Table 1. Primers and Antibodies Used in This Study

Gene	Primer for PCR	Reference	Antibody	Reference
MR	Forward: CAGTCTCCAGTCCCAATAATGT Reverse: GCAGTGTAGCTGAAGGCATTGT		H10E4C9F (Alexis Corp.)	Lombes et al[13]
11β-HSD type 2	Forward: ACGCAGGCCACAATGAAGTAG Reverse: GCAGCCAGGCTGGATGATGATG	Arcuri et al[12]	HUH23	Krozowski et al[14]
MDM2 (TG)	Forward: TGTAAGTGAACATTCAGGTG Reverse: TTCCAATAGTCAGCTAAGGA	Ohtani et al[16]	sc-965 (Santa Cruz)	Tan et al[19]
BIRC5	Forward: TCCGGTTGCGCTTTCCT Reverse: TCTTCTTATTGTTGGTTTCCTTTGC	Williams et al[17]		
RPL13A	Forward: CCTGGAGGAGAAGAGGAAAAG Reverse: TTGAGGACCTCTGTGTATTT			
GAPDH	Forward: TGAACGGGAAGCTCACTGG Reverse: TCCACCACCCTGTTGCTGTA			

It was determined that the target gene (TG) in this study was *MDM2* by microarray and real-time PCR analyses. The reason for this determination is described in detail in Results.

using microarray analysis.[1] Therefore, in this study, we first screened aldosterone-responsive genes involved in regulation of the cell cycle using microarray and quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analyses as a confirmation of the findings in the cell line derived from MR-positive human VSMCs. We then used immunoblotting and immunoflourescence analysis to further evaluate the expression level of protein of the mineralocorticoid-responsive gene to further confirm the results of microarray analysis above. Eplerenone, a specific MR blocker, has been demonstrated to protect extrarenal tissues from various aldosterone-induced damage.[1,3,9] Thus, in this study we also examined whether eplerenone may also inhibit an induction of this aldosterone-induced gene product in cultured human VSMCs. We then studied whether the gene product was involved in VSMC proliferation using small interfering RNA (siRNA) of the gene transfection. It then becomes important to examine relative abundance of the gene products in VSMCs of human cardiovascular system in correlation to serum aldosterone levels. So finally, we examined relative abundance of a gene product in VSMCs of human small resistance arteries obtained from patients of hypertension with primary aldosteronism and/or nonfunctioning adrenocortical tumors and normal or normotensive subjects using immunohistochemistry.

Materials and Methods

Cell Culture and Characterization

A cultured human VSMC cell line, ie, HASMC (derived from human abdominal aorta) was commercially obtained from Kurabo Corp. (Osaka, Japan). Characteristics of this cell line have been reported by Iseki et al.[10] It was cultured in a 75-cm^2 flask with F12-K medium containing 5% fetal bovine serum (FBS) at 37°C in a 5% CO_2 atmosphere. We examined whether these cells expressed both MR and 11β-hydroxysteroid dehydrogenases (11β-HSD) type 2 using RT-PCR, immunoblotting, and immunocytochemistry, as reported previously.[1,11] Primers and antibodies used in this study are summarized in Table 1.[12-14]

Gene Chip Microarray Assay and Real-Time PCR Study

HASMC was cultured until a subconfluent state was obtained. The medium was then replaced with FBS-free and phenol red-free medium (modified Eagle's medium) (Sigma, St. Louis, MO) to arrest cell proliferation. After 24 hours, the medium was replaced again with phenol red-free and FBS-free medium in the presence of aldosterone (10 nmol/L) or vehicle (0.1% ethanol). After incubation for 8 hours, the cells were subsequently subjected to total RNA extraction for microarray analysis. Total RNA was prepared as previously described.[11] Microarray analysis was performed using a Human 1A Oligo Microarray (Agilent Technologies, Palo Alto, CA) with *in situ* synthesized 60-mer oligonucleotides representing 17,086 unique human genes. The procedures were described in detail in a previous report.[15] In this study, the ratios represented the values up- or down-regulated by 10 nmol/L aldosterone treatment compared with control values. We independently repeated the same experiment twice, and the differences were calculated to further confirm aldosterone-related changes in gene expression obtained from microarray analysis. The ratios of genes increased by more than 2.0-fold by both replicates of 10 nmol/L aldosterone treatment were considered up-regulated via MR when compared with control values. When analyzing possible functions of these detected genes, we used the homepage of HUGO Human Gene Nomenclature Committee (*http://www.gene.ucl.ac.uk/nomenclature/*) for further examination. These results of microarray analyses were also confirmed by replicated quantitative RT/real-time PCR study. In this study, we regarded a gene that was also significantly up-regulated in RT/real-time PCR study as the target gene (TG) among the genes associated with mitogenesis and cell proliferation that were found to be significantly induced by aldosterone treatment in microarray analysis. Further RT/real-time PCR study was performed in the TG as follows. The VSMCs were further treated with aldosterone (100 pmol/L, 10 nmol/L), aldosterone (10 nmol/L) with eplerenone (100 nmol/L; Pfizer Inc., New York, NY), or vehicle. In addition, two additional flasks were treated with aldosterone (10

nmol/L) after pretreatment with inhibitors of RNA transcription, actinomycin D (ACD; 100 nmol/L; Sigma), or protein translation (Sigma), cycloheximide (CHX; 100 nmol/L; ICN Biomedicals Inc., Irvine, CA). After incubation for 8 hours, the cells were subsequently subjected to total RNA extraction for RT/real-time PCR analysis for target products mRNA expression. The detailed procedure of RT/real-time PCR analysis was previously described.[11] The mRNA levels for target product in each VSMC are summarized as a ratio of RPL13A and evaluated as a ratio (percentage) compared with that of each control cDNA. The information of primers used in this study is summarized in Table 1.[16,17] We independently triplicated the same experiments for confirmation.

Immunoblotting Analysis

HASMC was cultured in the phenol red-free medium containing 5% FBS in the presence of aldosterone (10 nmol/L) with and/or without eplerenone (100 nmol/L) or in the presence of vehicle (0.1% ethanol). After incubation for 48 hours, the cells were subsequently subjected to nuclear extraction for immunoblotting analysis. An immunoblotting analysis was performed using 60 μg of each protein. The detailed procedure was previously reported.[11,18] In brief, protein samples were electrophoretically transferred to a polyvinylidene difluoride membrane, immunoblotted with antibodies against TG product, and visualized using chemiluminescence techniques.[19] The relative amounts of TG protein levels for each band were standardized to the relative OD units as reported previously.[11] In addition, the relative amount of TG protein was evaluated as a ratio compared with control (percentage).[18] We also independently triplicated the same experiments to confirm the findings.

Immunoflourescence

HASMC was cultured in the phenol red-free medium containing 5% FBS in the presence of aldosterone (10 nmol/L) with and/or without eplerenone (100 nmol/L) or in the presence of vehicle (0.1% ethanol). After incubation for 48 hours, the cells were subsequently fixed, stained, and visualized using TG antibody (Table 1), fluorescein isothiocyanate-conjugated secondary antibody, 4,6-diamidino-2-phenylindole for nuclear staining, and conventional fluorescence microscopy according to the manufacturer's instructions. We also independently triplicated the same experiments for confirmation.

siRNA Preparation, Transfection, and Cell Count Assay

siRNAs against TG were commercially obtained from Qiagen (Valencia, CA) and transfected to HASMC. HASMC was then seeded in a 6-well plate at an initial concentration of 100,000 cells/well with F-12K medium containing 5% FBS and cultured until a subconfluent status was achieved. The medium was then replaced with phenol red-free and FBS-free medium to arrest the cell proliferation. After 24 hours, transfections of siRNA for endogenous gene targeting (0, 10, or 100 nmol/L) were performed with RNAiFect transfection reagent (Qiagen), respectively. After transfection, the cells were incubated in phenol red-free medium containing 5% FBS with aldosterone (10 nmol/L) with and/or without eplerenone (100 nmol/L) or in vehicle (0.1% ethanol). We measured the number of cells in each sample as described above after incubation for 48 and 72 hours, respectively, according to a previous report.[19] We also examined the number of cells treated by aldosterone (10 nmol/L) with and/or without eplerenone (100 nmol/L), respectively. To evaluate efficiency of transfection to the cells, we examined relative TG mRNAs levels in these cells at 24 hours after transfection of the specific siRNAs. As a positive control, transfections of siRNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 0, 10, or 100 nmol/L; Amersham Biosciences, Piscataway, NJ) were also performed, and relative GAPDH mRNAs were evaluated in the cells. The mRNA levels in each VSMC are summarized as a ratio of RPL13A and were normalized as the ratio at no treatment (0 nmol/L), respectively.

Human Adrenal Specimens

Thirty-six cases of adrenocortical tumors (24 primary aldosteronism associated with hypertension and 12 nonfunctioning adenomas associated with no clinical hormonal abnormalities, including normal aldosterone level but with hypertension) were retrieved from surgical pathology files of Tohoku University Hospital. In addition, 12 specimens of histopathologically normal adrenal glands were obtained from autopsy files of individuals without histories of hypertension (from Tohoku University Hospital, Sendai, Japan). These specimens were fixed in 10% formalin for 24 to 48 hours at room temperature and embedded in paraffin wax. The research protocols for this study were approved by the ethics committee at Tohoku University School of Medicine (2004-355).

Immunohistochemistry

Immunohistochemical analysis was performed using the specific antibody against TG protein (Table 1) by streptavidin-biotin amplification method using a Histofine kit (Nichirei, Tokyo, Japan), as previously described in detail.[11,19,20] After a thorough review of immunostained tissue sections, relative immunoreactivity for TG protein in VSMCs of at least 25 resistant small arteries (100 to 300 μm in diameter) adjacent to adrenal tumors or normal glands per case were evaluated by labeling index (LI), a quantitative value that evaluates the numbers of cells positive for TG immunoreactivity in VSMCs.[11] The LIs were independently and blindly evaluated by three of the authors (Y.N., T.S., and H.S.) to obtain immunohistochemical data objectively, and the mean of the three values was used for analysis.

