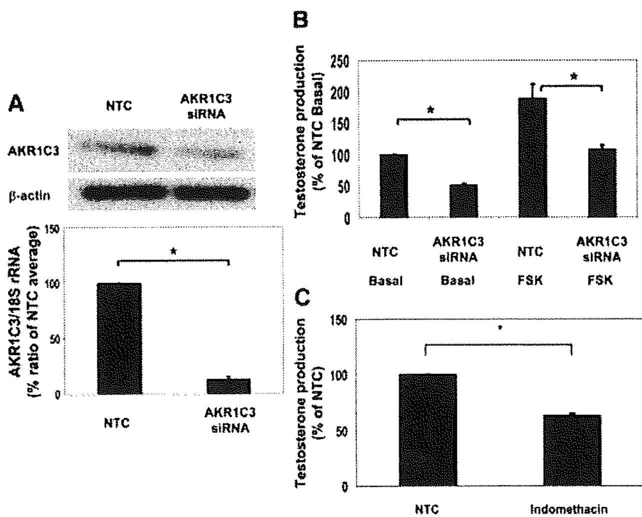

FIG. 4. Effects of siRNA depletion of AKR1C3 on adrenal cell production of testosterone. A, H295R adrenal cells were transfected with or without siRNA against AKR1C3 (AKR1C3 siRNA) or Stealth RNAi Negative Control Duplexes (NTC). After 96 h, mRNA and protein for AKR1C3 were detected by qPCR and Western analyses, respectively. The 18s rRNA and β-actin protein expression were used for normalization. Data are presented as mean ± SE of values from three independent experiments run and expressed as a percentage of NTC. *, $P < 0.05$, compared with NTC level. B, The level of testosterone in the media with H295R cells at 48 h after treatment with vehicle (basal) or forskolin (FSK) (10 μM) after AKR1C3 (AKR1C3 siRNA) or Stealth™ RNAi Negative Control Duplexes (NTC). Data are presented as mean ± SE of values from three independent experiments run and expressed as a percent of NTC basal. *, $P < 0.05$, compared with NTC basal level. C, The level of testosterone in the media with H295R cells treated for 48 h without (NTC) or with indomethacin (10 μM). Data are presented as mean ± SE of values from three independent experiments run and expressed as a percentage of NTC. *, $P < 0.05$, compared with NTC level.

actively synthesizes testosterone from androstenedione (2, 12, 23). This enzyme is not found in the ovary or the adrenal, leaving the method for ovarian and adrenal testosterone synthesis open. The ovary expresses both HSD17B1 and AKR1C3, giving this tissue two enzymes that could be involved in testosterone biosynthesis. A number of peripheral nonendocrine tissues have been shown to express high levels of AKR1C3, and it is this enzyme in muscle that is now believed to use circulating androstenedione to locally produce testosterone (24). Herein, we dem-

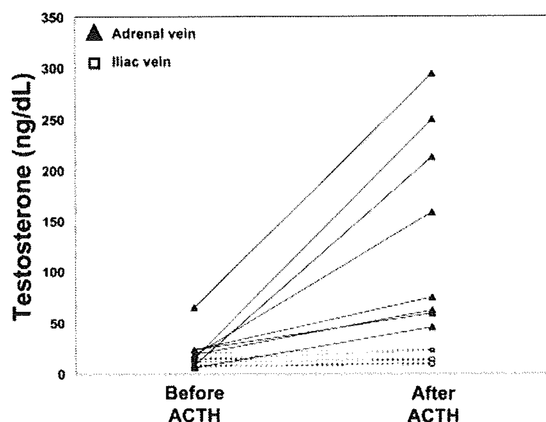

FIG. 5. Testosterone levels in the adrenal vein and iliac vein from women (n = 8) before and 15 min after iv ACTH administration (0.25 mg). As described in *Materials and Methods*, testosterone levels were measured using LC-MS-MS and values for both iliac vein (*squares*) and adrenal vein (*triangles*) are shown for each subject.

TABLE 1. Plasma aldosterone, cortisol, and testosterone levels detected in the adrenal vein and iliac vein of women (n = 8) before and 15 min after iv ACTH administration (0.25 mg)

	Aldosterone (ng/dl)	Cortisol (ug/dl)	Testosterone (ng/dl)
Adrenal vein			
Pre-ACTH	60.3	22.3	18.5
Post-ACTH	1717.6	612.5	116.3
Iliac vein			
Pre-ACTH	7.9	5.7	24.1
Post-ACTH	19.4	15.1	30.1

Testosterone and cortisol were measured using LC-MS-MS, and aldosterone was measured using RIA. Each value is shown as the median.

onstrated that the expression levels of HSD17B1 and HSD17B3 mRNA were very low in the adrenal gland when compared with ovary or testis, respectively. In addition, we also confirmed that these genes expression levels are much lower in H295R cells compared with AKR1C3 (data not show). However, it is true that these enzymes are important for testosterone production in human tissues, and further studies are needed to determine whether expression of very small amounts of these enzymes could contribute to testosterone production in human adrenal gland. Dufort and colleagues (25) reported that AKR1C3 protein is detectable in the human adrenal gland, liver, prostate, and prostate cancer cell line using Western analysis. To our knowledge, there are no previous studies regarding AKR1C3 localization in the human adrenal gland. Our results are intriguing in that they show that AKR1C3 mRNA and protein are predominantly expressed in ZR cells within the human adrenal gland. Pelletier *et al.* (26) previously reported that mice have an adrenal expression pattern of AKR1C3 that is restricted to the ZR of female adrenal gland but not in the male mouse adrenal gland. They also demonstrated that mouse AKR1C3 has some 20α-hydroxysteroid dehydrogenase (20α-HSD) activity (26). They suggested that adrenal cells released metabolites of progesterone, which would be effectively inactivated by the female adrenal ZR 20α-HSD (26). This does not appear to be the case in human adrenal gland because the levels of 20α-hydroxyprogesterone are very low in adrenal vein (data not shown). In this study, we also compared the different expression levels of AKR1C1, AKR1C2, AKR1C3, and AKR1C4. We confirmed that AKR1C1 mRNA expression level was relatively high in the adrenal gland as shown in a previous report (27). However, in our study, inhibition of AKR1C1 mRNA expression by 70% did not repress testosterone production in H295R cells (data not shown). It is reported that testosterone conversion from androstenedione occurs much more efficiently via AKR1C3 than through AKR1C1 (9). Our microarray and qPCR data suggested that AKR1C2 and AKR1C4 mRNA levels were very low in the adrenal gland, which seems to be compatible with a previous report (27). Therefore, it can be postulated that AKR1C3 is more likely to contribute to more testosterone production in the ZR compared with AKR1C1, AKR1C2, or AKR1C4. However, it awaits further study to clarify the exact role of each aldo-keto reductase family member in adrenal testosterone production in the future.

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Based on our findings, the expression and role of AKR1C3 appear to be different for human adrenals compared with mice. First, in humans, there is high expression of CYP17 in both the ZF and ZR (1). CYP17 acts to convert much of the progesterone produced within the adrenal to 17-hydroxy steroids including cortisol and decreases active progestin release from the adrenal gland. In addition, immunoreactivity and qPCR analysis for AKR1C3 suggests that its expression occurs in the ZR of adrenal gland from both men and women (data not shown). Moreover, our LC/MS analysis demonstrated that the amount of 20α-hydroxyprogesterone is much smaller than that of progesterone in the adrenal vein (data not shown). To better define the role of AKR1C3 in human adrenal cell steroid production, we made use of the H295R adrenal cell line. This cell line has characteristics of both the ZF and ZR adrenal zones, expresses AKR1C3, and also secretes testosterone (19, 28). Depletion of AKR1C3 expression using siRNA led to a significant drop in H295R testosterone production, whereas no decrease was observed after decreases in AKR1C1. Using a pharmacological approach, we also showed that indomethacin, which has been shown to inhibit AKR1C3 activity, partially blocks adrenal cell testosterone production (19). These data support a role of AKR1C3 in human adrenal testosterone biosynthesis.

Although most research focuses on adrenal production of aldosterone, cortisol, and DHEA, the human adrenal gland produces a wide range of steroids and steroid precursors. There is considerable evidence that the adrenal contributes to the circulating levels of testosterone, particularly in women, where normal gonadal testosterone production is considerably less than that seen in men. In addition, there have been several reports that examined direct secretion of testosterone from the ovarian and adrenal veins (3, 7, 29–33). These studies focused on women with androgen excess and demonstrated that the majority of these women have higher levels of adrenal testosterone production. Parker *et al.* (31) and Greenblatt *et al.* (3) measured testosterone levels in the adrenal vein after iv administration of ACTH. In their studies, ACTH administration tended to increase testosterone levels in the female adrenal vein (3, 31). Herein, we confirmed that ACTH significantly elevated testosterone levels in the female adrenal vein samples (6.3-fold compared with that before ACTH stimulation). In addition, there did not appear to be a significant difference in ACTH-stimulated adrenal testosterone production based on menopausal status; however, further studies with a larger patient population are needed to confirm our preliminary observation.

Quinkler *et al.* (34) reported that human adipose tissue plays an important role in production of active androgen through the activity of AKR1C3. Thus, the role of AKR1C3 in producing testosterone in extratesticular tissue appears clear. However, Goodarzi *et al.* (35) have also recently demonstrated that polymorphisms in the AKR1C3 gene are not associated with serum testosterone levels in women. Thus, currently, there does not appear to be a genetic component to link excess testosterone to AKR1C3. However, based on the current study, it is postulated that human adrenal AKR1C3 may well provide a mechanism for the adrenal to contribute to the circulating pool of testosterone in women.

In summary, we have confirmed that testosterone is directly secreted from the human adrenal under the control of ACTH. Of the primary enzymes previously shown to convert androstenedione to testosterone, AKR1C3 was found to be expressed in human adrenal gland. Within the adrenal gland, AKR1C3 expression was higher in the ZR than in the ZF. *In vitro* knockdown of AKR1C3 expression in adrenal cells decreased production of testosterone. These results indicate that the expression of AKR1C3 in the ZR plays a role in adrenal testosterone production.

