested that HSD3B2 ZR-specific downregulation would not be directly mediated by DNA methylation.

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1. Introduction

Androgen production in human adrenal cortex is zonally and developmentally regulated and is directly correlated with zone-specific lack of 3β-hydroxysteroid dehydrogenase type 2 (HSD3B2) enzyme expression. The developmental program that gives rise to the adrenal gland begins early in embryogenesis and continues into adult life (Xing et al., 2015). After birth, the fetal zone (FeZ) regresses by the 3rd postnatal month and the definitive and transition zones develop into the adult adrenal (Ishimoto and Jaffe, 2011). These morphological changes are accompanied by a rapid drop in the production of adrenal androgens DHEA and DHEA-S due to the involution of the FeZ. In pre-adrenarche children, the zona glomerulosa (ZG) and the zona fasciculata (ZF) are clearly present but only focal islands of reticularis cells can be identified. Adrenal production of DHEA and DHEA-S resumes at adrenarche (ages 6–8 years) when a continuous layer of reticularis cells develops and thickens forming the zona reticularis (ZR) (Auchus, 2011; Belgorosky et al., 2008; Xing et al., 2015). A major characteristic of steroidogenesis at adrenarche is a specific ZR downregulation of HSD3B2 expression (Dardis et al., 1999; Gell et al., 1998; Suzuki et al., 2000); however, the regulatory system controlling this functional differentiation is largely unknown.

The orphan nuclear receptor NR4A1 (nuclear receptor subfamily 4 group A member 1, also known as NGFIB or Nur77) has emerged as an essential transcription factor for human HSD3B2 expression. NR4A1 activates HSD3B2 transcription by binding to a specific response element (NBRE; AAAGGTCA) in the HSD3B2 promoter region (Bassett et al., 2004; Martin and Tremblay, 2005). Within the

Abbreviations: FeZ, Fetal zone; ZG, zona glomerulosa; ZF, zona fasciculata; ZR, zona reticularis; HAT, nondiseased human adrenal tissue; VAT, virilizing pediatric adrenocortical tumors; RARB, Retinoid Acid Receptor β; RABT, total RARB, RARB2, RARB isoform 2; NR4A1, nuclear receptor subfamily 4 group A member 1; HSD3B2, 3β-hydroxysteroid dehydrogenase type 2.

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http://dx.doi.org/10.1016/j.mce.2016.09.024
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Please cite this article in press as: Baquedano, M.S., et al., DNA methylation is not involved in specific down-regulation of *HSD3B2*, *NR4A1* and *RARB* genes in androgen-secreting cells of human adrenal cortex, Molecular and Cellular Endocrinology (2016), http://dx.doi.org/10.1016/j.mce.2016.09.024

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adult and fetal adrenal cortex, NR4A1 expression is also zone-specific, paralleling the expression of HSD3B2 (Bassett et al., 2004). Nevertheless, the mechanisms regulating this zonal expression pattern are unknown. Moreover, the expression of HSD3B2 and NR4A1 is markedly lower in adrenocortical tumors that arise in young children compared with normal adrenal cortex, supporting the thesis that the tumors originate from deregulation of either the fetal zone during embryogenesis or the developing ZR during the first few years of life (West et al., 2007). Consistently, more than 90% of pediatric patients present with a hyperandrogenic steroid profile with virilizing features (Michalkiewicz et al., 2004).

Methylation of DNA is an essential epigenetic signal involved in mammalian embryonic and postnatal development and cellular differentiation, while epigenetic disruption is a characteristic of cancer cells (Baylin et al., 1998). Cell-type-specific DNA methylation is involved in establishing or maintaining the expression pattern of specific genes and thus contribute to the stability of a specific differentiation state (Jaenisch and Bird, 2003). Methylation of cytosines in CpG islands leads to the binding of methylated CpG-binding domain proteins, transcription repressors, and/or histone deacetylases, thereby blocking gene transcription. In addition, DNA methylation may block the expression of specific genes by silencing essential transcription factors (Flück and Miller, 2004). We hypothesized that DNA methylation may be involved in the regulation of human adrenal functional zonation. However, little is known about the possible role of DNA methylation in adrenal zone-specific downregulation of *HS3B2* expression. Previous experiments altering DNA methylation patterns with the histone methyltransferase inhibitor 5-aza-2′-deoxycytidine in different steroidogenic cell lines yielded some conflicting data (Liu et al., 2004; Udhane et al., 2013). In this regard, the relevance of in vitro studies using cell lines is debatable, especially in the epigenetic field, as microenvironment and culture conditions differ dramatically from in vivo conditions and can modify the cell chromatin profile (Antequera et al., 1990). Therefore, in the present study we looked for CpG islands in *HSD3B2* and *NR4A1* gene promoters using normal human adrenal tissues of different ages and childhood virilizing adrenocortical tumors (VAT) to evaluate developmental and zone-specific changes in DNA methylation.

Recently, Udhane et al. showed evidence that Retinoid Acid Receptor, β (RARB) cooperates with NR4A1 to in-vitro regulate HSD3B2 transcription (Udhane et al., 2015). However, the role of RARB in the adrenal zonation remains to be explored. The *RARB* gene encodes several isoforms from two distinct promoters, P1 and P2. The RARB2 isoform is transcribed from the CpG-rich RARB P2 promoter and its expression is frequently silenced in human solid tumors (Virmani and Gazdar, 2003; Widschwendter et al., 2001). To obtain further insight into the relevance of RARB to human adrenal physiology, the ontogenesis of RARB mRNA expression in human adrenal tissues from early infancy to puberty and in VATs was studied. The methylation status of RARB P2 promoter was also determined.