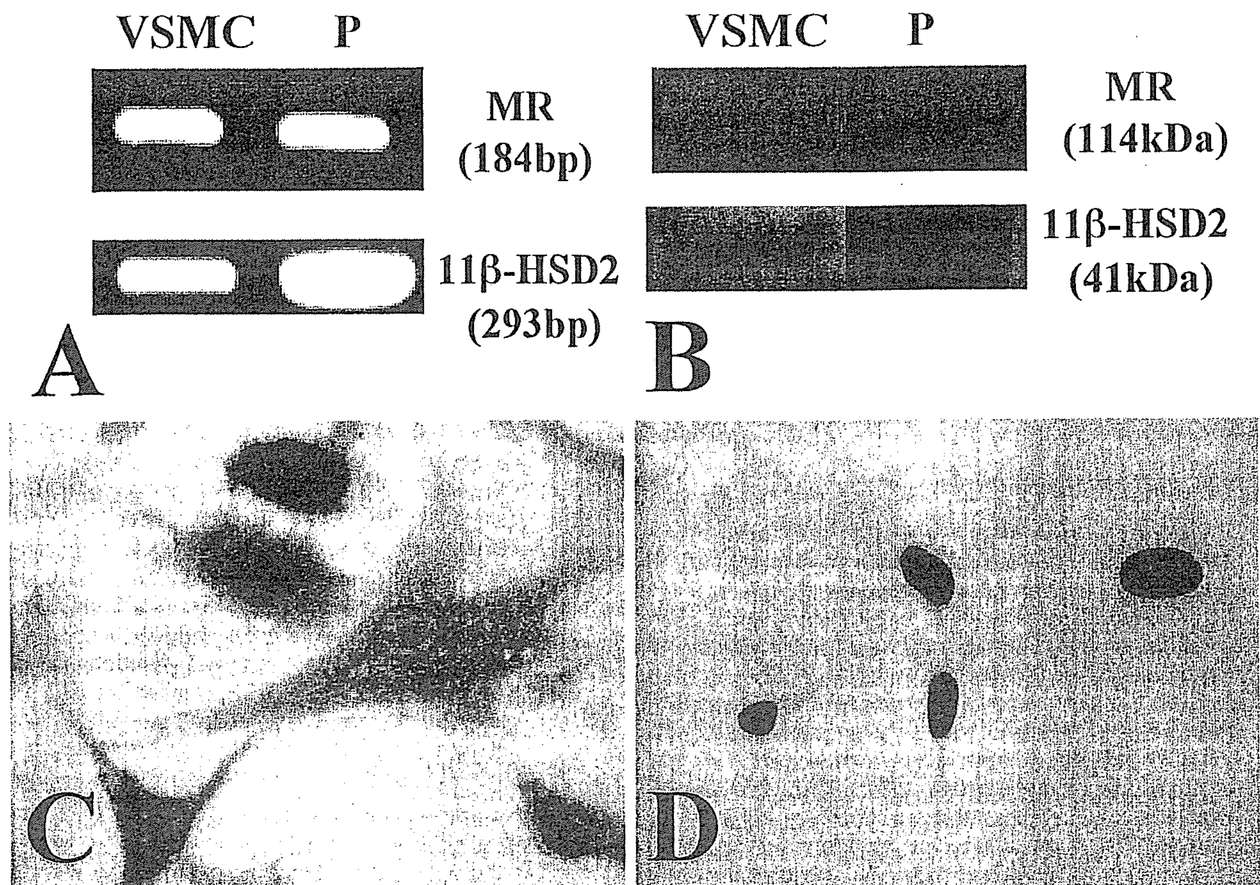


Figure 1. A: Representative RT-PCR analysis of total RNA from cultured human VSMCs (HASMC). RNA was amplified in the presence of oligonucleotide primers specific for MR (**top panel**) and 11β-HSD type 2 (**bottom panel**). Extracts from human kidney were used as a positive control (P). **B:** Representative immunoblotting studies demonstrating MR and 11β-HSD type 2 proteins in HASMC. Extracts from human kidney were used as a positive control (P). **C** and **D:** Immunocytochemical analysis demonstrated that immunopositive cells for MR appear brown as a result of diaminobenzidine colorimetric reaction in HASMC without ligand stimulation (**C**) or treated with 10 nmol/L aldosterone (**D**) (Original magnification, ×400).

Statistical Analysis

Values for all results are shown as mean ± SD. We used one-way analysis of variance followed by the Bonferroni test for comparisons between two different groups. A *P* value less than 0.05 was considered significant in this study.

Results

MR Expression in HASMC

RT-PCR study confirmed that both MR and 11β-HSD type 2 mRNAs were detected in HASMC (Figure 1A). Negative

Table 2. Aldosterone-Induced Genes Demonstrated by Microarray and Quantitative RT/Real-Time PCR (qRT-PCR) Analyses

Gene	Molecular function	Microarray fold change	qRT-PCR	
			Fold change	*P* value
MDM2	Cell growth/ inhibitor of apoptosis	2.2	2.3 ± 0.4	<0.05
BIRC5	Inhibitor of apoptosis	2.0	1.4 ± 0.5	Not significant
LCP1	Immune response/ inflammation	3.2	Not examined	
IGSF4B	Immune response /inflammation	3.0	Not examined	
CMRF-35H	Immune response/ inflammation	2.8	Not examined	
IL1R2	Immune response/ inflammation	2.8	Not examined	
SLC13A5	Citrate transporter	2.8	Not examined	
SLC22A8	Organic anion transporter	2.3	Not examined	

Ratios represent mean fold activation of gene expression in VSMCs treated by aldosterone for 8 hours compared with vehicle control. Results of qRT-PCR are shown as mean ± SD.

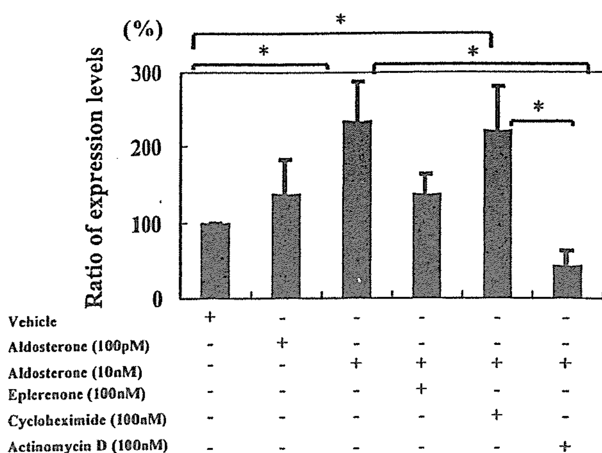
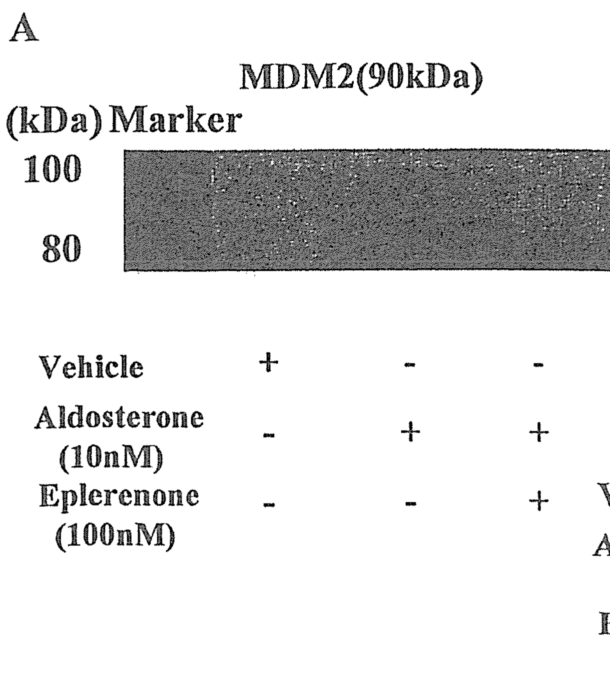


Figure 2. Results of RT/real-time PCR analysis for *MDM2* in HASMC among cells treated with vehicle (0.1% ethanol) (control), aldosterone alone (100 pmol/L, 10 nmol/L), aldosterone (10 nmol/L) with eplerenone (100 nmol/L), aldosterone (10 nmol/L) with CHX, and aldosterone (10 nmol/L) with ACD (100 nmol/L), respectively, after 8 hours. Data are shown as mean ± SD (*$P < 0.05$).

controls demonstrated no discernible bands (data not shown). MR and 11β-HSD type 2 proteins were also detected in HASMC (Figure 1B). Cellular localization of MR protein in the cells was also studied by immunocytochemical analysis. It was demonstrated that MR protein expression was detectable in both the nucleus and the cytoplasm without ligand stimulation (Figure 1C). After exposure to aldosterone, MR was solely found in the nucleus of the cells (Figure 1D). These findings were similar to results of previous study.[1]

GeneChip Microarray Assay and RT/Real-Time PCR Study

Table 2 summarized the genes associated with expression ratios of above 2.0 after 8 hours of duplicated 10 nmol/L aldosterone treatment in MR-positive HASMC. These genes were related to mitogenesis/cell growth, inflammation, and transporter of various substances. Among these genes, *MDM2* and *BIRC5* were known to be involved in cell proliferation and anti-apoptosis.[16,17,21] In addition, among these two genes, only *MDM2* was significantly promoted by aldosterone compared with control in a RT/real-time PCR study (Table 2). Therefore we further examined the features of *MDM2* as an aldosterone-responsive gene in HASMC and whether *MDM2* was associated with aldosterone-induced promotion of MR-positive VSMC proliferation using further quantitative RT/real-time PCR, immunoblotting analysis, immunoflourescence analysis, and siRNA transfection assay described above. Results of RT/real-time PCR study demonstrated that aldosterone with CHX also significantly increased *MDM2* expression in HASMC compared with controls ($P < 0.05$) (Figure 2). However, aldosterone with eplerenone and aldosterone with ACD did not increase expression of these mRNAs (Figure 2). Results of both