Acknowledgments

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Pathology

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Original Article

Peroxisome proliferator-activated receptor gamma in human prostate carcinoma

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Peroxisome proliferator-activated receptor (PPAR) is a member of the nuclear hormone receptor superfamily of transcription factors. Peroxisome proliferator-activated receptor gamma (PPARγ) plays an important role in the regulation of lipid homeostasis, adipogenesis, insulin resistance, and development of various organs. Agonists of PPARγ have been also reported to inhibit proliferation of prostate carcinoma cells as in other human malignancies, and these synthetic ligands have been used in differentiation-mediated therapy of various human carcinomas associated with high levels of PPARγ. The significance of PPARγ expression, however, was unknown in human prostate carcinoma tissues. The purpose of the present study was therefore to examine the immunolocalization of PPARγ in human prostate cancer tissues (40 cases) and correlate the findings with clinicopathological features of the patients in order to evaluate its possible biological significance. Twenty-nine patients were positive for PPARγ immunoreactivity (73%) and a significant inverse correlation was detected between PPARγ immunoreactivity, pT stage ($P = 0.036$), and serum concentration of prostate-specific antigen ($P = 0.0004$). In conclusion, PPARγ immunoreactivity is considered to be a new clinicopathological parameter of human prostate cancer.

Key words: immunohistochemistry, peroxisome proliferator-activated receptor gamma, prostate cancer, receptor

Peroxisome proliferator-activated receptor gamma (PPARγ) is a member of the nuclear hormone receptor superfamily, and has been designated NR1C3.[1,2] PPARγ functions as a transactivation factor following heterodimerization with retinoic X receptors and binds to its specific response elements termed 'peroxisome proliferating responsive elements' of various target genes.[3] PPARγ is one of the ligand-activated transcription factors, and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) is currently considered a naturally occurring PPARγ ligand.[4] It is well known that PPARγ plays an important role in adipogenesis, insulin resistance, and development of various organs.[5–7] Various *in vitro* studies have also demonstrated that PPARγ ligands have a potent antiproliferative activity against a wide variety of neoplastic cells.[4] Ota *et al.* reported a significant negative association between the body mass index and carcinoma PPARγ expression levels in patients with endometrial carcinoma, indicating that PPARγ is an important factor in forming a potential link between various aspects of so-called metabolic syndrome and development of some cancers.[8]

The expression of PPARγ has been extensively studied in breast and colon carcinoma. It has also been previously reported that PPARγ mRNA is expressed in primary human prostate cancer tissues and cell lines.[9,10] PPARγ is also reported to regulate E-cadherin expression and inhibit growth and invasion of prostate cancer.[11] It is thus very important to obtain a better understanding of the clinical and/or biological roles of PPARγ in human prostate cancer tissues in order to improve the potential clinical efficiency of PPARγ ligand therapy for prostate cancer patients. Little is known, however, about the clinicopathological features of PPARγ-positive prostate cancer. The aim of the present study was therefore to examine immunolocalization of PPARγ in 40 human prostate cancer patients, and correlate these findings with various clinicopathological parameters as a first step toward understanding the clinical significance of this nuclear receptor.

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MATERIALS AND METHODS

Patients and tissues

Forty surgical pathology specimens of prostate carcinoma were obtained from patients who underwent prostatectomy

between 1998 and 2003 at the Department of Urology, Tohoku University Hospital (Sendai, Japan). The mean age of the patients was 65.9 years (range, 54–77 years). All the patients examined in the present study did not receive radiation, chemotherapy, or hormone therapy prior to surgery. Clinical data, including patient age, serum prostate specific antigen (PSA) concentration, clinical stage, and lymph node status according to the International Union Against Cancer TNM classification (1987) were retrieved from detailed review of the patient charts. The histological grade and Gleason score of each tumor was evaluated by two of the authors (Y.N. and T.S.). All the specimens had been fixed in 10% formalin and embedded in paraffin wax at the Department of Pathology, Tohoku University Hospital. The Ethics Committee at Tohoku University School of Medicine approved the research protocol for the study.

Antibodies

Rabbit polyclonal antibody for PPARγ was raised against a synthetic peptide corresponding to amino acids 60–79 of mouse PPARγ1 (accession number AAA62110, GenBank), which also corresponds to amino acids 62-81 of human PPARγ1 (CAA62152, GenBank) or 90-109 of human PPARγ2 (AAB04028, GenBank).[12] This antibody therefore recognizes both human PPARγ1 and γ2.[12] Antibodies against androgen receptor (AR) and Ki-67 were purchased from Dako (Carpinteria, CA, USA) and Immunotech (Marseilles, France), respectively. Antibodies for estrogen receptor beta (ERβ) and progesterone receptor (PR) were also commercially obtained from Gene Tex (San Antonio, TX, USA) and NeoMarkers (Fremont, CA, USA), respectively.

Immunohistochemistry

Immunohistochemistry was done using the streptavidin–biotin amplification method using a Histofine Kit (Nichirei, Tokyo, Japan) as previously described.[13–15] For immunostaining the slides were heated in an autoclave at 120°C for 5 min in citric acid buffer (2 mmol/L citric acid and 9 mmol/L trisodium citrate dehydrate, pH 6.0) after deparaffinization for antigen retrieval. The dilutions of primary antibodies used in the present study were as follows: PPARγ, 1:1500; AR, 1:100; ERβ, 1:1500; and Ki-67, 1:50. The antigen–antibody complex was visualized with 3,3′-diaminobenzidine (DAB) solution (1 mmol/L 3,3′-DAB, 50 mmol/L Tris-HCl buffer (pH 7.6), and 0.006% H_2O_2) and counterstained with hematoxylin. Tissue sections of normal adrenal gland were used as a positive control for PPARγ; an invasive ductal carcinoma of the breast were used as a positive control for ERβ and PR; and normal prostate tissue was used as a positive control for AR. As for the negative controls, immunohistochemical preabsorption tests were performed for PPARγ, and normal rabbit IgG used instead of the primary antibody. No specific immunoreactivity was detected in these sections (data not shown).

Scoring of immunoreactivity

Evaluation of AR, ERβ, and Ki-67 immunoreactivity was performed in high-power fields (×400) using a standard light microscope. These results of nuclear immunoreactivity were independently reviewed by two of the authors (Y.N. and T.S.). We evaluated nuclear immunoreactivity of PPARγ based on our previous reports.[8,12] In all the cases examined, a total of more than 500 tumor cells from three different representative fields were counted independently by the two aforementioned authors, and the percentage immunoreactivity (i.e. labeling index (LI)), was determined in each case examined. After completely reviewing the immunostained sections of each lesion, all the examined cases were tentatively classified into the following two groups: +, >10% positive cells; –, <10% positive cells, for PPARγ immunoreactivity based on previous studies.[14,15]

Statistical analysis

Values for patient age, serum PSA levels, and LI for AR, ERβ, and Ki-67 are given as mean ± 95% confidence interval (95%CI), and associations between PPARγ immunoreactivity described here were evaluated using the unpaired *t*-test. Statistical differences between immunoreactivity for PPARγ and stage, lymph node status, and histological grade, and Gleason score were evaluated in a cross-table using the χ^2 test. $P < 0.05$ was considered significant.

RESULTS

PPARγ immunoreactive protein was predominantly detected in the nuclei of prostate carcinoma cells associated with weak cytoplasmic immunoreactivity (Fig. 1a). In normal prostate epithelium no cytoplasmic or nucleus immunoreactivity was detectable (Fig. 1b). In prostatic intraepithelial neoplasia (PIN) regions, weak immunoreactivity was detected in the cytoplasm but nuclear immunoreactivity was not confirmed (Fig. 1c).

Twenty-nine prostate cancers were defined as positive for PPARγ immunoreactivity (73%). There was a statistically significant inverse correlation between PPARγ immunoreactivity and pT stage ($P = 0.036$; Table 1). There was also a significant inverse correlation between PPARγ immunoreactivity

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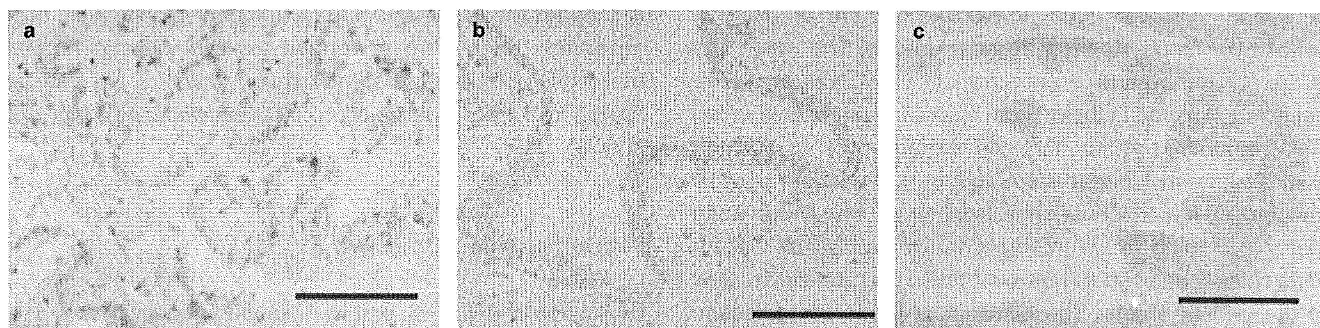

Figure 1 Immunoreactivity of peroxisome proliferator-activated receptor gamma (PPARγ) in human prostate cancer tissues. (**a**) PPARγ immunoreactive protein was detected in the nucleus of prostate carcinoma cells, but (**b**) it was negligible in the epithelial cells of non-neoplastic prostate glands. (**c**) In prostatic intra-epithelial neoplasia regions, weak staining was detectable in the cytoplasm but no nuclear staining was confirmed. Bars, 10 μm.