2. Methods

2.1. Human tissue samples

Twenty-six frozen nondiseased human adrenal tissue (HAT) samples collected for previous reports (Baquedano et al., 2007, 2005) were used, with local ethical approval from the Garrahan Pediatric Hospital, Argentina. Briefly, nonpathological human adrenal glands were obtained from multi-organ transplantation donors with a less than 24-h brain death diagnosis or patients who underwent resection of the kidney plus adrenal because of renal malignancy (n = 13), or from necropsies performed less than 6 h post-mortem (n = 13). Histological identification of the adrenal zones has previously been published (McNicol, 1992). Samples (n = 11, age 0.75–4.5yr) of frozen virilizing pediatric adrenocortical tumors (VAT) were obtained from the Tumor Bank of the Garrahan Pediatric Hospital. The tissues were used with the appropriate permission from the ethical review board of Garrahan Pediatric Hospital. Participants provided informed consent.

As previously described (Baquedano et al., 2007, 2005), to analyze the results, tissues were divided into three age groups (Gr): Gr1 less than 3 months old (n = 9), Gr2 between 3 months and 6 yr old (n = 9) and Gr3 older than 6 and up to 20 yr old (n = 8). This division was based on the mean age of postnatal FeZ involution (Ishimoto and Jaffe, 2011) for the first cut off, and on the mean age of adrenarche (Nakamura et al., 2009) for the second one. In Gr3, only those samples in which a continuous ZR was present were included.

2.2. Microdissection of adrenal tissues by laser capture microdissection (LCM)

Sections (5 μm) of frozen adrenal tissues (n = 7, from boys aged 14, 15, and 18 yr and girls aged 9, 11, 17, and 20 yr) were mounted on slides for LCM (steel frames with PEN [polyethylene naphthalate], Leica, Germany). Slides were fixed in methacarn (60% v/v absolute methanol, 30% v/v chloroform and 10% v/v glacial acetic acid), hematoxylin and eosin-stained, and dehydrated in rising ethanol and xylene.

Cells from the ZR and ZF were captured using the AS LMD microscope (Leica Microsystems, Germany). Captured cells were immediately processed for DNA and RNA extraction.

2.3. RNA isolation and gene expression analysis

Total RNA was extracted from each normal and tumor adrenal tissue sample by homogenization in TRIzol reagent (Invitrogen, Buenos Aires) following the manufacturer's instructions. Total RNA from the cells captured by laser capture microdissection was isolated and purified with the RNAqueous-Micro kit (AM-1931, Ambion Inc., USA) according to the manufacturer's instructions. Purity and integrity of each total RNA sample was evaluated spectroscopically and by gel electrophoresis before reverse transcription. Total RNA concentration was assessed by spectrophotometric absorbance at 260 nm.

1 μg of total RNA of intact normal and tumor adrenal tissues was reversely transcribed using oligo(dT)20 (Biodynamics) and MMLV reverse transcriptase (Amersham Biosciences. Buenos Aires, Argentina) following the manufacturer's instructions. First-strand cDNA was synthesized from 100 ng total RNA of ZR and ZF laser-captured cells primed with oligo(dT)20 and random primers (Biodynamics) (3:1, v/v) using Superscript III reverse transcriptase (Invitrogen, Applied Biosystems) according to manufacturer's instructions. Lack of genomic DNA contamination was confirmed by PCR amplification of RNA samples in the absence of cDNA synthesis.

cDNA from ZR and ZF laser-captured cells was first amplified by PCR with specific intron-spanning primer pairs for HSD3B2, P450c17, and chromogranin A (sequence primers listed in Table S1) for 35 cycles. PCR amplification of β-actin (30 cycles) was carried out to check the quality of cDNA generated from the laser-captured samples. The PCR reaction was performed using 1 μl cDNA as a template in 25 μl reaction volume containing 1.5 mM MgCl2, 200 μM dNTPs, 0.24 μM of each forward and reverse primer, and 1UTaq polymerase (Amersham Biosciences). Negative controls lacking cDNA were included in all PCR reactions. PCR products were analyzed on 2% agarose gels containing ethidium bromide and

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visualized using a UV transilluminator. The identities of RT-PCR products were verified by sequencing analysis.

Expression of total RARB (RABT), RARB isoform 2 (RARB2), HSD3B2, and NR4A1 was analyzed using SYBR-green-based real-time quantitative RT-PCR. Human *PBGD* gene and *β-actin* gene, which is expressed at stable levels regardless of age (Dardis et al., 1999), were used as low- and high-abundance housekeeping genes, respectively, to control PCR reactions and to normalize mRNA levels. Sequences of the specific intron-spanning primer pairs were previously published (Steckelbroeck et al., 2002; Udhane et al., 2015) and are listed in Table S1. The RARBT primers targeted all the RARB transcripts, while the RARB2 primers are specific to the RARB2 transcript isoform. The cDNA samples from intact tissues and laser-captured cells were diluted 1/50 and 1/5, respectively. Amplification reactions were run (in triplicate) on an iCycler Thermal Cycler (Bio-Rad Laboratories Inc.). qPCR amplification mixture (25 μL) contained 5 μl of diluted cDNA, 200 nM each of specific forward (F) and reverse (R) primers (Table S1), and 12.5 μl of iQ™ SYBR Green Supermix (Bio-Rad). The amplification protocol used was as follows: Initial 7-min denaturation and enzyme activation at 95 °C, 40 cycles of 95 °C for 30 s, X°C (annealing temperature, Table S1) for 30 s and 72 C for 30 s. This cycle was followed by a melting curve analysis, ranging from 55 °C to 95 °C, with temperature increasing steps of 0.5 °C every 2 s. Amplification curves and the mean Ct (threshold) values of triplicates were automatically determined using the Bio-Rad iQ5 Software 2.0.