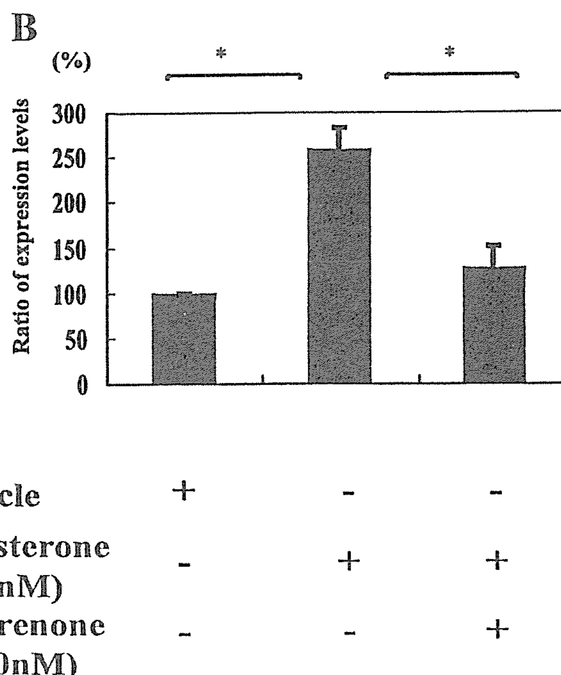


Figure 3. A: Representative immunoblotting studies demonstrating *MDM2* proteins in HASMC among cells treated with vehicle (0.1% ethanol) (control), aldosterone alone (10 nmol/L), and aldosterone (10 nmol/L) with eplerenone (100 nmol/L), respectively, after 48 hours. **B:** Relative levels of *MDM2* protein expression in HASMC among cells treated with vehicle (0.1% ethanol) (control), aldosterone alone (10 nmol/L), and aldosterone (10 nmol/L) with eplerenone (100 nmol/L), respectively, after 48 hours. Data are shown as mean ± SD (*$P < 0.05$).

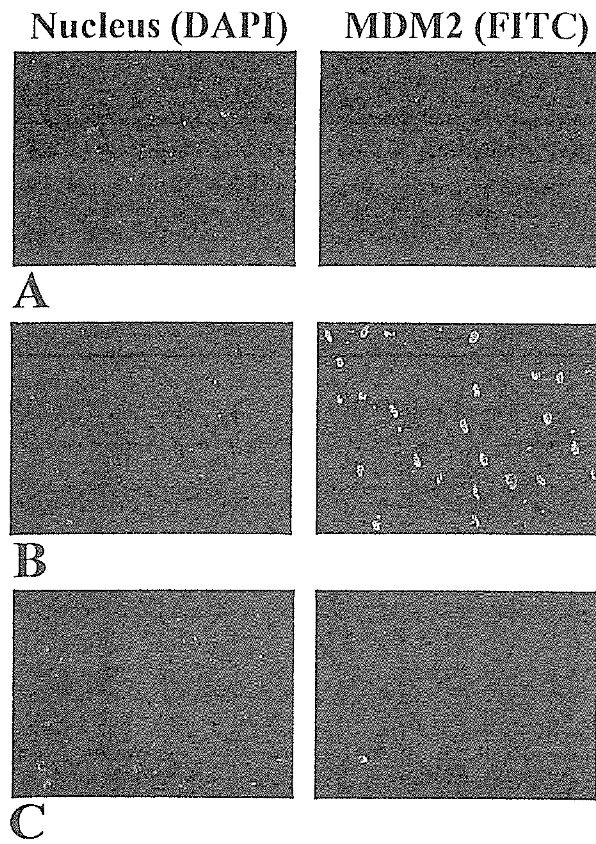


Figure 4. Representative immunofluorescence studies demonstrating *MDM2* proteins in HASMC among cells treated with vehicle (0.1% ethanol) (control) (**A**), aldosterone alone (10 nmol/L) (**B**), and aldosterone (10 nmol/L) with eplerenone (100 nmol/L) (**C**), respectively, after 48 hours. *MDM2* protein expression (green) was remarkably abundant in the nucleus of cells treated by aldosterone using fluorescein isothiocyanate (FITC)-conjugated secondary antibody (**B**), and its expression was blocked by eplerenone (**C**). 4,6-Diamidino-2-phenylindole (DAPI) was used for nuclear staining (blue).

microarray and quantitative RT/real-time PCR analyses also demonstrated that *MDM2* is one of the early genes induced by aldosterone via MR in HASMC.

MDM2 Protein Expression Study in Immunoblotting and Immunoflourescence

In the immunoblotting study, aldosterone significantly increased *MDM2* protein expression levels in HASMC compared with controls after 48 hours of incubation ($P <$ 0.05) (Figure 3). However, aldosterone with eplerenone did not promote expression of these proteins (Figure 3). In the immunoflourescence study, aldosterone extensively increased *MDM2* protein located in the nucleus of HASMC compared with controls after 48 hours of incubation (Figure 4, A and B). However, aldosterone with eplerenone did not induce *MDM2* protein expression (Figure 4C). Results of these analyses also demonstrated that *MDM2* protein was extensively induced by aldosterone via MR and inhibited by a specific MR blocker, eplerenone.

MDM2 siRNA Transfections and Cell Proliferation Assay

A down-regulation of *MDM2* and/or GAPDH mRNA levels was dose dependently confirmed in the cells by transfection of *MDM2* and/or GAPDH siRNAs (10 and 100 nmol/L) using RT/real-time PCR, respectively (Figure 5A). Under the absence of transfection of siRNA (0 nmol/L), aldosterone significantly promoted proliferation of HASMC compared with controls (approximately 1.3- to 1.4-fold after 48 and/or 72 hours) (Figure 5, B and C). However, under the transfection of *MDM2* siRNA (10 and 100 nmol/L), aldosterone did not promote the cell proliferation of HASMC compared with control (Figure 5, B and C). On the other hand, under eplerenone, aldosterone did not significantly promote cell proliferation of HASMC induced by aldosterone (Figure 5, B and C).

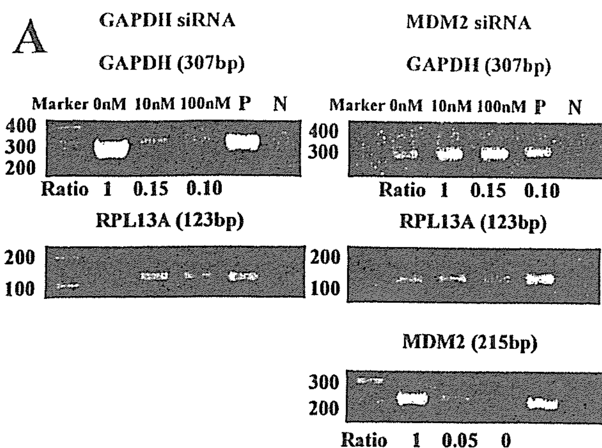
MDM2 Immunoreactivity in Human Resistance Artery

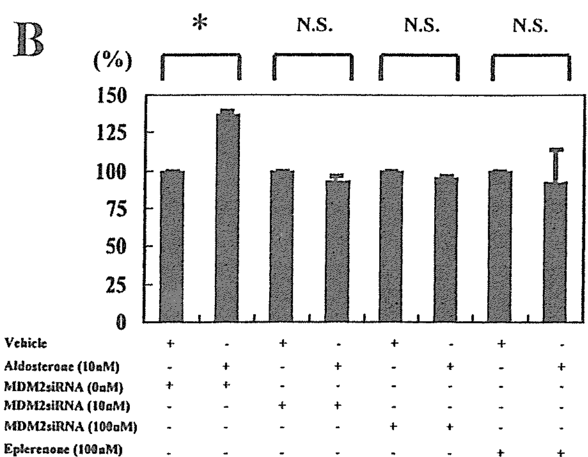
Figure 6 demonstrates representative examples of human small resistance arteries adjacent to normal adrenal gland or adrenal tumor specimen obtained by surgery. Relative immunoreactivities of *MDM2* were significantly higher in VSMCs of human small resistance arteries adjacent to adrenocortical adenoma of primary aldosteronism associated with hypertension than normal adrenal gland in normotensive patients and/or nonfunctioning adrenocortical adenoma with and/or without hypertension ($P < 0.05$).

Discussion

In our present study, results of both microarray and quantitative RT-PCR analyses and immunoblotting and immunofluorescence studies all suggest that *MDM2* is one of the genes induced by aldosterone via MR in cultured human VSMCs. In addition, results of siRNA analysis demonstrated that *MDM2* is possibly involved in VSMC proliferation through MR by aldosterone. Furthermore, results of immunohistochemical study in human arteries revealed that *MDM2* was markedly expressed in VSMCs of small arteries in the patients with aldosterone-producing adrenocortical adenoma compared with other groups including the hypertensive patients with nonfunctioning adrenocortical adenomas, ie, no clinical aldosterone abnormalities. These findings above indicate that an expression of *MDM2* is strongly induced by aldosterone itself via MR in VSMCs both *in vitro* and *in vivo*, and *MDM2* may be possibly involved in aldosterone-induced vascular structural remodeling of human resistance arteries, which may result in persistent hypertension even after resection of aldosterone-producing adrenocortical adenoma.[22]

VSMCs used in this study were also associated with 11β-HSD type 2 expression. It is known that both aldosterone and cortisol bind to human MR with equal affinity.[1,23] Plasma glucocorticoid concentrations are 100- to





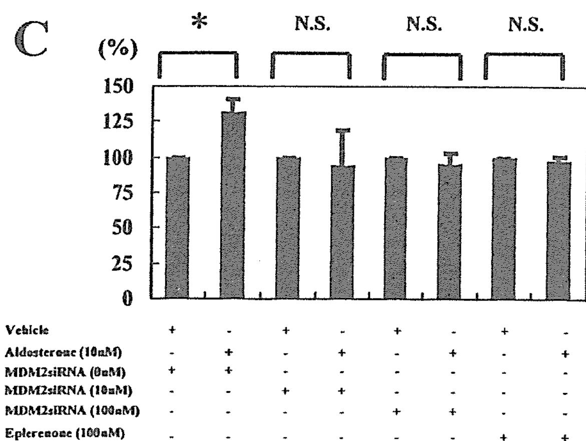


Figure 5. A: Expression of *MDM2*, GAPDH, and RPL13A mRNAs at 24 hours after the transfection of siRNA (0, 10, or 100 nmol/L) against *MDM2* and/or GAPDH in HASMC cells detected by real-time PCR, respectively. RPL13A expression was monitored as the control. The ratio of *MDM2* to RPL13A and/or GAPDH to RPL13A was calculated, and values were normalized as the ratio at no treatment (0 nmol/L), respectively. **B:** The levels of cell numbers in HASMC at 48 hours after treatment with vehicle (0.1% ethanol) (control) or aldosterone (10 nmol/L) after transfection of *MDM2* siRNA (0, 10, or 100 nmol/L) or under the presence of eplerenone (100 nmol/L). Data are presented as mean ± SD (*$P < 0.05$). **C:** The levels of cell numbers in HASMC at 72 hours after treatment with vehicle (0.1% ethanol) (control) or aldosterone (10 nmol/L) after transfection of *MDM2* siRNA (0, 10, or 100 nmol/L) or under the presence of eplerenone (100 nmol/L). Data are represented as mean ± SD (*$P < 0.05$). N.S., not significant.