Table 1 PPARγ immunoreactivity and clinicopathological parameters in human prostate cancer

	Positive (n = 29)	Negative (n = 11)	P
Age (years)	65.9 ± 0.8	65.8 ± 1.9	0.950
PSA (ng/mL)	9.9 ± 1.4	25.7 ± 5.5	0.0004*
Gleason score, n (%)			
2–6	8 (20.0)	0 (0)	
7	11 (27.5)	5 (12.5)	
8–10	10 (25.0)	6 (15.0)	0.139
Stage, n (%)			
pT2	16 (40.0)	2 (5.0)	
pT3	13 (32.5)	9 (22.5)	0.036*
Lymph node status, n (%)			
Positive	1 (2.5)	1 (2.5)	
Negative	28 (70.0)	10 (25.0)	0.465
AR LI (%)	77.3 ± 3.1	66.3 ± 9.1	0.149
ERβ LI (%)	42.0 ± 5.2	43.3 ± 9.7	0.903
PR LI (%)	9.5 ± 2.2	4.1 ± 2.3	0.165
Ki-67 LI (%)	7.2 ± 0.9	8.9 ± 2.2	0.395

*P < 0.05.

AR, androgen receptor; ERβ, estrogen receptor beta; LI, labeling index; PPARγ, peroxisome proliferator-activated receptor gamma; PR, progesterone receptor; PSA, prostate-specific antigen.

and the concentration of serum PSA (P = 0.0004; Table 1). There were no significant correlations, however, between PPARγ and AR, ERβ, or Ki-67 immunoreactivity (Table 1). The status of immunoreactivity for PPARγ was not significantly correlated with other clinicopathological parameters (Table 1).

DISCUSSION

In the present study 29 cases were defined as positive for PPARγ immunoreactivity (73%). PPARγ expression has been demonstrated in various kinds of human cancer cells and tissues.[16,17] In those reports PPARγ was considered to have suppressive effects through inducing growth inhibition, apoptosis, and differentiation of tumor cells.[16,17] These findings indicate that PPARγ immunoreactivity in human ovarian tumor tissues was significantly higher than in normal ovaries and benign ovarian tumors, which is consistent with our results.[18] Ota *et al.*, however, reported that PPARγ mRNA expression in carcinoma tissues was lower than that in normal tissues.[8] Similar results have also been reported in esophageal, lung, and ureter carcinomas.[19–21] Ikezoe *et al.* examined the expression of PPARγ in 339 clinical samples of various human malignancies and 71 cancer cell lines, including colon cancer, breast cancer, prostate cancer, lung cancer, osteosarcoma, glioblastoma, and leukemia.[22] They reported that all of the cell lines and clinical specimens that they examined expressed PPARγ, but the expression levels varied widely among different types of carcinoma.[22] Therefore, the aforementioned results indicate that the expression of PPARγ is dependent on tissue specificity and/or the mutational events that are required for cancer development.

In the present study PPARγ immunoreactivity was predominantly detected in the nuclei of prostate carcinoma cells, but relatively weak cytoplasmic immunoreactivity was

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detected in both prostate carcinoma and PIN regions. No cytoplasmic or nuclear immunoreactivity was detected in normal prostate epithelium. Matsuyama and Yoshimura also recently reported that PPARγ immunoreactivity in human prostate cancer tissues and PIN was more abundant than in benign prostatic hyperplasia tissues and normal prostate tissue.[23] Therefore, these findings suggest that cell differentiation may be part of the process in which PPARγ ligands mediate anti-proliferation in prostate cancer cells.[24] PPARγ has been also reported to act as a tumor suppressor through an involvement in cell cycle withdrawal and by promoting cell differentiation.[24] It is also true, however, that there have been studies reporting that PPARγ may act as a tumor promoter.[24–27] In addition, the tumor suppressor activity of PPARγ may be considered cell-type specific due to the presence or absence of co-factors in different cell types.[24,27] Therefore, all of these findings indicate that PPARγ expression may be associated with the process of cell differentiation of human prostate cancer, and that it plays an important role in regulating development of carcinoma cells, but that further investigation is required for clarification.

The present results also indicated a statistically significant inverse or negative correlation between PPARγ immunoreactivity and pT stage. PPARγ activation was previously demonstrated to inhibit the proliferation of prostate carcinoma cells, indicating that PPARγ expression level may be related to prostate cancer development and the potential application of its specific agonist.[10,28–31] Matsuyama and Yoshimura, however, also reported that there was a statistically significant positive correlation between PPARγ immunoreactivity and Gleason score.[23] In the present study there was no significant positive correlation between PPARγ immunoreactivity and Gleason score. The difference between the present results and those of the Matsuyama and Yoshimura study may be due to the different immunoreactivity evaluation system used. In addition, Matsuyama and Yoshimura considered both nuclear and cytoplasmic staining as positive.[23] In the present study we interpreted only equivocal presence of nuclear immunoreactivity as positive staining, as has been indicated in the majority of previous immunohistochemical evaluations of this nuclear receptor.[8,12,19–22,32] PPARγ protein has been reported to be localized mainly in the nuclei of various cells and has been postulated to have a genomic function as a nuclear receptor in human neoplastic tissues.[8,32] Results of a recent study suggested that PPARγ was also detected in the cytoplasm, and was reported to possess extra-nuclear/non-genomic actions in mammalian cells.[33] These results suggest that PPARγ protein is produced in the cytoplasm, but is predominantly present in the nuclei of prostate carcinoma cells. Therefore, PPARγ may play an important role in regulating development of carcinoma cells as a nuclear orphan receptor in human prostate cancer tissues. Further investigation is required, however, to clarify the significance of PPARγ expression in both the nucleus and cytoplasm in human prostate carcinoma cells. In the present study the number of cases examined was limited and did not include pT1 and pT4 due to tissue availability. In addition, Paltoo *et al.* reported that there was no direct association between the variant allele of PPARγ and both prostate cancer risk.[24] Therefore, further examination including the analysis of more cases is required for confirming the conclusions regarding the association between cancer development and PPARγ expression level.

The present results also demonstrated a significant inverse correlation between PPARγ immunoreactivity and serum PSA levels. It was recently reported that preoperative PSA levels are associated with increased inherent risks of biochemical progression of prostate carcinoma.[34] Hisatake *et al.* reported that troglitazone, a PPARγ agonist, suppressed PSA protein expression by inhibiting androgen receptor response element activation.[28] 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) is also currently considered an endogenous PPARγ ligand.[4] The aforementioned findings suggest that the high level of PPARγ expression possibly suppresses prostate cancer growth by binding its ligand, and its loss may result in the development of prostate carcinoma.

No significant correlations were detected between PPARγ and AR, ERβ, or PR immunoreactivity in the present study. Prostate cancer is known as a sex steroid-dependent tumor and it is also known that androgens play important roles in the pathogenesis and development via AR.[35] ERβ and PR were also reported to be present in prostate cancer tissues and are known to modify the biological course of prostate cancer.[36,37] Hisatake *et al.*, however, reported that PPARγ agonists did not repress expression of the AR in a human prostate cancer cell line.[28] Wang and Kilgore demonstrated that estradiol treatment inhibited the ligand-stimulated transactivation of PPARγ in human breast cancer cells expressing ERα but not ERβ.[38] These findings are consistent with the present results demonstrating no significant correlation between PPARγ and AR, or ERβ expression levels in human prostate cancer. Kim *et al.* also reported that PPARγ expression is regulated by PR in the granulosa cells of the preovulatory follicles during the ovulatory process.[39] The tissue levels of progesterone, however, are generally considered to be very low in human prostate compared to ovarian tissue. Therefore, progesterone is not considered to alter PPARγ expression levels in human prostate cancer.

In summary, we have demonstrated that PPARγ protein was detected in the majority of human prostate cancer tissue. In addition, immunoreactivity for PPARγ was inversely and significantly correlated with pT stage and serum PSA levels. These results all indicated that PPARγ immunoreactivity is considered a new biological marker of human prostate cancer, including the potential application of its specific agents in therapy.

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17β-hydroxysteroid dehydrogenase type 11 (Pan1b) expression in human prostate cancer

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Androgens are reported to be actively produced *in situ* in human prostate cancer. These locally produced androgens are also demonstrated to play important role in the pathogenesis and development of human prostate cancer. The status of locally produced androgen inactivation and metabolism, however, remains unclear. Therefore, it is important to examine the status of this *in situ* androgen metabolism and inactivation in order to improve clinical response to endocrine therapy in the patients diagnosed with prostate cancer. 17β-hydroxysteroid dehydrogenase type 11 (Pan1b) was demonstrated to display greatest activity with 5α-androstan-3α, 17β-diol (3α-diol) as substrate in several human androgen metabolizing tissues, suggesting that this enzyme may play important role in androgen metabolism. However, its details including the expression level of Pan1b have not been studied in human prostate cancer. In this study, we evaluated immunolocalization of Pan1b in human prostate cancer specimens obtained from surgery (n=40), and correlated the findings with clinicopathological features of the patients in order to study its clinical significance. Pan1b immunoreactivity was detected in 19 cases (48%) and was significantly associated with cancer of seminal vesicle invasion (P<0.05). These data suggest that Pan1b expression could be connected wth advanced prostate cancer.

Key words: 17ß-hydroxysteroid dehydrogenase type XI, prostate cancer, immunochemistry

Androgens play important role in the pathogenesis of human prostate cancer [1]. *In situ* production of androgens has been also suggested to play a pivotal role in the pathogenesis and/ or development of human prostate cancer [1, 2]. Suppression of androgen secretion and/or a blockade of their actions represent the basis for many forms of effective hormonal treatment of the patients diagnosed with prostate cancer [3]. However, the status of further metabolism or inactivation of these locally produced androgens still remains unclear. Therefore, it becomes very important to examine the levels of expression of androgens metabolizing and inactivating enzymes in the prostate cancer tissue in order to obtain a better understanding of the possible roles of *in situ* androgen metabolizing.

Human 17β-hydroxysteroid dehydrogenase type 11 (Pan1b) is known to display greatest activity with 5α-androstan-3α, 17β-diol (3α-diol) as substrate and to convert it to androsterone in several human androgen metabolizing tissues, suggesting its possible roles in human androgen metabolism [4, 5]. 3α-diol is known to be capable of stimulating cell proliferation in androgen-sensitive prostate cancer cell line (LNCaP) [6]. Laplante *et al.* demonstrated that Pan1b mRNA level was higher than that of 17β-hydroxysteroid dehydrogenase type 5 (AKR1C3), one of androgen-producing enzymes in LNCaP cells [6]. However, the status of this Pan1b in human prostate cancer has not been studied at all. Therefore, in this study, we examined Pan1b immunoreactivity in human prostate cancer, and correlated the findings with the status of androgen receptor (AR), estrogen receptor beta (ERβ), and other relevant clinicopathological findings in order to explore the possible biological significance of this androgen-matabolizing enzyme in human prostate cancer.