First, primer efficiency was validated with a standard curve of 5-fold serial dilution points of a cDNA pool from all samples and a no template control (NTC). After optimization of qPCR systems (correlation coefficients higher than 0.99 and the corresponding real-time PCR efficiencies in the range 0.95–1.02), relative expression levels were calculated as $2^{-(\text{mean Ct target gene} - \text{mean Ct housekeeping gene})}$ (Nolan et al., 2006; Schmittgen and Livak, 2008), following the MIQE guidelines (Lefever et al., 2009). Only a standard deviation of <0.20 in triplicate threshold cycle determinations was accepted for the analysis. A non-template control was carried out with every assay and no indication of PCR contamination was observed. Gene-specific amplification was confirmed by a single peak in the melting-curve analysis and a single band on a 2% agarose gel stained with ethidium bromide.

For all of the statistical analyses, the expression data, expressed in arbitrary units (AUs), were converted to logarithmic values so that values approximated a normal distribution. Normality was assessed using the Shapiro-Wilk test. Results are expressed as the mean ± SEM. Statistical significance among age groups was determined by ANOVA followed by the Bonferroni *post hoc* test. The paired student's *t*-test was used to compare ZF and ZR gene expression levels. Differences were considered statistically significant when P was <0.05.

2.4. Immunohistochemistry of RARBT

Immunohistochemistry staining was performed employing the streptavidin-biotin and peroxidase method using the manufacturer's protocol (DAKO Catalyzed Signal Amplification (CSA) System, HRP. DAKO Cytomation. Carpinteria, CA 93013 USA), as previously described (Baquedano et al., 2007). Briefly, after deparaffinization, sections (5 μm) were subjected to antigen retrieval and proteinase K (20 μg/ml) treatment (10 min). Endogenous biotin activity was blocked and endogenous peroxidase activity was quenched. The sections were further blocked (Protein Block Serum-Free, DAKO X0909, DAKO Corp. Carpinteria, CA 93013 USA) for 15 min. Sections were then incubated with the primary antibody for RARB (2 μg/mL, RARβ Antibody (C-19): sc-552, Santa Cruz Biotechnology, Inc CA) for 18 h, at 4 °C. After washing, tissues were incubated for 15 min with biotinylated goat anti-rabbit immunoglobulin. Bound antibodies were visualized with 3,3′-diaminobenzidine tetrahydrochloride. As negative controls, normal rabbit serum was used instead of primary antibodies.

2.5. In silico screening for CpG islands

A sequence starting 2000 bp upstream from the transcriptional start site of *NR4A1* and *HSD3B2* was used in Methyl Primer Express Version 1.0 and CpG Island Searcher (http://www.cpgislands.com) (Takai and Jones, 2003) programs to check the presence of CpG islands or CpG rich regions in the *NR4A1* and *HSD3B2* promoter region. The criteria used to define CpG islands were: DNA sequence length ≥500bp, GC content ≥55%, and a ratio of the observed CpG frequency versus the expected one ≥0.65 (Takai and Jones, 2002). DNA sequence information of human CpG islands, including flanking sequences was obtained via the UCSC Genome Browser with the following ID numbers: NC_000012.12, Chr12: 52,037,715–52,059,507 for NR4A1, and NG_013349.1, chr1:119,414,931–119,423,035 for HSD3B2.

2.6. DNA isolation and sodium bisulfite treatment

Genomic DNA (gDNA) was extracted from 9 HAT (Gr1 (n = 3), 3,15, and 26 days; Gr2, (n = 3), 1.6, 2 and 4 yr and Gr3, (n = 3), 14, 16 and 20 yr) and 4 VAT with a QIAamp DNA Mini Kit (QIAGEN, USA), and 400 ng of gDNA from each sample was then subjected to bisulfite conversion using the EZ DNA Methylation-Direct Kit (Zymo Research Corporation, Orange, CA) according to the manufacturer's standard protocol.

gDNA from adrenal ZR and ZF laser-captured cells was bisulfite converted directly from 3 microdissected tissues (n = 3, from boys aged 14 and 15 and a girl aged 17 yr) using the EZ DNA Methylation-Direct Kit (Zymo Research Corporation, Orange, CA) according to the manufacturer's instructions.

Purified, non-methylated and methylated human DNA (D5014-1, Zymo Research Corporation, Orange, CA) was used as a positive and negative control to assess the efficiency of bisulfite-mediated conversion of DNA.