1000-fold higher than those of aldosterone, but aldosterone-responsive tissues express the cortisol-inactivating enzyme 11β-HSD type 2, which converts cortisol to its 11-keto derivatives that have a lower affinity for MR.[1,24] 11β-HSD type 2 is abundant in the kidney, making renal cells highly responsive to aldosterone.[1,25,26] It is known that VSMCs also express a functional 11β-HSD type 2 capable of inactivating cortisol, as in other aldosterone target tissues.[1] In addition, the presence of both MR and 11β-HSD type 2 has been reported in human aorta.[1,27] Therefore, HASMC is considered an appropriate model for *in vitro* examination of direct effects of aldosterone in human cardiovascular system.

Results of our present study did demonstrate that aldosterone itself may possibly exert cell proliferative effects on VSMCs *in vitro* through MR by aldosterone to some extent (approximately 1.3- to 1.4-fold compared with control). Angiotensin II is well known to be required for mitogenic effects of aldosterone in a lower dose in rat VSMCs, and the pathway via extracellular signal-regulated kinase activation has been considered to be involved in aldosterone-induced VSMC mitogenesis under the presence of angiotensin II.[4,5] In addition, Ishizawa et al also reported that aldosterone itself possibly induced proliferation of VSMCs (less than 1.5-fold compared with control), which suggest that proliferative effects of aldosterone alone on VSMCs are not necessarily marked as demonstrated in our present study.[8] However, higher plasma aldosterone levels have been demonstrated to be associated with the status of in-stent restenosed coronary arteries in patients with angina, which suggest that aldosterone is involved in VSMC proliferation and vascular remodeling in these patients.[28] In addition, inflammation, fibrosis, and calcification have been considered to play important roles in vascular injuries caused by aldosterone.[1] Therefore, these findings all suggest that VSMC proliferation induced by aldosterone itself is one of the important factors on vascular remodeling and is possibly accelerated by other factors including inflammation and angiotensin II.

MDM2 is a nuclear protein that forms a complex with p53.[21] *MDM2* is known to regulate the biological activity of p53 by preventing p53-mediated apoptosis or reversing p53-induced G1 block of the cell cycle and thus promoting the entry of cells into S phases through formation of these complexes.[21,29,30] In addition, *MDM2* has been also considered to be involved in promoting the entry of cells from G2 into M phases by reversing p53-mediated G2 block of the cell cycle.[21,29,31] *MDM2* is also known to interact both physically and functionally with the RB protein, which is also involved in VSMC growth.[21,32–34] *MDM2* was also reported to be regulated by the Ras-driven Raf/MEK/

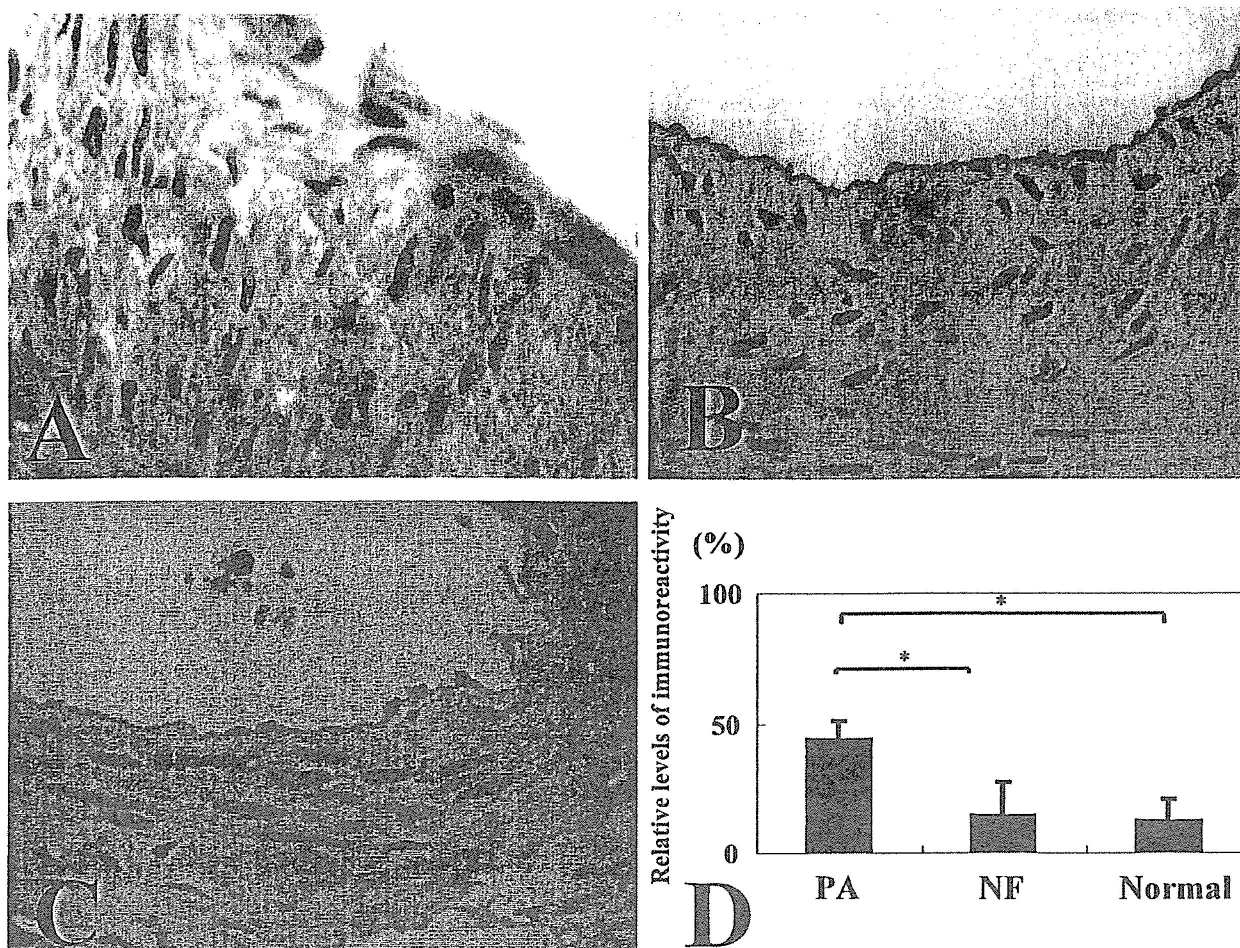


Figure 6. A–C: Immunohistochemistry for *MDM2* in VSMCs of small resistance arteries adjacent to adrenal tumors or normal adrenal glands. Immunoreactivity for *MDM2* in VSMCs of small resistance arteries adjacent to adrenal tumor of primary aldosteronism (**A**), nonfunctioning adrenal tumor (**B**), and non-neoplastic adrenal gland (**C**). Immunoreactivity appears brown as a result of diaminobenzidine colorimetric reaction. Original magnification, ×400. **D:** The relative immunoreactivity was evaluated by the LI in each group (0 to 100). Data are represented as mean ± SD ($^*P < 0.05$). PA, aldosterone-producing adrenocortical adenoma; NF, nonfunctioning tumor; normal, non-neoplastic adrenal gland.

MAP kinase pathway, in a p53-independent manner.[35] Several investigators reported that *MDM2* may also play an important role in atherogenesis in human atherosclerotic plaques.[21,32,33,36]Results of our present study suggest that *MDM2* may be involved in regulation of a cell cycle and cell proliferation by aldosterone itself in human VSMCs, which is consistent with these previous studies.[21,29–36]

In our present study, the patients associated with both increased plasma aldosterone levels and hypertension demonstrated significantly more *MDM2* immunoreactivity in VSMCs of small arteries than those with hypertension but normal plasma aldosterone levels. Therefore, *MDM2* is considered to be at least induced by relatively high levels of plasma aldosterone but not necessarily by effects of hypertension alone and to be involved in vascular remodeling frequently detected in the patients with primary aldosteronism.[7] However, it is also true that VSMC proliferation may occur in response to hypertension itself.[37] Therefore, the *MDM2* pathway demonstrated in this study is certainly not the only factor involved in vascular structural remodeling associated with elevated levels of plasma aldosterone. Further investigations of direct aldosterone effects toward human cardiovascular system are required for clarification.

The MR-mediated effects of aldosterone are considered genomic ones through binding of aldosterone to the intracellular MR and the translocation of the steroid-MR complex to the nucleus, where it acts as a transcriptional regulator, resulting in its biological effects after several hours.[38] In addition, quantitative RT-PCR analysis in our present study demonstrated that ACD suppressed aldosterone-induced *MDM2* mRNA expression, but CHX did not inhibit its expression. Therefore, these findings all demonstrated that *MDM2* is considered one of the first established responsive genes in MR-positive VSMCs. However, it awaits further investigations to clarify the exact mechanisms of *MDM2* induction pathway.

Results of real-time PCR, immunoblotting, immunofluoresence, and cell proliferation studies also demonstrated that eplerenone may inhibit *MDM2* induction by aldosterone in MR-positive VSMCs. Eplerenone may also confer cardiovascular protective effects on aldo-

sterone-induced VSMC proliferation and vascular remodeling through this pathway above. Aldosterone antagonists have been demonstrated to inhibit both of these vascular injuries by aldosterone in some clinical and experimental studies in cardiovascular system.[1,3,9] Eplerenone is a selective aldosterone blocker that provides clinical efficacy in the treatment of hypertension and heart failure compared with spironolactone, a relatively nonselective aldosterone blocker in view of its side-effect profile.[9] These findings, including results of our present study, all demonstrated that eplerenone may prevent vascular damages through this *MDM2* pathway at least in the patients with primary aldosteronism. In conclusion, *MDM2* is considered one of the mineralocorticoid-responsive genes involving MR-mediated VSMC proliferation and may play important roles in aldosterone-related vascular structural alterations of human cardiovascular system.