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Materials and Methods

Patients and tissues. Forty surgical specimens of prostate carcinoma were obtained from the patients who underwent prostatectomy from 1998–2003 at the Department of Urology, Tohoku University Hospital (Sendai, Japan). The mean age of the patients was 65.9 y (range: 54–77 y). All patients examined in this study did not receive radiation, chemotherapy, or hormone therapy before surgery. Clinical data, including patient age, serum prostate specific antigen (PSA) concen-

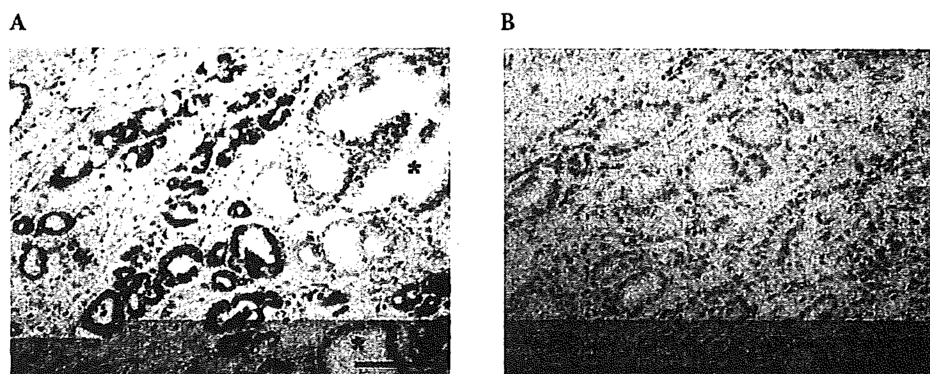

Figure 1. Immunoreactivity of Pan1b in human prostate cancer. A: Pan1b immunoreactive protein was detected in the cytoplasm of prostate carcinoma cells, but it was negligible in the epithelial cells of non-neoplastic prostate glands (*). B: No specific immunoreactivity was observed in the negative control section. Bar = 100 μm, respectively.

tration, clinical stage, and lymph node status according to the International Union Against Cancer TNM classification (1987), and Gleason score were retrieved from detailed patient charts describing individual patient histories. The histological grade of each tumor was evaluated by two of the authors (Y.N. and T.S.). All specimens were fixed with 10% formalin and embedded in paraffin wax at the Department of Pathology, Tohoku University Hospital. The Ethic's Committee at Tohoku University School of Medicine approved the research protocol for this study.

Antibodies. Rabbit polyclonal antibody for HUP1 (Pan1b) was kindly provided by Dr. Krozowski (Baker Heart Research Institute, Central Melbourne, Australia) [4, 5]. Antibodies against AR and Ki-67 were purchased from DAKO Corporation (Carpinteria, CA) and Immunotech (Marseilles, France), respectively. Antibodies for ERβ were also commercially obtained (Gene Tex, San Antonio, TX)

Immunohistochemistry Immunohistochemical analysis was performed employing the streptavidin-biotin amplification method using a Histofine Kit (Nichirei, Tokyo, Japan) and has been previously described in detail [2]. The dilutions of primary antibodies used in our study were as follows: Pan1b, 1: 200; AR, 1: 100; ERβ, 1: 1,500; and Ki-67, 1: 50. The antigen–antibody complex was visualized with 3,3'-diaminobenzidine (DAB) solution [1 mmol/l 3,3'-DAB, 50 mmol/l Tris-HCl buffer (pH 7.6), and 0.006% H_2O_2] and counterstained with hematoxylin. Tissue sections of normal adrenal glands were used as positive controls for Pan1b, an invasive ductal carcinoma of the breast were used as positive controls for ERβ, and normal prostate tissue was used as a positive control for AR. As for negative controls, immunohistochemical preabsorption tests were performed for Pan1b, and normal rabbit IgG used instead of the primary antibody. No specific immunoreactivity was detected in these sections (data not shown).

Scoring of immunoreactivity. Scoring of immunoreactivity was performed based on previous reports [2, 7, 8]. For statistical analyses of Pan1b immunoreactivity, the carcinoma cases were tentatively classified into the following two groups: +, positive, more than 10% positive cells; and –, no immunoreactivity, less than 10% positive cells [2]. The evaluation (+, positive carcinoma cells; and – no immunoreactivity) was performed by two of the authors (Y. N. and T. S.). Scoring of AR, ERβ, and Ki-67 in carcinoma cells was performed on high power fields (X400) using standard light microscopy. In each case, more than 500 carcinoma cells were counted independently by two of the authors above, and the percentage of immunoreactivity, i.e. labeling index (LI), was determined [2, 7, 8]. We evaluated all the slides containing carcinoma cells in each individual case.

Statistical analysis. Values for patient age, serum PSA levels, and LI for AR, ERβ, and Ki-67 were presented as the mean±95% confidence interval (95% CI), and associations between Pan1b immunoreactivity and the parameters described above were evaluated using the unpaired-t test. Statistical differences between Pan1b immunoreactivity and other clinicopathological factors were evaluated in a cross-table using the χ^2-test. $P<0.05$ was considered significant.

Results

Pan1b immunoreactive protein was detected in the cytoplasm of prostate carcinoma cells (Figure 1). Its immunoreactivity was, however, not detected or very weakly detectable in human normal prostate (Figure 1). Nineteen cases were defined as positive for Pan1b immunoreactivity (48%). There was a statistically significant positive correlation between Pan1b immunoreactivity and the status of seminal vesicle invasion ($P<0.05$) (Table 1). However, Pan1b immunoreacitivity was not significantly correlated with other clinicopathological parameters including patient age, concentration of serum PSA levels, Gleason score of carcinoma, extracapsular extension, or lymph node status (Table 1). There were no significant cor-

relations between Pan1b immunoreactivity and AR, ERβ, or Ki-67 immunoreactivity (Table 2).

Discussion

In this study, we demonstrated Pan1b immunoreactivity which was detected in human prostate cancer in approximately 50% of examined cases. Values of this immunoreactivity were significantly correlated with the status of seminal vesicle invasion.

We previously reported that androgen-producing enzymes were co-expressed in human prostate cancer, and were involved in the local production of testosterone and 5α-dihydro-testosterone (DHT), which may play important roles in biological behaviors of prostate carcinoma cells [2]. These locally produced androgens are also possibly metabolized and inactivated in human prostate cancer tissue. For example, aromatase was also reported to be expressed and in human prostate cancer tissue, possibly metabolizing testosterone into estrone (E1) [9]. In addition, AKR1C2, one of the human members of the aldo-keto reductase (AKR) 1C gene family, was reported to be expressed and convert DHT to 3α-diol in both human prostate cancer cell line and tissue [10, 11]. Therefore, results of our present study suggest that Pan1b may contribute to conversion of 3α-diol to androsterone in human prostate cancer tissue.

Chai *et al.* previously reported high levels of Pan1b mRNA expression in the human pancreas, kidney, liver, lung, adrenal, ovary, and heart [5]. They also demonstrated that Pan1b immunoreactivity is detectable in steroidogenic cells such as syncytiotrophoblasts, sebaceous glands, Leydig cells, and granulosa cells of the dominant follicle and corpus luteum [5]. The steroidogenic Y1 mouse cell is also demonstrated to express Pan1b protein [5]. Therefore, it is reasonably postulated that Pan1b plays an important role in steroid metabolism of these tissues. The lowest expression of Pan1b has been, however, reported in the normal prostate as well as in human skeletal muscle, brain, stomach, thymus, and colon [5]. Pan1b is reported to be expressed in LNCaP cell line known as androgen-dependent prostate cancer cell line [6]. Results of our present study demonstrated that Pan1b is abundantly expressed in human prostate cancer tissue, but not in the normal prostate. All these findings suggest that Pan1b expression is possibly induced by the malignancy in human prostate cancer tissue.

In our present study, immunoreactivity for Pan1b was positively correlated with the status of seminal vesicle invasion. The prostate cancer patients with seminal vesicle invasion are considered unlikely to experience long-term biochemical (PSA) remission from cancer recurrence [12]. We previously reported that AKR1C3 immunoreactivity was positively associated with extracapsular extension in human prostate cancer tissues, which suggest that locally produced testosterone may play a role in etiology and development of human prostate cancer [2, 13]. However, androgen metabolism in advanced prostate cancer tissue may also be accelerated because of increasing testosterone production. 3α-diol is also known to be capable of stimulating cell proliferation in LNCaP cells [6]. Therefore, the elevated Pan1b expression level is considered to be associated with acceleration of androgen production and metabolism in advanced human prostate cancer tissues, and may be related to adverse clinical outcome of the patients with prostate cancer with seminal vesicle invasion. However, Pan1b immunoreactivity was not correlated with other malignant parameters including, lymph node involvement, Gleason score, or Ki-67 LI in our present study. Therefore, further studies are required to clarify the degree of involvement of the expression of Pan1b in cell proliferation and development of prostate cancer. In contrast, Pan1b immunoreactivity was not correlated with AR and ERβ LIs in our present study. Pan1b is well-known

Table 1. Correlation between Pan1b immunoreactivity and clinicopathological parameters in human prostate cancer specimens.

	Positive (n=19)	Negative (n=21)	*P* value
Age (years)	66.1±1.2	65.7±1.1	0.812
PSA (ng/ML)	15.1±3.1	13.5±2.9	0.705
Gleason score			
2–6	3 (7.5%)	5 (12.5%)	
7	8 (20.0%)	8 (20.0%)	
8–10	8 (20.0%)	8 (20.0%)	0.818
Stage			
pT2	8 (20.0%)	10 (25.0%)	
pT3	11 (27.5%)	11 (27.5%)	0.726
Extracapsular extension			
Positive	7 (17.5%)	9 (22.5%)	
Negative	12 (30.0%)	12 (30.0%)	0.698
Seminal vesicle invasion			
Positive	6 (15.0%)	1 (2.5%)	
Negative	13 (32.5%)	20 (50.0%)	0.026
Lymph node status			
Positive	2 (5.0%)	0 (0%)	
Negative	17 (47.5%)	21 (47.5%)	0.127

Table 2. Correlation between Pan1b and AR, ERβ, and Ki-67 immunoreactivity in human prostate cancer specimens.