2.7. Amplification and sequencing of bisulfite-treated DNA

A 361 bp fragment (Chr12: 52,051,168–52,051,168) was amplified from bisulfite-treated DNA using bisulfate sequencing primers designed with Methyl Primer Express® Software Version 1.0 (primer sequences listed in Table S1). The amplified products were cloned into the pCR2.1-TOPO vector according to the manufacturer's protocol (Invitrogen) and 10 clones were randomly selected for bisulfite sequencing to analyze the methylation status in the promoter region of *NR4A1* gene. The cloned PCR fragments were sequenced with the M13 reverse and forward primers in an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Buenos Aires, Argentina) using the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Buenos Aires, Argentina). BiQ analyzer software was used for quality control and to derive DNA methylation patterns from the sequencing results (Bock et al., 2005). The BDPC (Bisulfite sequencing Data Presentation and Compilation) program was used to present the methylation pattern of single amplicons, create the figures and compile methylation data (Rohde et al., 2008).

2.8. Methylation-specific polymerase chain reaction (MSP)

RARB2 methylation (an isoform of RARB transcribed by P2

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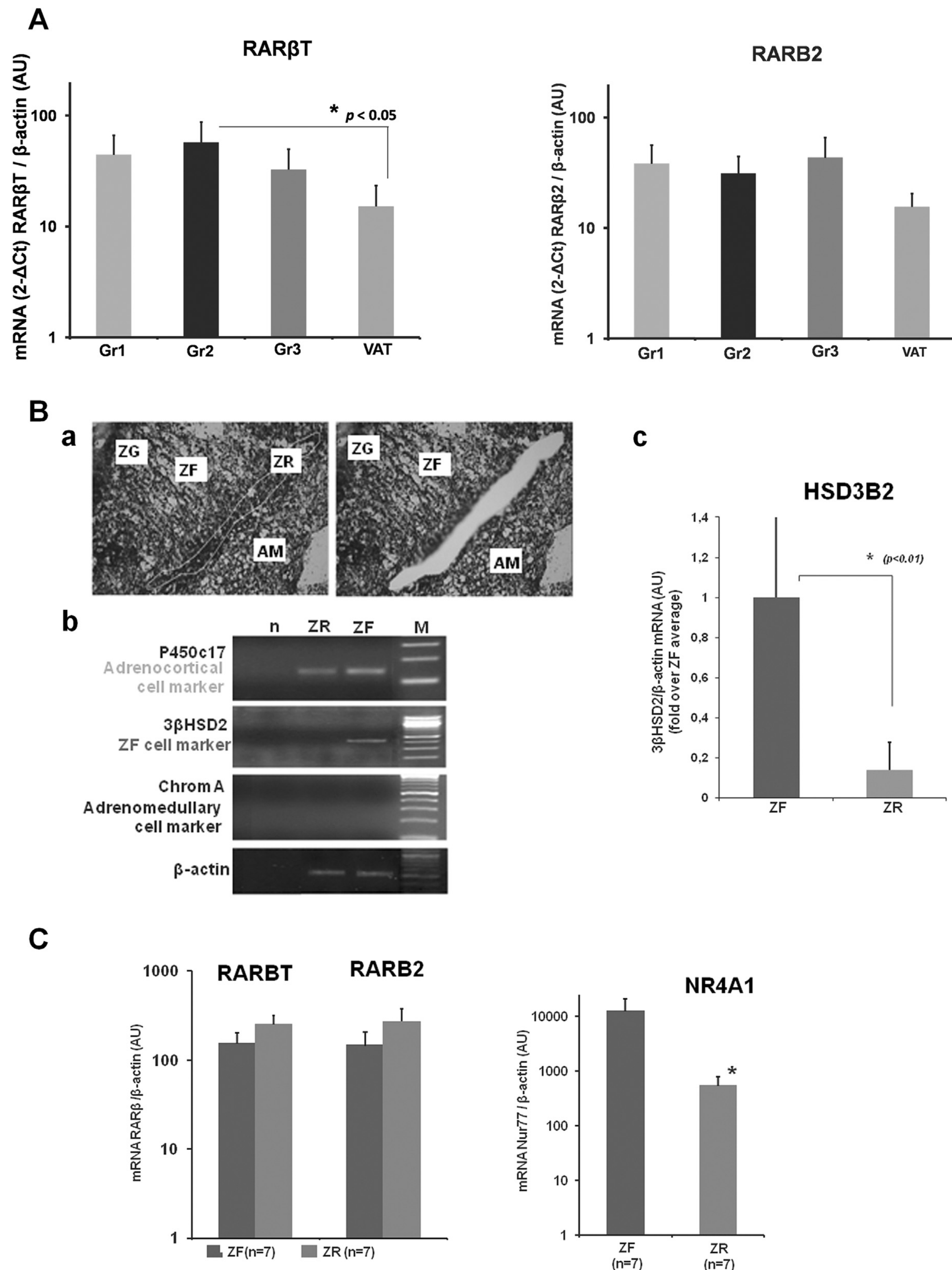