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REVIEW

Nuclear Receptor Mediated Gene Regulation through Chromatin Remodeling and Histone Modifications

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Abstract. Nuclear steroid/thyroid vitamin A/D receptor genes form a gene superfamily and encode DNA-binding transcription factors that control the transcription of target genes in a ligand-dependent manner. It has become clear that chromatin remodeling and the modification of histones, the main components of chromatin, play crucial roles in gene transcription, and many distinct classes of NR-interacting co-regulators have been identified that perform significant roles in gene transcription. Since NR dysfunction can lead to the onset or progression of endocrine disease, elucidation of the mechanisms of gene regulation mediated by NRs, as well as the identification and characterization of co-regulator complexes (especially chromatin remodeling and histone-modifying complexes), is essential not only for better understanding of NR ligand function, but also for pathophysiological studies and the development of therapeutic interventions in humans.

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I. The Nuclear Receptor Gene Superfamily

A. Biological functions of nuclear receptors (NRs)

Fat-soluble ligands, such as steroid/thyroid and vitamin A/D, exert a wide variety of biological effects through the transcriptional regulation of target genes via cognate NRs that exhibit specific ligand binding (see Fig. 1). Therefore, NRs are associated with cellular proliferation/differentiation events and are involved in a variety of functions in different cell types. Indeed, NRs are thought to be central to homeostasis as well as the development of clinical pathology in human beings. The physiological importance of the 48 NR family members currently recognized in human beings has been verified in mouse genetic models (Table 1) [1–37]. For instance, peroxisome proliferator-activated receptor (PPARγ), a principal factor in the regulation of adipocyte differentiation and fat storage, has been shown to control glucose tolerance via the general regulation of insulin sensitivity [38, 39]. Indeed, the PPARγ agonist thiazolidinedione has been used successfully in the clinical treatment of Type II diabetes mellitus. Likewise, fibrate, an agonist of the PPARγ-

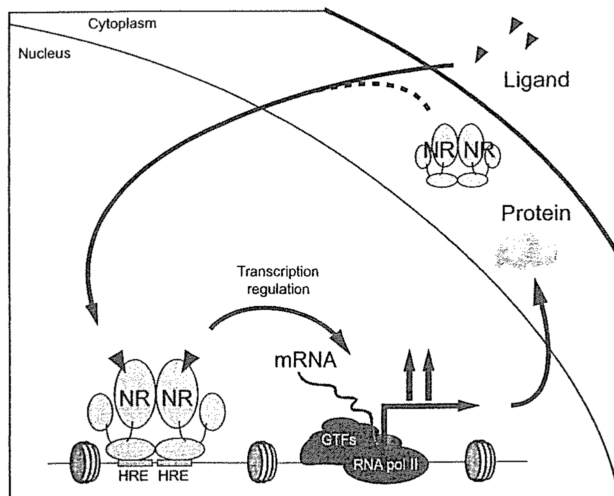

Fig. 1. Nuclear receptor controls expression of target genes in a ligand-dependent manner.
Lipophilic ligands, such as fat-soluble vitamins A and D, as well as thyroid/steroid hormones, are thought to exert their physiological effects through transcriptional control by the cognate nuclear receptors (NRs). NRs recognize and bind with the specific recognition sites, termed hormone responsive elements (HREs). Ligand binding to NRs induces association with general transcription factors (GTFs) and the target genes are transcribed by RNA polymerase II (RNApol II).

related protein PPARα, has been used to treat hyperlipidemia, as PPARα regulates the expression of genes associated with lipid metabolism, including the lipoprotein lipase (LPL) gene that promotes the catabolism of chylomicron, very low density lipoprotein (VLDL), and intermediate density lipoprotein (IDL), with suppression of fatty acid synthesis in the liver and suppression of triglyceride (TG) production [40–42]. Sex hormone antagonists are also effective against sex hormone-dependent tumors of reproductive organs, breast, and prostate [43–45].

Reflecting the significance of NR functions in the biological effects of NR ligands, NR gene mutations underlie a range of genetic diseases. For example, mutations in coding regions of the androgen receptor (AR) can lead to complete loss of the androgen response, resulting in testicular feminization (Tfm) [46], and unusually expanded polyglutamine repeats within the AR A/B domain lead to spinal and bulbar muscular atrophy (SBMA) [47]. Genetic mutations in the vitamin D receptor (VDR) that result in the loss of vitamin D responsiveness cause hereditary vitamin D-resistant rickets type II (HVDRR) [48], while thyroid hormone resistance syndrome (RTH) is due to mutation of the thyroid hormone receptor (TR)-β gene [49]. Genetic disease also results from the malfunction of nuclear orphan receptors (*i.e.* those with unknown ligands). For example, several hepatocyte nuclear factor (HNF)-4α gene mutations are known to cause maturity onset diabetes mellitus of the young (MODY)-1 [50], while photoreceptor-cell specific NR (PNR) mutations lead to enhanced S-cone syndrome [51].

B. NR structure

All NR genes are thought to have evolutionarily developed from a single ancestor gene, such that in metazoans, the NR gene superfamily is found in all genomes from *C. elegans* to human. As all NR superfamily members share structural and functional characteristics that reflect this evolutionary relationship, NR proteins contain five functional domains designated A to E [52, 53] (Fig. 2). The A/B domain contains the activation function (AF)-1 region that is constitutively active even without ligand binding. The highly-conserved DNA binding domain (DBD) is located within the C domain, while the D domain contains the nuclear localization signal (NLS) [54]. The moderately-conserved ligand binding domain (LBD) is mapped to the E domain, and consists of approximately 250 mostly hydrophobic amino acids that form a ligand-binding pocket made up of 12 α-helixes present in most of NRs. This domain plays a critical role in activation function (AF)-2 activity, which is induced by ligand binding, and results in clear shifting of the C terminal-most α-helix 12 [55]. As the ratio between AF-1 and AF-2 is dependent on the tissue and cell type, AF-1 and AF-2 activities are probably controlled through a diverse range of molecular mechanisms. Of the 12α-helixes encoded by the LBD E domain, specific ligands bind to a hydrophobic cave formed by α-helices 3, 4 and 5. Ligand binding induces a structural alteration in the E domain, mainly in terms of movement of the α-helix 12. For ERα the angle of this α-helix 12 shift has been reported to vary according to ligand type [56, 57], and appears to define the transactivation function. In contrast, NR AF-1 domains appear to mediate specific intracellular functions as the conservation of A/B domain amino acid sequences between NRs is low. While intramolecular interaction between AF-1 and AF-2 functions in gene regulation has been well described [58, 59], its molecular basis with respect to

Table 1.

- glucocorticoid receptor (GR)
  - impaired lung development (most of the mutant mice died during the perinatal period), [increase of corticosterone and ACTH in heterozygous mice] (33)
- mineralocorticoid receptor (MR)
  - hyperkalemia, hyponatremia (pseudohypoaldosteronism) (4)
- androgen receptor (AR)
  - testicular feminization (Tfm) and osteopenia in the male mutant mice, abnormal brain masculinization (16, 32)
- progesterone receptor (PR)
  - pleiotropic reproductive abnormalities (25)
- estrogen receptor (ER) α
  - infertility (22)
- ER β
  - reduction in fertility (12, 19)
- retinoic acid receptor (RAR) α
  - high postnatal lethality, testis degeneration (23)
- retinoic acid receptor (RAR) β
  - no abnormality (14)
- retinoic acid receptor (RAR) γ
  - growth deficiency, early lethality, male infertility (21)
- thyroid hormone receptor (TR) α
  - reduced linear growth, bone maturation delay, moderate hypothermia, reduced thickness of the intestinal mucosa (9)
- thyroid hormone receptor (TR) β
  - resistance to thyroid hormone (8), deficit in auditory function (7)
- Vitamin D receptor (VDR)
  - growth retardation, alopoecia, hypocalcemia, impaired bone formation (36)
- peroxisome proliferator-activated receptor (PPAR) α
  - lipid accumulation in the livers of fasted or high fat diet mutant mice (17, 20)
- peroxisome proliferator-activated receptor (PPAR) β/δ
  - embryonic lethal, growth retardation in surviving mice (30)
- peroxisome proliferator-activated receptor (PPAR) γ
  - embryonic lethal, [protection from high fat diet induced adipocyte hypertrophy and insulin resistance in heterozygous mice] (38, 39)
- liver X receptor (LXR) α
  - loss of normal response to dietary cholesterol (28)
- liver X receptor (LXR) β
  - no apparent abnormal phenotype (2)
- farnesoid X receptor (FXR)
  - elevation of serum bile acid, cholesterol, and triglycerides levels (34)
- pregnenolone X receptor (PXR)/steroid and xenobiotic receptor (SXR)
  - loss of normal response to xenobiotic treatment (18)
- retinoid X receptor (RXR) α
  - embryonic lethal, [growth deficiency in heterozygous mice] (13)
- retinoid X receptor (RXR) β
  - embryonic lethal, male infertility in surviving mice (15)
- retinoid X receptor (RXR) γ
  - central resistance to thyroid hormone (5), less weight gain when fed a high fat diet (11)
- photoreceptor-specific nuclear receptor (PNR)
  - retinal degeneration (1)
- TLX
  - reduction in the size of rhinencephalic and limbic structures, including the olfactory, infrarhinal and entorhinal cortex (26, 37)
- hepatocyte nuclear factor (HNF) 4α
  - embryonic lethal (6)
- retinoid-related orphan receptor (ROR) α
  - cerebellar defects (10)
- retinoid-related orphan receptor (ROR) β
  - retinal degeneration (3)
- retinoid-related orphan receptor (ROR) γ
  - absence of lymph node (35)
- adrenal-4 binding protein (Ad4BP)/steroidogenic factor (SF)-1
  - lack of adrenal glands and gonads, structural and functional abnormalities in spleen (24, 27)
- chicken ovalbumin upstream promoter-transcription factor (COUP-TF) I
  - defects in morphogenesis of the glossopharyngeal ganglion, axonal projection, and arborization (31)
- COUP-TF II
  - embryonic lethal (29)