	Positive (n=19)	Negative (n=21)	P value
AR LI (%)	70.5±5.5	77.7±4.1	0.292
ERβ LI (%)	42.8±7.0	41.9±6.1	0.921
Ki-67 LI (%)	7.2±1.2	8.1±1.3	0.583

to convert 3α-diol to androsterone as described above [4, 5]. However, to the best of our knowledge, no studies reported the association between these steroids and steroid hormone receptor expression in human prostate cancer. On the other hand, Tchédam-Ngatcha *et al.* reported that androsterone derivatives do not bind to AR or ER [14]. In addition, Yan *et al.* recently reported that 3α-diol contributed to androgen-independent prostate cancer progression [15]. Therefore, Pan1b expression may not influence AR or ERβ expression levels in human prostate cancer. However, in this study, the number of cases examined was rather limited due to tissue availability and further investigation including the analysis of more cases may be required for confirming the reliability of conclusions obtained in this study.

In summary, we demonstrated that Pan1b protein was detected in approximately 50% of prostate cancer specimens that we examined, and Pan1b expression may have an effect on the character of advanced prostate cancer.

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The Mediator Complex Subunit 1 Enhances Transcription of Genes Needed for Adrenal Androgen Production

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There are three enzymes involved in the biosynthesis of the adrenal androgen dehydroepiandrosterone (DHEA) sulfate. Cholesterol side-chain cleavage (CYP11A1) and 17α-hydroxylase/17,20-lyase (CYP17) metabolize cholesterol into DHEA, whereas steroid sulfotransferase family 2A1 (SULT2A1) is responsible for conversion of DHEA to DHEA sulfate. We previously examined the mechanisms regulating CYP11A1, CYP17, and SULT2A1 transcription and found that each is regulated, in part, by the transcription factor GATA-6. Previous studies suggested that mediator complex subunit 1 (MED1, also called PPARBP or TRAP220) is a cofactor involved in not only the regulation of nuclear receptors but also the activation of GATA-6 transcription. Herein we demonstrated a role for MED1 in the regulation of CYP11A1, CYP17, and SULT2A1 transcription. Transient transfection assays with SULT2A1 deletion and mutation promoter constructs allowed the determination of specific the GATA-6 binding *cis*-regulatory elements necessary for transactivation of SULT2A1 transcription. Binding of MED1 and GATA-6 was confirmed by coimmunoprecipitation/Western analysis and chromatin immunoprecipitation assay. We demonstrated expression of MED1 mRNA and protein in the human adrenal and determined that knockdown of MED1 expression via specific small interfering RNA attenuated CYP11A1, CYP17, and SULT2A1 expression levels in H295R cells. In addition, we demonstrated that MED1 enhanced GATA-6 stimulated transcription of promoter constructs for each of these genes. Moreover, the activity of MED1 for SULT2A1 promoter was mediated by GATA-6 via the −190 GATA-binding site. These data support the hypothesis that MED1 and GATA-6 are key regulators of SULT2A1 expression, and they play important roles in adrenal androgen production. **(*Endocrinology* 150: 4145–4153, 2009)**

The production of dehydroepiandrosterone sulfate (DHEAS) within the adrenal cortex relies on three steroid-metabolizing enzymes. Cholesterol side-chain cleavage (CYP11A1) and 17α-hydroxylase/ 17,20-lyase (CYP17) are involved in the conversion of cholesterol to dehydroepiandrosterone (DHEA) (1). CYP11A1 performs the conversion of cholesterol to pregnenolone, whereas CYP17 catalyzes the 17α-hydroxylation of pregnenolone and, subsequently, the 17,20-lyase reaction on its 17α-hydroxy derivative to DHEA. CYP11A1 and CYP17 are expressed in both the zona fasciculata (ZF) and zona reticularis (ZR) of the human adrenal cortex (1). On the other hand, steroid sulfotransferase family 2A1 (SULT2A1), commonly known as steroid sulfotransferase, has been localized by immunohistochemistry to the DHEA-producing adrenal ZR, in which it catalyzes the conversion of DHEA to DHEAS (1–3). Although the enzymatic activity of SULT2A1 has been studied in some detail, little is known about the regulation of human SULT2A1 expression. We previously reported the role of

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Abbreviations: ChIP, Chromatin immunoprecipitation; CYP11A1, cholesterol side-chain cleavage; CYP17, 17α-hydroxylase/17,20-lyase; CYP21, 21-hydroxylase; DHEA, dehydroepiandrosterone; DHEAS, DHEA sulfate; HSD3B2, 3β-hydroxysteroid dehydrogenase type 2; MED1, mediator complex subunit 1; qPCR, quantitative real-time RT-PCR; RNAi, RNA interference; SF-1, steroidogenic factor 1; siRNA, small interfering RNA; StAR, steroidogenic acute regulatory protein; SULT2A1, steroid sulfotransferase family 2A1; ZF, zona fasciculata; ZR, zona reticularis.

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three transcription factors, steroidogenic factor 1 (SF-1 or NR5A1), GATA-6, and estrogen-related receptor-α (or NR3A1) in the regulation of SULT2A1 transcription (4, 5). However, the potential for transcription factor coactivator enhancement of adrenal ZR SULT2A1 expression has not been studied.

The mediator complex subunit 1 (MED1; also called PPARBP or TRAP220) was originally identified as a coactivator for peroxisome proliferator-activated receptor-γ but later shown to participate in the transactivation of other nuclear receptors (6–11). In addition, it is suggested that MED1 is a cofactor involved in not only the activation of nuclear receptors but also the activation of other groups of transcription factors (6). Crawford *et al.* (6) demonstrated that MED1 interacts with five GATA family transcription factors, including GATA-4 and GATA-6. The MED1 coactivator activity was independent of the nuclear receptor recognition sequence motif LXXLL, in which L is leucine, and *X* is any amino acid (6). GATA-6 is highly expressed in the adult and fetal adrenal cortex and is capable of regulating transcription of steroidogenic enzymes, including CYP11A1, CYP17, and SULT2A1 (12–14). Saner *et al.* (4) previously reported that GATA-6 is a positive regulator of SULT2A1 transcription through a GATA-binding site in the proximal promoter.

Whereas MED1 is widely expressed in normal and tumor tissues, its potential role in the adrenal gland has not been examined (8, 15–19). Herein we demonstrated that MED1 was expressed in the human adrenal gland and that it enhanced adrenal cell CYP11A1, CYP17, and SULT2A1 gene expression. MED1 enhancement of SULT2A1 was through interactions with GATA-6, which bound to its respective response elements on the regulatory region of the SULT2A1 gene. The current study supports a potential role for MED1 in the regulation of adrenal DHEAS production.

Materials and Methods

Human tissue attainment

Human adult adrenal gland, brain, heart, kidney, liver, lung, premenopausal ovary, salivary gland, skeletal muscle, and thymus were obtained through the Cooperative Human Tissue Network (Philadelphia, PA), CLONTECH (Palo Alto, CA), University of Texas Southwestern Medical Center (Dallas, TX), and Tohoku University School of Medicine (Sendai, Japan). The use of these tissues was approved by the Institutional Review Boards of the University of Texas Southwestern Medical Center, Medical College of Georgia (Augusta, GA), and Tohoku University School of Medicine.

Cell culture

The human adrenocortical cell line (H295R) was used for all transfection experiments and was routinely cultured in DMEM/Ham F12 medium (Life Technologies, Inc., Carlsbad, CA) supplemented with 2.5% Ultroser G (Life Sciences, Cergy, France), penicillin, streptomycin (Life Technologies), gentamicin (Sigma-Aldrich, St. Louis, MO), and 1% insulin, human transferrin, selenious acid + universal culture supplement premix (BD Biosciences, Bedford, MA) (20). HEK293T cells were routinely cultured in DMEM/Ham F12 medium (Life Technologies, Inc.), 10% cosmic calf serum (HyClone, Logan, UT), and antibiotics consisting of penicillin, streptomycin, and gentamicin.

RNA extraction, cDNA synthesis, and quantitative real-time RT-PCR (qPCR)

The protocol for cDNA synthesis was previously described in detail (21). We measured the relative expression levels of MED1 mRNA in H295R cells and human tissues described above. Analysis of MED1 was performed using primers and probes from TaqMan gene expression assays (Applied Biosystems, Foster City, CA). qPCRs were performed using the ABI 7500 fast real-time PCR system (Applied Biosystems). Quantitative normalization of cDNA in each tissue-derived sample was performed using expression of 18S rRNA as an internal control. Transcript expression was normalized as previously described (21).

Immunohistochemistry

Immunohistochemical analysis was performed on serial sections of adrenal tissue, using the streptavidin-biotin amplification method using a U.T.R. Vectastain kit (Vector Laboratories, Burlingame, CA). Antibodies used included SULT2A1 (rabbit polyclonal; Abcam, Cambridge, MA), GATA-6 (rabbit polyclonal; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and MED1 (rabbit polyclonal, kindly provided by Dr. R. G. Roeder, Laboratory of Biochemistry and Molecular Biology, Rockefeller University, New York, NY) (9). Antigen retrieval was performed by heating the slides in a microwave oven for 15 min in citric acid buffer [2 mM citric acid and 9 mM trisodium citrate dehydrate (pH 6.0)]. The dilutions of the MED1 and GATA-6 primary antibodies used in this study were 1:200. The antigen-antibody complex was visualized with 3.3′-diaminobenzidine solution [1 mM 3.3′-diaminobenzidine; 50 mM Tris-HCl buffer (pH 7.6); and 0.006% H_2O_2] and counterstained with hematoxylin. For positive control, a normal rat liver section was used (19). For negative control, normal rabbit or mouse IgG was used instead of primary antibodies.