Fig. 1. mRNA expression analysis of RARB, RARB2, and NR4A1 in human adrenal tissues. (A) qRT-PCR analysis of RARB and RARB2 in normal human adrenal tissues from the three age groups and in pediatric virilizing adrenocortical tumors. Gr 1, n = 9; Gr 2, n = 9; Gr 3, n = 8 and VAT, n = 11. **(B) Laser-capture microdissection of ZR and ZF in normal human adrenal tissues from Gr3 (n = 7). (a)** Representative photomicrograph of a 14-year-old human adrenal gland before and after LCM of the ZR. **(b)** Representative result of mRNA zonal expression. Total RNA was extracted from cells of ZR and ZF and analyzed by RT-PCR. PCR reactions with specific primers for 3βHSD2 (ZF cell marker), P450c17 (adrenocortical cell marker), and chromogranin A (adrenomedullary cell marker) were performed to analyze the purity of each dissected adrenal zone. Each panel is labeled according to the primers used. **(c)** Zonal expression of HSD3B2 mRNA by qRT-PCR. **(C)** Zonal expression of mRNA encoding RARBT, RARB2 and NR4A1 by qRT-PCR. The bars show mean mRNA levels, quantified in relation to β-actin mRNA (arbitrary units, AU); the error bars represent SEM in each group. Results are expressed as mean ± SEM; $*p < 0.05$. NTC, no template control; MW – molecular weight.

promoter) was determined by using reported primers to discriminate methylated and unmethylated 5′-promoter region on bisulfite-treated DNA (Côté et al., 1998; Pirouzpanah et al., 2015; Youssef et al., 2004), Table S1. The amplified region contained appropriate numbers of CpG that could be analyzed by MSP of *RARβ2* gene. It was suggested that the methylated status of this region inversely associates with the expression of *RARB2* (Youssef et al., 2004). PCR was carried out in a total volume of 25 μl containing 1 × PCR buffer, dNTPs (0.2mMeach), 2 mM MgCl2, 0.5 μM of each primer, 1U of Taqpolymerase (Amersham), and sodium bisulfite-treated DNA (100–150 ng). Purified, non-methylated and methylated human DNA (D5014-1, Zymo Research Corporation, Orange, CA) was used as positive and negative methylation control. Negative-control samples without DNA were included for each set of PCR. PCR products were analyzed on 2% agarose gels containing ethidium bromide (Life Technologies, Inc.). The PCR for untreated DNA with a set of methylated and unmethylated primers showed no false-positive results caused by incomplete bisulfite treatment.

3. Results

3.1. NR4A1 and RARB expression in human adrenal tissues

Mean ± SEM (AU) RARBT and RARB2 mRNAs levels in Grs 1–3 and in VAT is shown in Fig. 1A. Data normalized to β-actin are presented. The data were subsequently normalized to the relative amount of PBGD and β-actin amplified (low and high abundance housekeeping genes, respectively) and similar patterns of expression were seen by normalization to either gene.

Mean RARBT and RARB2 mRNAs levels (mean ± SEM, AU) were similar among HAT from the 3 age Grs (Gr1 (n = 9), 44.2 ± 22.1 and 38.3 ± 0.36; Gr2 (n = 9), 57.25 ± 29.57 and 31.21 ± 13.32; and Gr3 (n = 8), 34.42 ± 17.2 and 43.41 ± 22.4, respectively) without significant differences among them ($p > 0.05$). mRNA levels of RARBT (15.09 ± 8.14) were lower in VAT compared to age-matched HAT ($p < 0.05$). RARB2 mRNA levels (15.53 ± 4.95) tended to be lower in VAT compared to HAT without statistical significance.

LCM was used to obtain cDNA exclusively from the ZR and ZF cells (Fig. 1B). In seven experiments with different human adrenal tissues from Gr 3, RARBT and RARB2 mRNA levels were detected in both micro-dissected ZF (155.39 ± 48.76 and 146.94 ± 69.59 AU, respectively) and ZR (250.66 ± 60.71 and 270.65 ± 102.11 AU, respectively) cells with no significant difference between them (Fig. 1C).

By immunohistochemistry, RARB protein was strongly expressed in the whole adrenal cortex. Both cytoplasmic and nuclear RARB staining was observed in adrenal tissues regardless of age. Representative results of RARB cell immunolocalization in Gr2 and in Gr3 are shown in Fig. 2.

As expected, the relative transcript abundance of NR4A1 was significantly lower in ZR (551.48 ± 123.2) than in ZF (12911.41 ± 1203.2) cells (Fig. 1C). Representative results of mRNA zonal expression are shown in Fig. 1Bb. PCR reactions with specific primers for HSD3B2 (ZF cell marker), P450c17 (adrenocortical cell marker), and chromogranin A (adrenomedullary cell marker) were performed to analyze the purity of each dissected adrenal zone. SYBR Green based qRT-PCR assays indicated that HSD3B2 mRNA levels in micro-dissected ZR were dramatically lower than those in ZF cells (n = 7, $p < 0.01$) (Fig. 1Bc).

3.2. DNA methylation profile of the CpG island at the NR1A4 promoter region

In silico screening of *HSD3B2* and *NR4A1* gene sequences, including 2-Kb upstream of the respective start sites, revealed that CpG islands are not present in the *HSD3B2* gene, while *NR4A1* promoter was found to be embedded within a CpG island, suggesting that *NR4A1* gene expression might be related to the methylation status of the CpG island (Fig. 3A). Methylation status of a total of 40 CpG sites across a 361-bp region within the NR4A1 CpG island was characterized by clonal bisulfite genomic sequencing. The DNA methylation pattern of this region was studied in nine HAT samples (Gr1, n = 3; Gr2, n = 3 and Gr3,n = 3) and in four VATs. As is shown in Fig. 3B, practically all of the CpG sites were