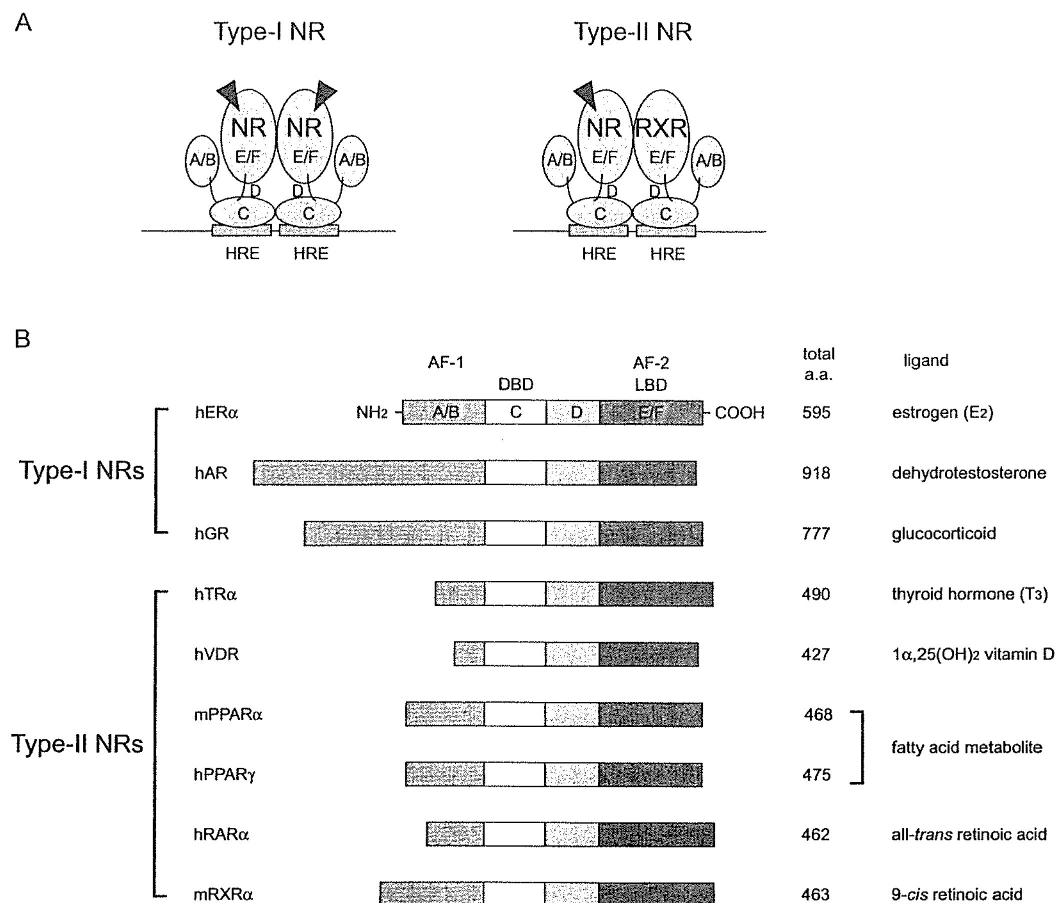

Fig. 2. Structure and function of nuclear receptors.
A. Classes of ligand-dependent NRs. NRs are subdivided into subfamilies in terms of their partnership, *e.g.* homodimer (Type-I NRs), and RXR heterodimer (Type-II NRs). B. Functional domains of the NR superfamily. Total number of amino acids (a. a.) and the ligand for each receptor are shown on the right. The receptors are specific for estrogens (ER), androgens (AR), glucocorticoids (GR), thyroid hormone (TR), vitamin D (VDR), fatty acid metabolite (PPAR), and retinoic acid derivatives (RAR, RXR).

structure alterations of the entire NR molecule following ligand binding remains largely unknown.

Nevertheless, it is hoped that an understanding of the molecular mechanisms that regulate AF-1 and AF-2 activities will facilitate the development of new therapies targeted to NRs, especially as it may be possible to minimize the risk of side effects of endogenous ligands through the development of synthetic ligands. For example, estrogen replacement therapy for post-menopausal women can cause adverse effects such as uterine bleeding, mastodynia, and weight gain, as well as increased risk of endometrial cancer, breast cancer, and coronary heart disease [60]. Thus, the development of a selective estrogen modulator (SERM) with beneficial effects on bone and the cardiovascular system, but without adverse effects on the uterine tract or mammary glands, would be highly desirable. For example, tamoxifen is a SERM originally designated as a pure estrogen antagonist in the treatment of estrogen-dependent breast cancer. However, it became obvious from clinical applications over 30 years that tamoxifen in fact served not only as an AF-1 agonist in bone, lipid metabolism, and the cardiovascular system, but also as an AF-2 antagonist in mammary glands and female reproductive organs [61]. Raloxifen, a SERM now marketed in Japan, has been reported to be very effective in improving osteoporosis in post-menopausal women [62, 63], but cannot prevent hot flashes. Thus, based on detailed knowledge of the mechanisms of hERα AF-1 and AF-2 functions, it is anticipated that the desirable effects of SERMs can be further improved.

II. Decoding of Histone Signals and the Regulation of Gene Expression

A. Chromatin structure and histone modifications

Each human cell contains approximately 2 meters of DNA. As DNA is acidic (*i.e.* negatively charged), chromatin structures are maintained in an electrically neutral state through association with histone proteins that are basic (*i.e.* positively charged). Two molecules of each of the four histone types (H2A, H2B, H3, and H4) interact to form a histone octamer. DNA is coiled around this octamer, which forms a nucleosome, considered to be the minimum and basic structure of chromatin. One nucleosome subunit contains approximately 146 base pairs of DNA. However, not all DNA is coiled around histone octamers, as stretches of protein-free DNA serve as linker DNA between regions of coiled nucleosomal DNA. Repeated nucleosome units then form chromatin structures.

With regard to gene regulation, DNA regions contained within histone octamers are thought to be transcriptionally repressed. In contrast, linker DNA regions may play a leading role in gene activation, as these areas are easily accessible to transcription factors. To decode genetic information within the chromosome via transcription, it is now thought that histone octamers have to slide along the chromosomal DNA. However, the signals encoded on the chromosome that guide this process have long remained a mystery.

Recently, a revolutionary hypothesis was proposed that these chromatin signals are in fact related to post-translational modifications on the histones. This breakthrough was based on observations from the crystal structure of the nucleosome, that the N-terminal tails of histones extended out from the regions of coiled DNA (see Fig. 3) [64, 65]. It has since become clear that these histone N-terminal tails can be post-translationally modified by processes such as acetylation, deacetylation, methylation, phosphorylation, ubiquitination, and sumoylation, that are targeted to specific amino acid residues [66, 67]. Furthermore, it has been shown biochemically that enzymes associated with these post-translational histone modifications exist as large protein complexes in the nucleus [68, 69]. It appears that each histone octamer is uniquely modified according to different combinations of post-translational modifications. In other words, the nucleosome, long thought to have a relatively simple repetitive structure, may actually contain arrangements of modifications that reflect specific signals. Thus, the nucleosomal array may contain information on chromosomal position, such that the decoding of specific DNA sequences can only occur when certain chromosomal nucleosome arrays are reorganized.

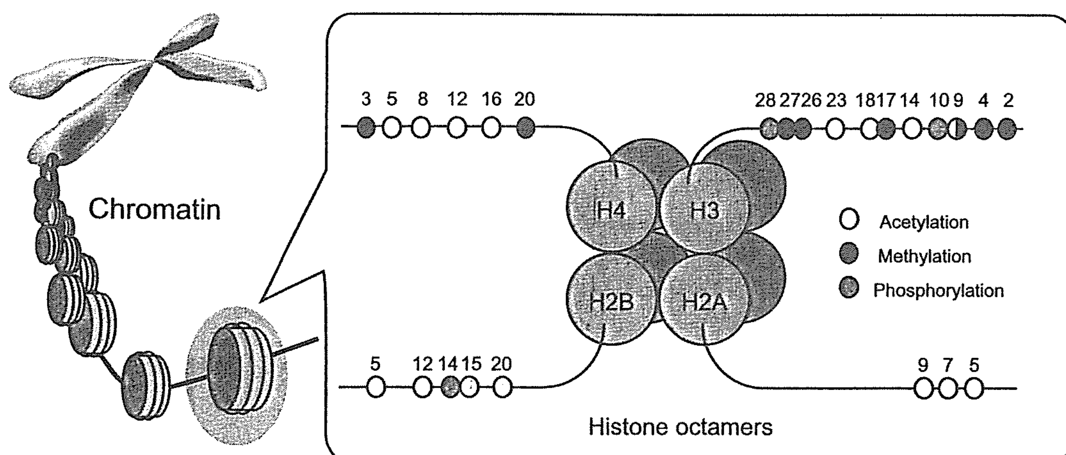

Fig. 3. Representative post-translational modifications of N-terminal tails of histones.
Chromatin is constituted with linker DNA and a nucleosome structure, which forms a complex between histone octamers surrounded by about 150 base pairs of DNA. N-terminal histone tails are protruded from the nucleosome core and are modified by several histone-modifying enzymes. Numerous combinations of post-translational histone modifications generates "histone code" to define the chromatin state and mark the addresses upon chromatin.

B. Chromatin remodeling

ATP-dependent chromatin remodeling complexes are primarily responsible for the rearrangement of nucleosomal arrays, according to signals defined by histone modifications [70, 71]. The sliding of histone octamers, around which contacting DNA is coiled, is facilitated by ATP-dependent chromatin remodeling complexes, thereby exposing new naked DNA regions. Chromatin structures are also formed by ATP-depending chromatin remodeling complexes during DNA replication. Histone octamers are transferred to newly synthesized DNA by these complexes, and nucleosomal arrangements adjusted. Thus, chromatin remodeling factors and/or complexes play a major role in tertiary chromatin structure.