Transfection of MED1 small interfering RNA (siRNA) and examination of adrenal steroidogenic enzyme expression in H295R cells

siRNA of MED1 was commercially obtained from Dharmacon (Chicago, IL). As a negative control, Stealth RNA interference (RNAi) negative control duplexes were also used (Invitrogen Life Technologies, Inc., Carlsbad, CA). Electrical transfection assays were performed using the Nucleofector system (AMAXA, Gaithersburg, MD). Briefly, H295R cells were cultured to 80–90% confluence in growth medium and then trypsinized and resuspended in Nucleofector Solution R (AMAXA) at a ratio of 5 million cells per 100-μl solutions. Indicated amounts of MED1 siRNA or Stealth RNAi negative

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control duplexes (100 nM at final concentration) were added to the solution, and the mixture was run under program T20 in the Nucleofector system. Cells were allowed to recover for 48 h before treatment. Both RNA and protein were isolated from the cells for qPCR and Western analysis. For qPCR, primers and probes for the amplification of the selected human SULT2A1 sequence were done using TaqMan gene expression assays (Applied Biosystems). The primer/probe sets for human steroidogenic acute regulatory protein (StAR), CYP11A1, 3β-hydroxysteroid dehydrogenase type 2 (HSD3B2), CYP17, and 21-hydroxylase (CYP21) were designed using Primer Express 3.0 (Applied Biosystems) and purchased from Integrated DNA Technologies, Inc. (Coralville, IA). For Western analysis, a polyclonal human anti-MED1 (Abcam) and a monoclonal human anti-β-actin antibody (Sigma-Aldrich) were used. In addition, after transfection, the cells were incubated for 72 h and then incubated for 6 h in a low-serum medium containing 0.1% cosmic calf serum and 10 μM 22(R)-hydroxycholesterol (Sigma). The medium was collected for DHEA measurement using ELISA (ALPCO Diagnostics, Salem, NH) and DHEAS measurement using RIA (Diagnostic Systems Laboratories, Webster, TX).

Preparation of reporter constructs and expression vectors

The 5′-flanking DNA from the human genes for CYP11A1, CYP17, and SULT2A1 were inserted upstream of the firefly luciferase gene in the reporter vector pGL3basic (Promega, Madison, WI) (4). Mutations to the previously defined GATA-binding site (−190) in the SULT2A1 promoter were created by completely removing this site with a −332 deletion construct. Empty pGL3basic served as the control vector to measure basal reporter activity in all transfections. The human MED1 vector was also kindly provided by Dr. R. G. Roeder (Laboratory of Biochemistry and Molecular Biology, Rockefeller University, New York, NY) (9). The construct pMALT, encoding the full-length human GATA-6, was a gift from Dr. Christopher Gove (King's College, London, UK) (22). The plasmids for GATA-6 short (MYQ) and long form (M147L) have been described previously (4).

Transfection study

Transient transfection assays were performed using Transfast (Promega) in a ratio of 4 μl Transfast per microgram of DNA and the indicated amounts of expression vectors. Cells were harvested 24 h after recovery and assayed for luciferase activity using the luciferase assay system (Promega). To normalize luciferase activity, cells were cotransfected with 50 ng/well of β-galactosidase plasmid (Promega).

Coimmunoprecipitation and Western analysis

H295R cells were grown to confluence on 10-cm dishes and lysed by passive lysis buffer (Promega). After centrifugation, supernatant was then incubated with Protein A/G PLUS-Agarose (catalog no. sc-2003; Santa Cruz Biotechnology) and rabbit IgG (Santa Cruz Biotechnology) for 1 h. After centrifugation, supernatant was then incubated with Protein A/G PLUS-Agarose and a polyclonal MED1 antibody for 24 h. The pellets were washed four times. The isolated protein complexes were denatured for 5 min at 95 C and followed by Western blotting analysis with the polyclonal GATA-6 antibody at a 1:200 dilution.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using H295R cell lysates; EZ-ChIP (Upstate, Charlottesville, VA), MED1 polyclonal antibody, and GATA-6 polyclonal antibody. DNA was diluted into 20 μl of nuclease-free water, and 5 μl were used for each PCR of 32 cycles. The PCR primer sequences were designed based on a previous report showing the primary GATA-binding *cis*-element in the SULT2A1 promoter region (−190 site) (4). The PCR primer sequences used were as follows: SULT2A1 promoter, forward, 5′-ACTCTCAGGAACGCAAGCTC-3′, reverse, 5′-ACCTTGTCCCAGCATGTCAC-3′. Products were then separated on a 4% agarose gel for detection of promoter region amplification.

Statistical analysis

Results are given as the mean ± SE where appropriate. Statistical analyses were done by unpaired t test or one-way ANOVA, followed by *post hoc* test for comparisons between two groups dependent on the data types. Significance was accepted at the 0–0.05 level of probability ($P < 0.05$).

Results

Human adrenal tissue expresses MED1 mRNA and protein

qPCR was performed using mRNA isolated from several types of human tissues and H295R cells (Fig. 1). The level of MED1 transcript was significantly higher in H295R cells compared with all other human tissues that were examined ($P < 0.05$) (Fig. 1). The expression level of MED1 mRNA was not significantly different among human tissues examined in this study (Fig. 1). It is likely that MED1 mRNA is widely expressed in human organs including both steroidogenic and nonsteroidogenic tissues.

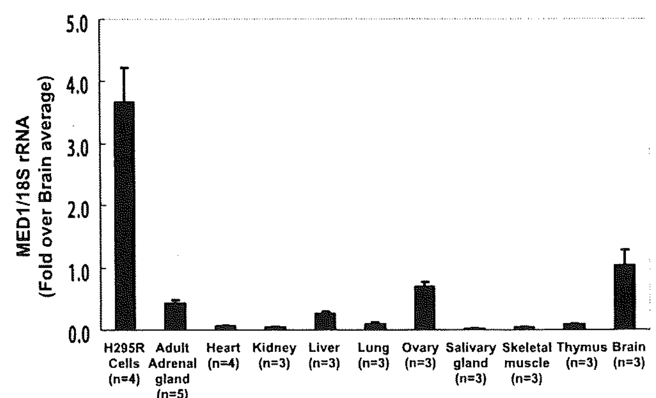

FIG. 1. Quantification of MED1 transcript levels in human tissues and H295R cells. Levels of MED1 mRNA were compared in human adult adrenal gland, brain, heart, kidney, liver, lung, premenopausal ovary, salivary gland, skeletal muscle, thymus, and H295R cells using qPCR and normalized to 18s rRNA. Data points are expressed as the fold over the average expression levels seen in the brain (19). The level of MED1 transcript was significantly higher in H295R cells compared with any types of human tissues that were examined ($P < 0.05$). However, the expression level of MED1 mRNA was not significantly different among human tissues examined.

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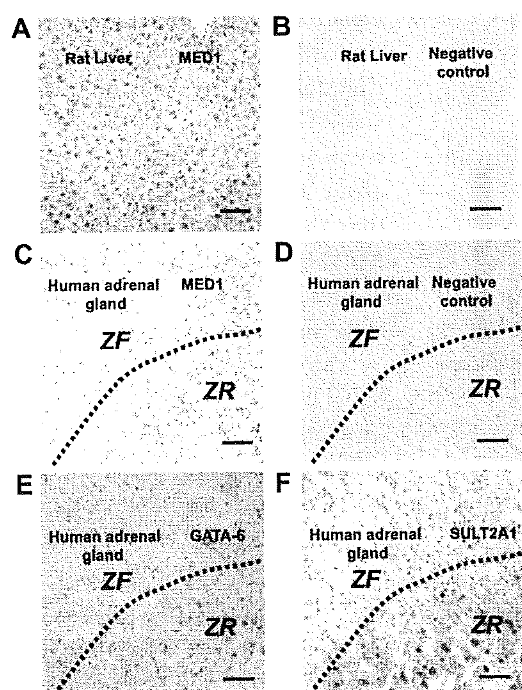

FIG. 2. Immunohistochemical localization of MED1 in the human adrenal gland. MED1 immunoreactivity was detected in the nuclei of rat liver cells (positive control) (A). As a negative control, primary antibody was replaced with buffer only, and no specific immunoreactivity was detected in liver cells (B). MED1 immunoreactivity was also detected in the nuclei of cortical cells of the adrenal cortex (C). When primary antibody was replaced with buffer alone, there was no specific immunoreactivity (D). Immunohistochemical analysis of GATA-6 (E) and SULT2A1 (F) in human adult adrenal gland. *Bar*, 10 μm.

MED1 immunoreactivity was examined in rat liver (positive control) and human adrenal gland (Fig. 2). MED1 immunoreactivity was detected in nuclei of both the liver and adrenocortical cells, including ZF and ZR (Fig. 2C). No staining was observed in the absence of MED1 antibody (Fig. 2D). We also confirmed that GATA-6 was expressed in both the ZF and ZR (Fig. 2E), whereas SULT2A1 was predominantly expressed in the ZR (Fig. 2F), consistent with previous reports (4, 23).

Effects of MED1 down-regulation on adrenal cell steroidogenic enzyme transcript levels and steroid production

To determine whether MED1 plays a role in the expression of steroid-metabolizing enzymes, we used siRNA to decrease its expression in H295R adrenal cells. Seventy-two hours after transfection of H295R cells with MED1-specific siRNA, the levels of MED1 mRNA dropped by 80% and protein level by 40%, compared with control cells (NTC) (Fig. 3A). SULT2A1 mRNA levels were significantly decreased by 40% in the H295R cells after MED1 siRNA transfection (Fig. 3B). CYP11A1 and CYP17 mRNA levels were also significantly decreased in the H295R cells after MED1 siRNA transfection (Fig. 3B). StAR, HSD3B2, and CYP21 mRNA levels did not significantly decrease afterMED1 siRNA transfection (Fig. 3B).

To examine the effects of MED1 knockdown on H295R cell production of DHEA and DHEAS, cells were incubated with 22(R)-hydroxycholesterol (10 μM) for 6 h. This steroid precursor is rapidly metabolized by steroidogenic cells and provides a measure of steroidogenic capacity (24). Seventy-two hours after transfection of cells with MED1 siRNA, there was a decrease in the production of both DHEA and DHEAS (~40% for each) (Fig. 3, C and D).