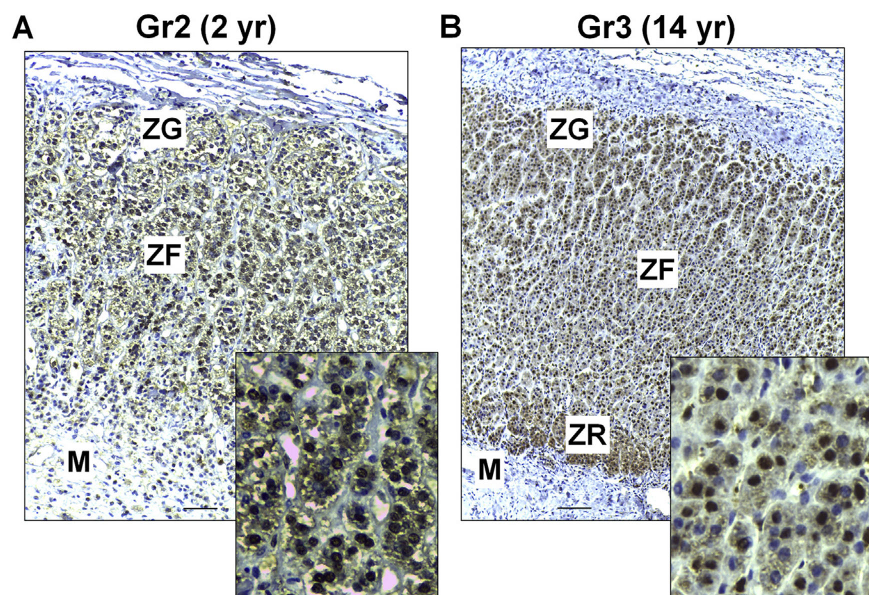

Fig. 2. Immunohistochemistry localization of RARB in paraffin sections of normal human adrenal glands of Gr2 (A) and Gr3 (B). Immunorreactivity for RARB was seen in the whole adrenal cortex of the three age groups. Immunopositive cells were visualized with the DAB colorimetric reaction that results in a brown colored precipitate at the antigen site. Counterstaining was performed using hematoxylin, which stains cell nuclei blue. Scale bar = 100 μm. ZG, zona glomerulosa; ZF, zona fasciculate; ZR, zona reticularis; M, adrenal medulla. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