III. Chromatin Regulation by NRs

A. Ligand-induced co-regulator switching by NRs

Unliganded NRs are transcriptionally silent even when bound to specific DNA elements. Upon ligand binding, NR transactivation functions through AF-1 and AF-2 are induced together along with co-regulator switching (Fig. 4). Co-regulators that associate with unliganded or liganded NRs are classified into two

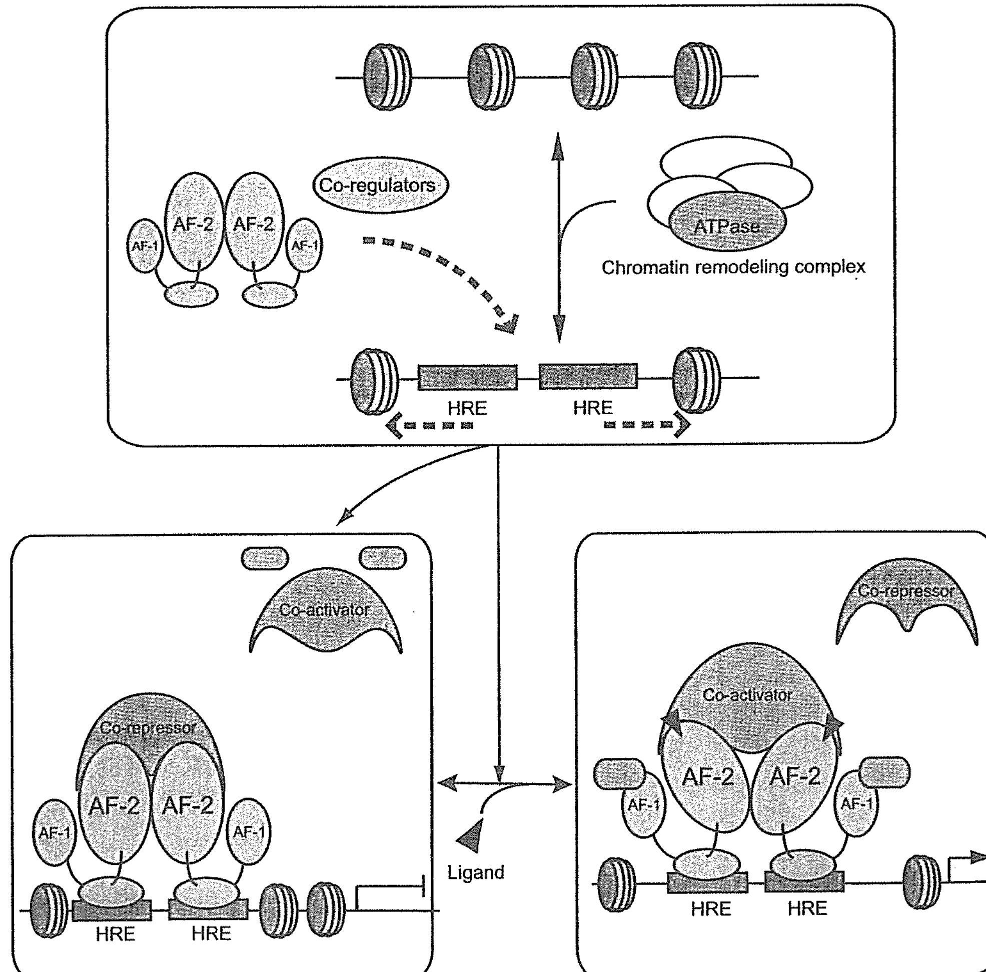

Fig. 4. Co-regulators support ligand-dependent transcriptional controls by NRs through chromatin remodeling and histone modifications.
Ligand binding positively and negatively controls gene expressions of target genes by NRs through switching of co-regulators; most of them form histone modifying enzyme complexes, together with histone remodeling by ATP-dependent chromatin remodeling complexes.

functionally-opposite groups according to their impact on transactivation function. While co-repressors repress the transactivation function of unliganded NRs by physically associating with the LBD, particularly through α-helix 12, liganded NRs are co-activated by a number of co-activators through physical association with both the AF-1 and AF-2 domains. The switching between co-regulator classes is induced by ligand binding. Most co-regulators appear to form complexes, and their roles in gene regulation are most likely linked to histone modification and chromatin remodeling.

B. Chromatin modifications by NRs

Gene regulation is controlled by epigenetic modifications that define the chromatin state, mainly via histone modification. The best characterized of the histone modifications mediated by NRs is histone acetylation and deacetylation. Co-regulator complexes with histone acetyltransferase (HAT) activity activate the transcription of target genes through the acetylation of histones, while histone deacetyltransferase (HDAC) complexes deacetylate histones and serve as co-repressors for unliganded NRs. Histone methylation is also induced by co-regulator complexes that associate with NRs. The methylation of lysine at amino acid position 4 (K4) in histone H3 appears to induce an active state in chromatin that leads to transcriptional activation. In contrast, methylation of K9 in histone H3 is thought to lead to transcriptional suppression by inducing the adjacent chromosomal region to adopt an inactive state [72–75].

C. Chromatin remodeling by ATP-dependent chromatin remodeling complexes and NRs

ATP-dependent chromatin remodeling complexes use ATP hydrolysis to rearrange nucleosomal arrays in a non-covalent manner, thereby rendering chromosomal DNA accessible to DNA-binding transcription factors, including NRs (see Fig. 4). ATP-dependent chromatin remodeling complexes with distinct subunit combinations are classified into three major complex types (SWI/SNF, ISWI, and Mi-2) according to the ATPase that forms the main component of the complex [70]. Some of these complexes are known to physically associate with NRs [76–79]. For example, WINAC, a human multi-protein complex that directly interacts with VDR through the Williams syndrome transcription factor (WSTF), exhibits ATP-dependent chromatin remodeling activity, and contains both SWI/SNF components and DNA replication-related factors. WSTF is highly homologous to hACF1, which together with hSNF2h are involved in the formation of well-characterized ISWI-based chromatin remodeling complexes. While WINAC mediates the recruitment of VDR to target gene promoters in the absence of ligand, the subsequent binding of co-activators to VDR requires ligand binding (Fig. 5) [80]. WINAC dysfunction seems to be at least partly responsible for some of the phenotypes associated with Williams syndrome, a rare autosomal dominant hereditary disorder with multiple symptoms, typically including congenital vascular lesions, elfin face, mental retardation, growth deficiency, and transient appearance of infantile aberrant vitamin D metabolism, including hypercalcemia [79]. Although some of the biological roles of ATP-dependent chromatin remodeling factors remain to be investigated, defects or mutations in Ini1, hBrg1, or hBrm, which are subunits of the SWI/SNF type ATP-dependent chromatin remodeling complex subtype, have been found in several cancers [81–85]. Furthermore, an SWI2/SNF2-like ATPase motif is present in ATRX, a protein produced by a causative gene for myelodysplasia associated with α-thalassemia (ATMDS) [86], and an SNF2-like domain is present in SMARCAL1, a protein that when defective leads to Schimke immuno-osseous dysplasia [87]. Hence, upon confirmation that these factors do act as chromatin remodelers, their related syndromes could be referred to as "chromatin remodeling factor diseases", and therefore considered as part of the "co-regulator disease" category. As chromatin remodeling is an essential step in gene regulation, ATP-dependent chromatin remodeling complexes presumably directly and indirectly support the ligand-induced transactivation of NRs. However, the functional interplay between NRs and chromatin modifying enzyme complexes remains to be clarified.

IV. NR Co-regulators and Co-regulator Complexes

A. NR AF-2 co-activators

It is well understood that ligand-induced transcriptional activation by NRs consists of two activation

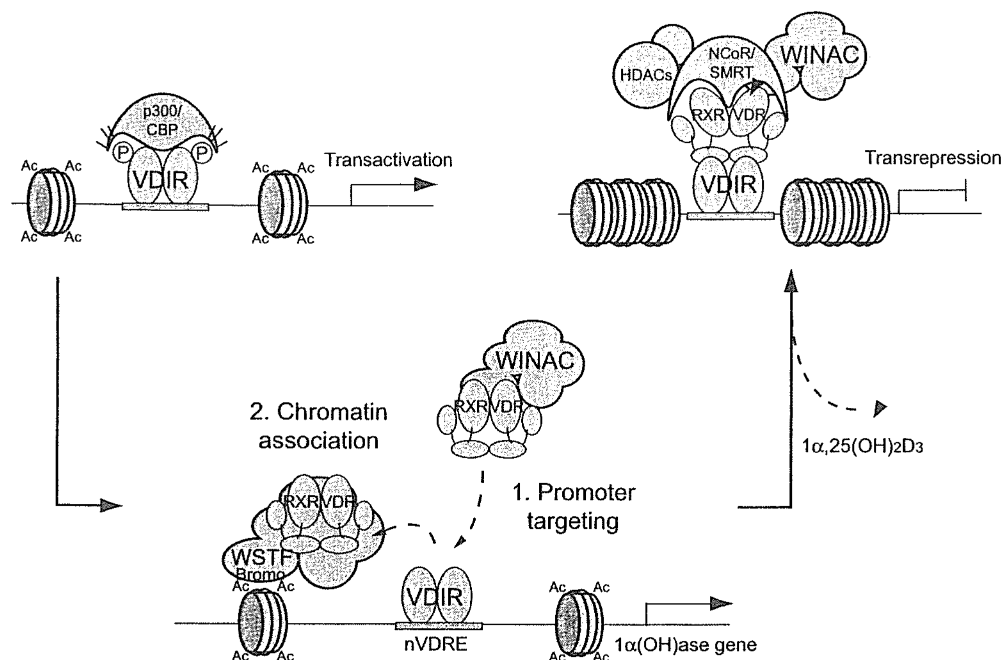

Fig. 5. Ligand-induced transrepression by VDR mediates functions of a novel chromatin remodeling complex (WINAC) and co-regulator complexes of histone modifying enzymes in the gene promoter of a vitamin D biosynthesis enzyme [1α(OH)ase].

steps [88, 89]. The first step is coupled with histone modifications such as acetylation, and is then followed by the formation of multi-complexes with general transcription factors that constitute a transcription initiation complex. Two such complexes, the p160/p300 HAT complex and the DRIP/TRAP complex, have been well characterized. Some components of these complexes have been shown to bind to liganded NR AF-2 domains through consensus LXXLL and related motifs present on some components [90]. p160/p300 complexes harbor one of three p160 family members (SRC-1 [91], TIF2/GRIP-1/SRC-2 [92, 93], pCIP/RAC3/ACTR/AIB1/TRAM-1/SRC-3 [94–98]) and CBP/p300 [99]. All these components are in fact HAT proteins presumably required by NRs either as single factors or, more likely, as multisubunit complexes with other components in a ligand-dependent manner. HAT activity facilitates transcription by loosening chromatin structures through the acetylation of histone N-terminal tails.