MED1 activates the transcription of CYP11A1, CYP17, and SULT2A1

We previously showed that transcription of SULT2A1, CYP11A1, and CYP17 are increased by GATA-6 and SF-1 (4, 13). To determine whether these genes were regulated by MED1 with GATA-6 or SF-1, we used promoter constructs for each of these steroidogenic genes in transient transfection of H295R cells. We cotransfected each of the reporter constructs with SF-1 or GATA-6 alone or in combination with MED1. Compared with transfected SF-1 alone, there was no significant additive effect of MED1 on the induction of any of the reporter gene activities (Fig. 4). However, GATA-6 showed an additive stimulation of reporter gene activities for CYP11A1, CYP17, and SULT2A1 when cotransfected with MED1 (Fig. 4).

Both isoforms of human GATA-6 increases SULT2A1 gene transcription with MED1

It has been reported previously that both the human and mouse genomes have two isoforms of GATA-6, which use two distinct promoters and initiation codons (22). Both isoforms are present in the fetal and adult mouse tissues in which GATA-6 is normally present (25, 26). In humans, both the long and short isoforms of GATA-6 are highly expressed in ovarian theca cells and adrenal cells (4, 27).

Our Western analysis demonstrated that input of H295R cell nuclear protein had both the long and short GATA-6 proteins, with the short isoform being the predominantly expressed protein, as previously reported (Fig. 5A) (4). In our coimmunoprecipitation and Western analysis, the complex of cell lysates and MED1 antibody could detect distinct bands that corresponded to both short and long GATA-6 isoforms (Fig. 5A). However, the negative control with nuclear extract of H295R cells and rabbit IgG instead of MED1 antibody did not lead to distinct bands for either isoform (Fig. 5A). The results indicate that MED1 interacts with both short and long isoforms of GATA-6 in H295R cells (Fig. 5A).

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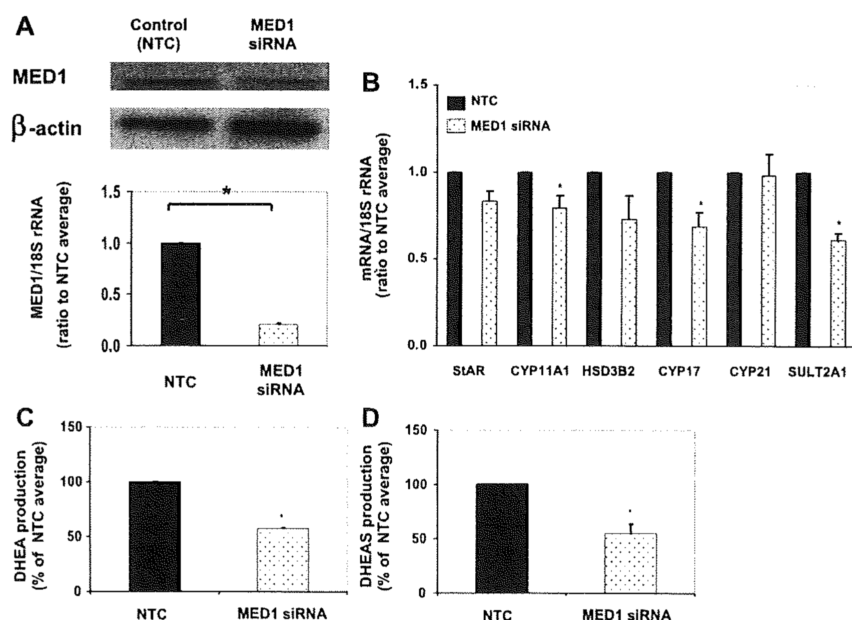

FIG. 3. Effects of siRNA depletion of MED1 on the transcript levels of adrenal steroidogenic enzymes. A, H295R adrenal cells were transfected with or without siRNA against MED1 (MED1 siRNA) or Stealth RNAi (negative control; NTC). After 48 h, mRNA and protein for MED1 were detected by qPCR and Western analyses, respectively. 18S rRNA and β-actin protein were used for normalization. Data are presented as mean ± SE for three independent experiments. *, $P < 0.05$. B, Transcript levels for adrenal steroidogenic enzymes were determined using qPCR. StAR, CYP11A1, HSD3B2, CYP17, CYP21, and SULT2A1 mRNAs were examined 48 h after H295R cells were transfected with or without siRNA against MED1 (MED1 siRNA) or Stealth RNAi (NTC). Data are presented as mean ± SE for three independent experiments. *, $P < 0.05$. C and D, Production of DHEA (C) and DHEAS (D) by H295R cells 72 h after MED1 siRNA transfection. To examine the capacity to produce DHEA and/or DHEAS, cells were treated with MED1 (MED1 siRNA) or Stealth RNAi (NTC). After this treatment, the cells were incubated for 6 h with 10 μM 22(R)-hydroxycholesterol. Data are presented as mean ± SE of values from three independent experiments and expressed as a percent of NTC. *, $P < 0.05$, compared with NTC.

To determine whether MED1 increased SULT2A1 transcription by either the long or short isoforms of GATA-6, we cotransfected the SULT2A1 reporter constructs with both isoforms of GATA-6 in combination with MED1. Transient transfection analyses were performed in H295R cells, with expression vectors encoding either the long isoform (MALT), the short isoform (MYQ), or a mutated form that could encode only the long isoform (M147L). Both forms of GATA-6 additively stimulated transcription of SULT2A1 with MED1 (Fig. 5, B–D).

MED1 enhances SULT2A1 transactivation by GATA-6 via a specific DNA binding site on the SULT2A1 promoter

We previously reported that GATA-6 transactivation of SULT2A1 was lost only when the −190 GATA-binding site was deleted or mutated. In this study, we used both deletion and mutation analysis to determine whether the −190 GATA-binding site was necessary for optimal MED1 activation of SULT2A1. As in our previous study, transient transfections were done in HEK293T cells to avoid interactions with adrenal cell SF-1 (13). We demonstrated that deletion or mutation of the −190 GATA-binding site blocked transactivation of SULT2A1 by GATA-6 alone and with MED1. This suggests that the −190 site was necessary for MED1- and GATA-6-mediated activation of SULT2A1 gene transcription (Fig. 6, A and B).

The binding of MED1 to the SULT2A1 promoter region was demonstrated using ChIP assay in H295R cells. ChIP assay using MED1 antibody demonstrated interaction with the −190 site in the SULT2A1 promoter (Fig. 6C). ChIP assay without antibody (negative control) did not show the band corresponding to MED1 (Fig. 6C, *upper panel*). MED1 is known to lack a DNA binding domain (6). However, we have previously shown that GATA-6 directly binds the SULT2A1 promoter (4). In this study, ChIP assay using GATA-6 antibody also demonstrated interaction with the −190 site in the SULT2A1 promoter (Fig. 6C, *lower panel*). Taken with our current coimmunoprecipitation and Western analysis, these findings suggest that MED1 association with the SULT2A1 promoter is likely through GATA-6 and its binding to the SULT2A1 promoter.

Discussion

In this study, we demonstrated that MED1 mRNA and protein were expressed in the human adrenal cortex. In addition, MED1 was found to additively increase the effects of GATA-6 on the transcription of CYP11A1, CYP17, and SULT2A1. Moreover, the ability of MED1 to increase SULT2A1 promoter activity was mediated by GATA-6 via its −190 DNA binding site. These data support the hypothesis that adrenal androgen production can be regulated by GATA-6 and MED1.

MED1 is known to be ubiquitously expressed in many mouse and rat tissues (17–19, 27–29). MED1 expression is also seen in the human brain and uterus as well as breast and lung cancer cells (8, 15, 19). However, to our knowledge, there is no previous study demonstrating the expression of MED1 in the adrenal gland. In this study, we demonstrated abundant expression MED1 mRNA in H295R

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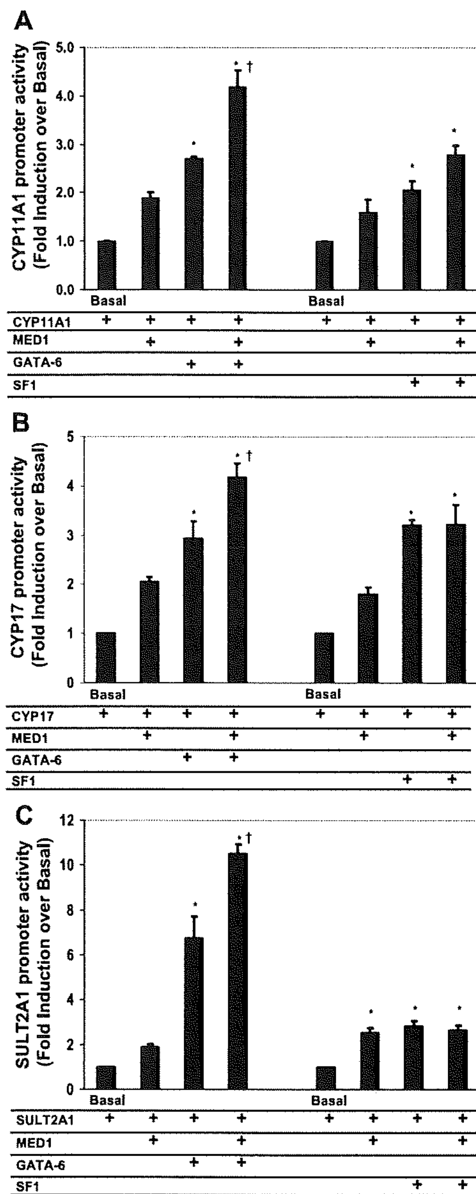

FIG. 4. Comparison of the effects of MED1 and GATA-6 or SF-1 on the transcriptional activity of CYP11A1 (A), CYP17 (B), or SULT2A1 (C) reporter gene activity after transfection with reporter gene constructs. Luciferase promoter constructs containing CYP11A1, CYP17, or SULT2A1 (1 μg/well) was cotransfected in H295R cells with MED1 expression plasmid (0.1 μg/well) and GATA-6 plasmid (0.1 μg/well). H295R cells were lysed and assayed for luciferase activity 24 h after transfection. Data were normalized to cotransfected β-galactosidase expression vector, and data are expressed as fold induction over basal reporter. Results represent the mean ± SE of data from at least three independent experiments, each performed in triplicate. *, $P < 0.05$, compared with basal level; †, $P < 0.05$, compared with only GATA-6-transfected group.