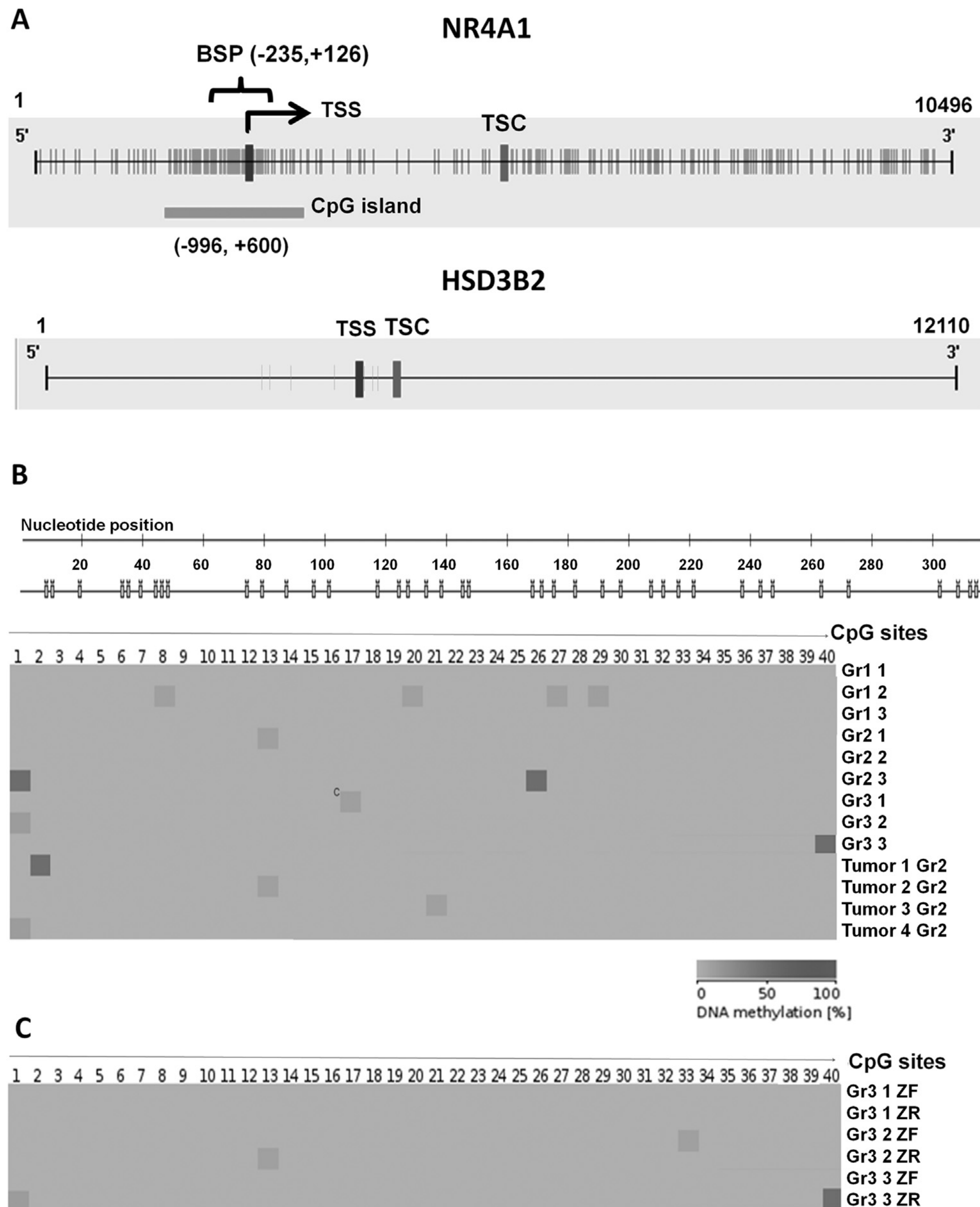

Fig. 3. DNA methylation patterns in the NR4A1 promoter region (A). Schematic structure of the design for BSP assay on the NR4A1 promoter CpG Island. **B and C. Schematic display of the methylation status of 40 CpG sites across a 361-bp region within the CpG island at the NRA1 promoter investigated by bisulfite sequencing**. Each column represents a single CpG site. Each row corresponds to a single tissue and represents the average methylation of a CpG site encoded in a continuous grey scale code. **B,** Compilation of NR4A1 DNA methylation results in normal human adrenal tissues from the three age groups and in virilizing pediatric adrenocortical tumors. Gr1, n = 3; Gr 2, n = 3; Gr3, n = 3, and VAT, n = 4. **(C),** Compilation of NR4A1 DNA methylation results in ZR and ZF from 3 microdissected adrenal tissues from Gr3.

unmethylated in normal HAT from the three age Grs and in VAT.

Next, the zonal methylation status of the *NR4A1* gene was studied in three microdissected samples. No CpG methylation was detected in the *NR4A1* promoter in either ZR or ZF adrenal zones (Fig. 3C).

3.3. DNA methylation profile of the CpG island at the RARB P2 promoter

Methylation-specific PCR was performed on bisulfite-modified DNA samples from Gr2 (n = 3) and Gr3 (n = 3) HATs and in VATs (n = 4) by simultaneous use of primers for the methylated and unmethylated forms of the *RARB* gene promoter P2. RARB P2 promoter was found to be unmethylated in all studied VATs and HATs and any significant methylation difference between tumor and age-matched normal tissue was observed (Fig. 4).

4. Discussion

Several lines of evidence indicate that DNA methylation plays a regulatory role in developmental cell type-specific gene expression (Fan et al., 2005; Futscher et al., 2002; Hoivik et al., 2013a, 2013b; Lee et al., 2010; Oda et al., 2006). Although the contribution of DNA methylation to the adrenocortical cell-type specific steroidogenic activity has not been thoroughly studied, DNA methylation has been shown to regulate certain genes involved in steroidogenesis (Hoivik et al., 2013a; Missaghian et al., 2009; Zhang and Ho, 2011). *In vitro* experiments using the human adrenocortical NCI-

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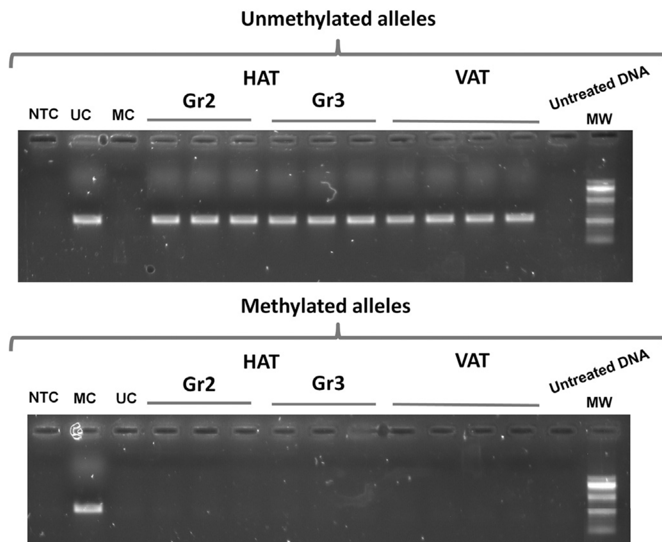

Fig. 4. Methylation of the RARB P2 promoter in normal human adrenal cortex from Gr1 and Gr2 and in pediatric virilizing adrenocortical tumors. All DNA samples were bisulfite treated except those designated untreated. MS-PCR was performed using unmethylation-specific and methylation-specific primers for RARB2. MS-PCR shows that the RARB2 promoter was clearly unmethylated in normal and tumors adrenal tissues. Purified non-methylated (UC) and methylated (MC) human DNA (D504-1), Zymo Research, was used as positive and negative methylation control. U, unmethylated; M, methylated; NTC, no template control; UC non-methylated human DNA. MW – molecular weight.

H295R cell line showed that inhibition of DNA methylation by 5-aza-2′-deoxycytidine increased basal HSD3B2 expression (Liu et al., 2004). Typically, CpG islands are associated with regions that are involved in transcriptional regulation (Jaenisch and Bird, 2003). The absence of a CpG island in the human *HSD3B2* gene suggested that direct epigenetic regulation of the *HSD3B2* promoter was not essential. In the human adrenal cortex, NR4A1 was shown to determine zonal expression of HSD3B2 (Bassett et al., 2004). Thus, we hypothesized that epigenetic regulation of human adrenal H*SD3B2* could be tied to the DNA methylation-mediated ZR-specific downregulation of *NR4A1*.