SRC-1, one of the three p160 protein family members, contains three LXXLL motifs essential for ligand-dependent binding to NR AF-2 domains. SRC-1 has been shown to assemble with CARM1, an enzyme with dual histone methyltransferase [100, 101] and HAT activities [102]. From structural analyses, it appears that SRC-1 binds to a groove formed by NR α-helixes 3, 4, 5, and 12 via the LXXLL motifs upon ligand binding. However, the three SRC-1 LXXLL motifs are not equivalent with respect to NR interactions. Altered amino acid sequences around the LXXLL motifs demonstrated altered NR interaction efficiencies dependent on the NR being used, which suggested that amino acids around the LXXLL motifs are essential for recognition and specific interactions with liganded NRs.

p300 was originally identified as a protein that bound adenovirus E1A [103], while the p300-related CBP protein was initially characterized as a co-activator of cAMP responsive element binding protein (CREB), a transcription factor activated by cAMP signaling [104]. CBP is thought to be the causative gene for the developmental disorder Rubinstein-Taybi syndrome characterized by multiple abnormalities, including broad thumbs and halluces, mental retardation, growth retardation, developmental delay, microcephaly, and craniofacial abnormalities [105]. p300/CBP exhibit high structural homology to each other, and are ubiquitously expressed in a variety of cells and tissues. It is likely that p300/CBP bind to other classes of DNA-binding transcriptional factors in addition to NRs, functioning as common co-activators for these factors

[106–109]. Although p300/CBP physically bind in a ligand-dependent manner to NR AF-2 domains to activate transcription [110], they also function as co-activators for AF-1 [58], which suggests that the p300/CBP co-activators bridge AF-1 and AF-2.

Many other co-activators have been identified that may be important in NR functions. PGC-1 was initially described as a PPARγ co-activator [111], and reported to dock with p160 member co-activators to NRs. PGC-1 has more recently been shown to be important for energy homeostasis. Indeed, a single nucleotide polymorphism (SNP) of the PGC-1 gene (Gly482Ser) is associated with the conversion from impaired glucose tolerance to Type II diabetes [112, 113]. Other NR co-activators include PRIP/ASC-2/AIB3/RAP250/NRC that contains a single LXXLL motif and may act as a bridging factor between p300/CBP and DRIP130, as well as being a component of the DRIP complex. Interestingly, its gene is known to be amplified in breast cancer [114–117]. Another example is GT-198. While its gene is localized to a breast cancer susceptibility locus, GT-198 protein exhibits kinase activity and acts as a tissue-specific NR co-activator through interaction with NR DNA-binding domains [118]. Hydrogen peroxide-inducible clone-5 (Hic-5), which belongs to the group III LIM domain protein family, contains four carboxyl-terminal LIM domains (LIM1–LIM4) and acts in the nucleus as a co-activator for steroid hormone receptors such as GR and AR [119, 120].

Following histone modification and chromatin remodeling, a mediator-like complex that forms a bridge between the NR-associated histone modifying complexes and the RNA polymerase II/transcription initiation complex is believed to be recruited to NRs. One such mediator-like complex is the DRIP/TRAP complex. This complex appears to contain no HAT activity, and was identified independently by two groups as a protein complex that interacted with VDR and TRα in a ligand-dependent manner [121, 122]. The DRIP/TRAP complex enhances the transcriptional activity of NRs on naked DNA templates in cell-free, ligand-dependent transcription assays [123, 124], and also appears to activate transcription mediated by several transcriptional factor classes in addition to NRs. The complex component DRIP205/TRAP220 exhibits ligand-dependent binding to NRs via two LXXLL motifs, NR1 and NR2 [125]. In the presence of thyroid hormone, the TR-RXR heterodimer recruits the DRIP/TRAP complex through the binding of RXR and TR to the NR1 and NR2 motifs, respectively. Mice heterozygous for a defective TRAP220 gene display pituitary hypothyroidism, whereas humans with TRAP230 abnormalities develop hypothyroidism [126]. Such conditions might also be classified as "co-regulator diseases" as might Rubinstein-Taybi syndrome that is caused by abnormal CBP function [105].

It is noteworthy that a third class of NR co-activator complex, the TFTC-type HAT complex, has also been identified [127]. This co-activator complex class harbors HAT activity, like p160/p300 complexes, but functionally resembles the DRIP/TRAP complex as a mediator complex. The TFTC-type HAT complex contains GCN5 HAT, the c-Myc interacting protein TRRAP/PAF400, and TAFII30, which are common factors shared with HAT complex subclass members including hTFTC, hPCAF, and hSTAGA HAT co-activator complexes. Three LXXLL motifs located in the central region of the TRRAP protein serve as the direct ligand-dependent surface for several NRs, including ERα. Surprisingly, antisense mRNA molecules for TRRAP inhibit the estrogen-dependent cell growth of breast cancer cells, which indicates that TRRAP might represent a new therapeutic target in the treatment of estrogen-dependent breast cancer [126, 127].

B. AF-1 co-activators of NRs

While the above factors and complexes act as NR AF-2 co-activators, a number of NR AF-1 co-activators have also been documented. Amino acid sequences of NR A/B domains, which contain AF-1 activity, vary among NRs, which suggests that tissue-specific AF-1 functions of particular NRs are supported by unique co-activators. For instance, ERα is phosphorylated by mitogen-activated protein (MAP) kinase activated by growth factor signaling. This phosphorylation occurs at the serine residue at position 118 in the A/B domain, and potentiates hERα AF-1 function [128]. DEAD box helicases p68 and p72 form a p160/p300 co-activator complex with the RNA co-activator SRA. This complex appears to bind more strongly to phosphorylated than non-phosphorylated ERα A/B domains, and serves as an ERα AF-1 co-activator [129, 130]. Thus, p68 and p72 AF-1 co-activators may mediate cross-talk between the growth factor and estrogen signaling pathways. Besides p68/p72, an RNA

splicing complex also appears to be preferentially recruited to phosphorylated Ser[118] in hERα [131]. In this case, the hERα AF-1 domain appears to also serve as an interacting domain for the activated dioxin receptor (AhR), establishing another potential cross-talk between estrogen- and AhR-mediated signals [132]. "Co-regulator diseases" due to AF-1 co-activators abnormalities have also been reported, such as defects in an AR-specific AF1 co-activator that results in Tfm [133], and mice genetically deficient in Cnot 7 (CAF1), an AF-1 co-activator of retinoid X receptor (RXR) β, have recently been reported to exhibit oligo-asetheno-teratozoospermia [134].

C. Co-repressors of NRs

Generally, NRs activate the transcription of target genes through the recruitment of co-activators in a ligand-dependent manner, while in the absence of ligand NRs suppress transcription by recruiting co-repressors. These co-repressors can include histone modifying complexes such as the NR-co-repressor (N-CoR) and silencing mediator for retinoic acid receptor and thyroid hormone receptor (SMRT) complexes [135–137]. NCoR and SMRT complexes share a number of proteins such as HDAC 1/2/3, the Mad presumptive co-repressor mSin3, and transducin (beta)-like (TBL) 1, and a WD-40 repeat-containing protein, the gene for which was found to be mutated in human sensorineural deafness [138–142]. These co-repressor complexes deacetylate the N-terminal tails of histones, thereby "locking" the chromatin structure, leading to suppression of target gene transcription.

TBL1 and the homologous TBLR1 are thought to serve as factors that exchange co-repressors for co-activators. The ubiquitin/proteasome system includes the 26S proteasome, a complex composed of a 20S catalytic core involved in protein proteolysis and two ATPase-containing 19S regulatory particles that recognize polyubiquitin-tagged substrates [143]. TBL1 and TBLR1 are thought to function as adaptors for the recruitment of ubiquitin/19S proteasome complexes, thereby mediating the proteasomal degradation of co-repressors, and inducing the recruitment of coactivators [144].

The mechanisms of transcriptional repression by nuclear orphan receptors remain largely unknown. However, it has been reported that the nuclear orphan receptor chicken ovalbumin upstream promoter-transcription factors (COUP-TF) I represses the transcriptional activity of target genes that interact with NCoR and SMRT [145]. Another nuclear orphan receptor, PNR, which is the causative gene for enhanced S-cone syndrome, acts as a sequence-specific repressor that controls neuronal differentiation in the developing retina. A PNR co-repressor complex has been identified that includes E2F/Myb-associated proteins, NCoR/HDAC complex-related components, TBL3 (part of the same protein family as TBL1), and the DEVH-box co-repressor (Dev-CoR) that belongs to the DEAD/DEVH protein family. This co-repressor directly interacts with PNR and functions as a platform protein. Notably, the PNR-associated Dev-CoR complex appears to function as a negative cell cycle repressor via inhibition of cell cycle-related gene promoters, indicating that co-repressors may have similar biological importance as co-activators in gene regulation (S. T., H. K., S. K., unpublished results).

V. Perspectives

NRs require a number of distinct classes of factors and/or complexes for their ligand-independent and -dependent functions in gene regulation. From the most current views on the molecular mechanisms of gene regulation by DNA-binding transcription factors, it appears that a number of complexes and factors associate with a given transcription factor in a sequential and highly regulated manner. However, while NRs appear to recruit a number of factors/complexes, it is still unclear whether particular NR molecules require many factors/complexes or only limited numbers of factors/complexes depending on the promoter/chromatin context. Also, it is likely to become clear in the near future how many of the numerous diseases and pathophysiologies related to NR functions are linked to malfunctions within co-regulators or co-regulator complexes. Such advances in the understanding of molecular mechanisms that underlie NR function in a variety of physiological and pathophysiological situations will contribute to drug discovery and new clinical applications.

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