adrenal cells and that MED1 protein is expressed in the nuclei of human adrenocortical cells. MED1 was previously shown to participate in the transactivation of both nuclear receptors and transcription factors (6–11). MED1 also has a function in the regulation of growth, differentiation and metabolism of the central nervous system through nuclear receptors (19). Therefore, we postulated

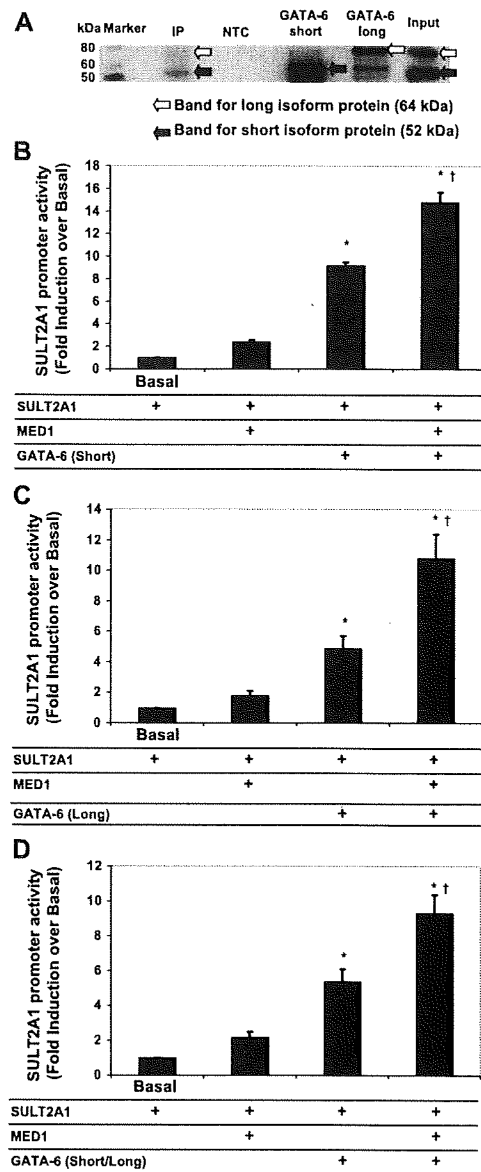

FIG. 5. Comparison of GATA-6 isoforms on MED1-enhanced SULT2A1 gene transcription. A, Coimmunoprecipitation with nuclear extract of H295R cells using a MED1 antibody. The complex of cell lysates and MED1 antibody (IP) allowed detection of distinct bands for both short (52 kDa) and long (64 kDa) GATA-6 isoforms, which were also seen in the input sample (input). The negative control (NTC) was prepared with nuclear extract of H295R cells and rabbit IgG instead of MED1 antibody. *In vitro*-prepared short isoform (GATA-6 short) and long isoform (GATA-6 long) represented lysates obtained from H295R cells that had elevated expression of each isoforms after transient transfection with the respective expression vector and acted as positive controls. B–D, *In vitro*-prepared short isoform (GATA-6 short), long (GATA-6 long) isoform, and both short and long isoforms (GATA-6 short/long) proteins were also included as positive controls. MED1 effects on SULT2A1 reporter gene activity with GATA-6 short (B), GATA-6 long (C), or GATA-6 short/long (D) isoforms are shown. Luciferase promoter constructs containing SULT2A1 (1 μg/well) were cotransfected in H295R cells with MED1 expression plasmid (0.1 μg/well) and GATA-6 short (B), GATA-6 long (C), or GATA-6 short/long (D) isoforms plasmid (0.1 μg/well). Cells were lysed and assayed for luciferase activity 24 h after transfection. Data were normalized to cotransfected β-galactosidase expression vector, and data shown are expressed as the fold induction over basal reporter. Results represent the mean ± SE of data from at least three independent experiments, each performed in triplicate. *, $P < 0.05$, compared with basal level; †, $P < 0.05$, compared with only GATA-6-trasfected group.

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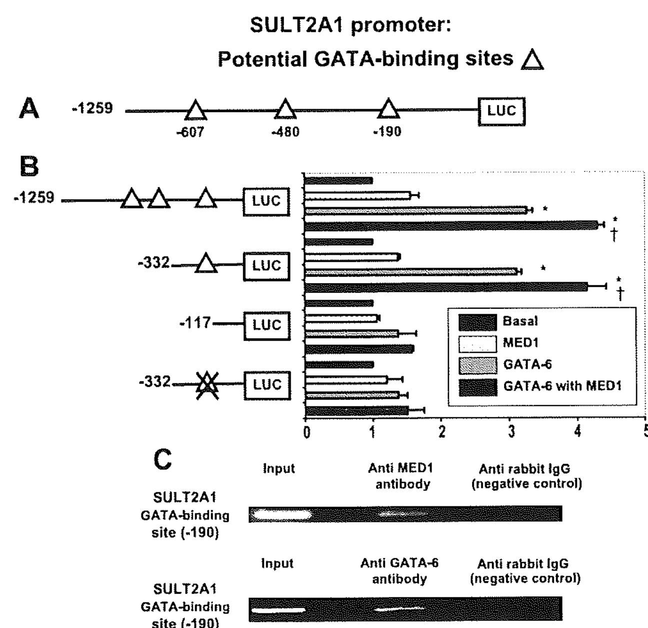

FIG. 6. The role of the −190 GATA binding *cis*-element in the regulation of SULT2A1 transcription. A, A schematic representation of SULT2A1 promoter with potential GATA binding sites. *Triangles* represent potential binding sites, and the *numbers below* represent the base pair at which the site begins (based on the translational start site). B, Deletion and mutation analysis in HEK293T cells. A series of pGL3 reporter constructs containing progressively smaller amounts of SULT2A1 5′-flanking DNA (1 μg/well) were cotransfected in HEK293T cells with MED1 expression plasmid (0.1 μg/well) and GATA-6 plasmid (0.1 μg/well). Data were normalized to cotransfected β-galactosidase expression vector. Results represent the mean ± SE of data from at least three independent experiments, each performed in triplicate. *, $P < 0.05$, compared with basal level; †, $P < 0.05$, compared with the GATA-6 only transfection group. C, ChIP assay using H295R nuclear lysate demonstrated a distinct band corresponding to the −190 site of the SULT2A1 promoter (input). Immunoprecipitation with either GATA-6 or MED1 antibodies showed that both proteins were associated with the −190 site in the SULT2A1 promoter. ChIP assay was performed as described in *Materials and Methods*.

that adrenal MED1 acts as a transcriptional coactivator that regulates adrenal differentiation.

CYP11A1 and CYP17 are known to play critical roles in cholesterol conversion to DHEA in the adrenal ZR. Both genes are also required for production of cortisol and therefore are present in both the adrenal ZF and ZR (4, 30). On the other hand, SULT2A1 is predominantly expressed in the cytoplasm of adrenocortical cells in the ZR, in which it acts to convert DHEA to DHEAS. Herein we demonstrated a role for MED1 in regulating expression of CYP11A1, CYP17, and SULT2A1 in H295R cells. MED1 knockdown decreased expression of these genes, whereas increased expression of MED1 augmented transcription of reporter constructs driven by their promoters. In addition, we demonstrated that knockdown of MED1 in adrenal cells decreased production of DHEA and DHEAS. Together these experiments suggest a role for MED1 in adrenal androgen biosynthesis.

MED1 participates in the transactivation of both nuclear receptors and transcription factors (6, 28, 29). Crawford *et al.* (6) previously demonstrated that MED1 interacts with five GATA factors using a mouse model: GATA-1, GATA-2, GATA-3, GATA-4, and GATA-6. GATA-6 and the nuclear hormone receptor SF-1 are highly expressed in the adrenal cortex and have previously been shown to have a role in regulating expression of CYP11A1, CYP17, and SULT2A1 (4, 13, 14, 31, 32). In this study, we examined the ability of MED1 to influence SF-1 and GATA-6 regulation of the enzymes needed for DHEAS biosynthesis. Whereas MED1 had no significant effect on SF-1 induction of these genes, we demonstrated that MED1 enhanced the transcriptional activation of CYP11A1, CYP17, and SULT2A1 by GATA-6. Based on these findings, our results also indicate that MED1 interacts with GATA-6 protein to enhance the capacity for adrenal androgen production.

To further detail the mechanisms of MED1 action, we demonstrated that deletion or mutation of the SULT2A1 −190 GATA-binding site resulted in a loss of GATA-6 as well as MED1/GATA-6 transactivation. We previously reported that GATA-6 transactivation of SULT2A1 was due to its direct interaction with the −190 GATA-binding site (4). On the other hand, MED1 is known not to have a direct DNA binding domain (6). However, our current coimmunoprecipitation and Western analysis showed that GATA-6 and MED1 interact with each other. These findings along with the results of our ChIP assay suggest that MED1 does not directly bind to the SULT2A1 promoter; rather, it enhances GATA-6 mediated transcription. Human GATA-6 consists of two isoforms transcribed using two distinct promoters (22). The long isoform of GATA-6 (MALT) encodes a protein of 595 amino acids, whereas the short isoform (MYQ) encodes a protein of 449 amino acids. We previously reported that in normal human adrenal gland, the long form was preferentially expressed, whereas in the H295R cells, the short isoform was the major isoform present (4). These observations agree with the results of our current coimmunoprecipitation showing the difference of intensity between the bands of short and long forms. However, both our previous and current studies suggest that there was no significant difference in the effects of either isoform on SULT2A1 transcription. MED1 interactions with the two GATA-6 isoforms have not been previously studied. Herein we demonstrate that the transcriptional activation of SULT2A1 by both isoforms is enhanced by MED1.

Compared with transcription factors, the potential roles for transcription factor coactivators in adrenal androgen production have not been well studied (4, 5). In the current study, we demonstrated that the stimulatory effect

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