As in previous studies of human fetal adrenals (Bassett et al., 2004; Goto et al., 2006) we detected that mirroring HSD3B2 expression, the level of NR4A1 mRNA was low in DHEA-secreting ZR cells of pubertal HAT. This observation agrees with earlier immunohistochemistry studies (Kelly et al., 2004; Lu et al., 2004). To determine whether this differential expression is reflected in the methylation status, clonal bisulfite sequencing on DNA isolated from laser-microdissected ZR and ZF cells was performed. To our knowledge, this is the first demonstration of the utility of laser microdissection to explore adrenal zone-specific methylation status. Similar unmethylated clone profiles in ZF and ZR were found, suggesting that the modulation of DNA methylation is not involved in the gene-expression profile of ZR functional differentiation. Consistent with this, the *NR4A1* promoter remained virtually completely unmethylated in normal HAT from the three age Grs. No developmental changes in methylation status were observed either in the youngest Gr, in which the FeZ is still present, or in the oldest Gr, in which the ZR is fully developed.

A recent study showed that human adrenal corticocarcinoma NCI-H295R cells grown under starvation conditions acquire a hyperandrogenic steroid profile by repressing HSD3B2 expression which resembles the human adrenal during adrenarche (Kempná et al., 2010; Udhane et al., 2015). RARB was shown to be down-regulated in this experimental tumor-cell model and the authors provided evidence that RARB regulated *HSD3B2* transcription in cooperation with NR4A1 (Udhane et al., 2015). To provide a clue to RARB biological actions at adrenarche, we studied the ontogenesis of RARB mRNA expression and zonal localization in human adrenal tissues from early infancy to puberty. Our expression data demonstrated that *RARB* is indeed expressed in the human adrenal cortex with similar expression levels in ZR and ZF. In contrast to previous speculations (Udhane et al., 2015), no ZR-specific decrease in RARB mRNA levels at the age when adrenarche occurs was observed, suggesting that starved, hyperandrogenic H295R cells would not be a good model for understanding adrenal functional zonation during human development. Nevertheless, the relative transcript abundance of RARBT was significantly lower in childhood hyperandrogenic VAT than in the age-matched non-pathological HAT group. These results are consistent with those in the adrenal corticocarcinoma H295R cells, suggesting that in virilizing adrenocortical tumor tissues, both the lack of RARB and NRA4A1 might cooperate in the regulatory mechanisms underlying tumor-specific HSD3B2 down-regulation in favor of a hyperandrogenic steroid profile (Udhane et al., 2015; West et al., 2007).

Epigenetic changes, such as aberrant DNA methylation are a hallmark of human neoplasia including adrenocortical neoplasms (Baylin et al., 1998; Bielinska et al., 2009; Feinberg et al., 2006). Therefore the methylation status of the CpG-rich NR4A1 and RARB P2 promoters in VATs was analyzed. Similar unmethylated promoter profiles were evidenced in VAT and HAT, indicating that promoter methylation could not account for the downregulation of RARB and NR4A1 mRNA expression in VATs compared to age-matched HATs. Further studies are required to address if epigenetic mechanisms other than DNA methylation are associated with human adrenal cell-specific transcript regulation underlying developmental or pathophysiological androgen production. Interestingly, a very recent study about the role of NR4A1 in hypercholesterolemia-induced circulating inflammation revealed that the DNA methylation levels in the *NR4A1* promoter are very low both in hypercholesterolemia patients and healthy individuals, while histone H3 acetylation played a major role in epigenetic regulation of NR4A1 by hypercholesterolemia (Xie et al., 2015). In recent years, microRNAs have emerged as key regulatory molecules that regulate 30–80% of human genes (Lewis et al., 2005); hence, it might be important to consider a role of microRNAs in the regulation of adrenal androgen production.

In summary, to our knowledge, this is the first comprehensive report on the expression of RARB in the human adrenal gland during childhood and puberty. Our results suggest that RARB is not associated with the ZR-specific down-regulation of adrenal HSD3B2 expression of postnatal human adrenal cortex zonation. Taken together, the expression and methylation profiles in non-pathological and tumor specimens of human adrenal cortex and in laser-microdissected ZR and ZF cells documented in this study demonstrated that DNA methylation would not be involved in the zone-specific and/or VAT downregulation of adrenal NR4A1 and RARB expression. Furthermore, the lack of CpG islands in *HSD3B2* promoter region suggests that the known downregulation of *HSD3B2* gene expression in human ZR cells would not be directly mediated by DNA methylation.

Acknowledgements

We thank Dr Marina I Flamini (Institute of Medicine and Experimental Biology of Cuyo (IMBECU), National Research Council of Argentina, Mendoza, Argentina) for kindly supplied anti-RARB antibody.

This study was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) (Subsidio

Please cite this article in press as: Baquedano, M.S., et al., DNA methylation is not involved in specific down-regulation of *HSD3B2*, *NR4A1* and *RARB* genes in androgen-secreting cells of human adrenal cortex, Molecular and Cellular Endocrinology (2016), http://dx.doi.org/10.1016/j.mce.2016.09.024

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Florencio Fiorini 2012), Fundación Florencio Fiorini (PIP 2014-2016) and Fondo para la Investigación Científica y Tecnológica(FONCYT) (PICT-2010-0555).

Disclosure Summary: All authors have nothing to declare.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mce.2016.09.024.

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