

**Current Topics****Recent Advances in the Understanding of Nuclear Receptors- and Drug-Metabolizing Enzymes-Mediated Inter-Individual Differences****Review****Identification of the ROR $\alpha$  Transcriptional Network Contributes to the Search for Therapeutic Targets in Atherosclerosis**

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The retinoic acid receptor-related orphan receptor  $\alpha$  (ROR $\alpha$ ) is involved in the regulation of several physiological processes, including development, metabolism, and circadian rhythm. ROR $\alpha$ -deficient mice display profound atherosclerosis, in which hypoalphalipoproteinemia is reportedly associated with decreased plasma levels of high-density lipoprotein, increased levels of inflammatory cytokines, and ischemia/reperfusion-induced damage. The recent characterization of endogenous ligands (including cholesterol, oxysterols, provitamin D<sub>3</sub>, and their derivatives), mediators, and initiation complexes associated with the transcriptional regulation of these orphan nuclear receptors has facilitated the development of synthetic ligands. These findings have also highlighted the potential of application of ROR $\alpha$  as a therapeutic target for several diseases, including diabetes, dyslipidemia, and atherosclerosis. In this review, the current literature related to the structure and function of ROR $\alpha$ , its genetic inter-individual differences, and its potential as a therapeutic target in atherosclerosis is discussed.

**Key words** retinoic acid receptor-related orphan receptor  $\alpha$ ; metabolism; cholesterol; statin; ischemia; atherosclerosis

**1. INTRODUCTION**

Lipid-soluble signaling molecules, including steroid hormones, lipids, and fatty acids, regulate the expression of target genes at the transcriptional level *via* cytoplasmic or nuclear receptors.<sup>1)</sup> As these receptors act as lipophilic ligand-induced transcriptional regulators, they play a central role in the mechanism of action of these lipophilic signaling molecules.<sup>2)</sup> Steroid hormones were first recognized as being mediated by nuclear receptors and the action of lipophilic ligand molecules.<sup>2)</sup> Following the cloning of nuclear receptors for estrogen and glucocorticoids in the 1980s, nuclear receptors for progesterone, mineralocorticoids, thyroid hormone, and vitamin D were cloned.<sup>3,4)</sup> Based on their amino acid sequence homology, these nuclear receptors were defined as a highly homologous, structurally similar, and molecularly evolved superfamily.<sup>2)</sup> Subsequently, homology screening was conducted using a synthetic DNA probe corresponding to five amino acid residues with extremely high homology in the DNA-binding region. This process identified retinoic acid receptor  $\alpha$  (RAR $\alpha$ ) and retinoid X receptor (RXR) with all-*trans* retinoic acid (RA) and its isomer 9-*cis* RA as ligands, respectively.<sup>5,6)</sup> Other orphan receptors, such as retinoic acid receptor-related orphan receptor  $\alpha$  (ROR $\alpha$ ), were also identified with unknown ligands.<sup>7,8)</sup>

Nuclear receptors have a common structural organization; the N-terminal region (A/B domain) is highly variable and contains at least one constitutively activated activation function region 1 (AF-1), whereas the DNA-binding domain (DBD, C domain), a short motif responsible for DNA-binding speci-

ficity that contains two highly conserved zinc finger motifs, is conserved.<sup>9)</sup> Between the DBD and ligand-binding domain (LBD, E domain) is a less conserved region (D domain) that behaves as a flexible hinge between the C and E domains and contains the nuclear localization signal, which may overlap with the C domain. The characterization of the unliganded or liganded domains increases our understanding of the mechanisms involved in ligand binding. The C-terminal region of the LBD is the ligand-dependent AF-2.<sup>1)</sup> Thus, by elucidating the commonality and diversity of the structure and function of nuclear receptors, a better understanding of physiology is expected. This may be applicable to the medical field, as ligands can be used to target nuclear receptors involved in diseases (Fig. 1A).

**2. STRUCTURE, DISTRIBUTION, AND FUNCTION OF RORS**

ROR $\alpha$ , also called NR1F1 for orphan monomeric receptors of unknown ligands, is a member of the NR1F superfamily of nuclear receptors, including receptors for thyroid and steroid hormones, retinoids, and vitamin D.<sup>10,11)</sup> The ROR $\alpha$  gene is encoded on human chromosome 15q21–22 and mouse chromosome 9.<sup>12)</sup> It has three subtypes, each of which has a unique tissue-specific distribution; ROR $\alpha$  is highly expressed in the brain, liver, thymus, heart, vessels, testes, and skin,<sup>10,13,14)</sup> whereas ROR $\beta$  and ROR $\gamma$  are highly expressed in the brain and skeletal muscle, respectively.<sup>15,16)</sup> The tissue-specific distribution of ROR subtypes affects the development of these expressed tissues.<sup>14)</sup> Owing to alternative promoter usage and exon splicing,

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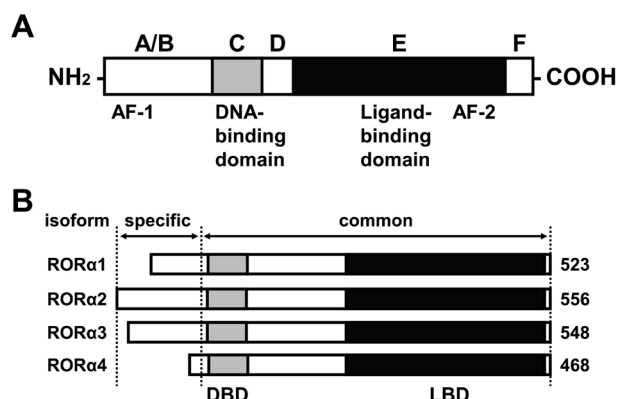


Fig. 1. Structure of ROR $\alpha$  Isoforms, Members of the Nuclear Hormone Receptor Superfamily

A. Domain structure of the nuclear receptor. B. Structure of the human ROR $\alpha$  isoforms. In their isoforms (Protein database accession numbers; ROR $\alpha$ 1; NP\_599023, ROR $\alpha$ 2; NP\_599022, ROR $\alpha$ 3; NP\_002934, ROR $\alpha$ 4; NP\_599024), the NH<sub>2</sub>-terminus of each ROR $\alpha$  generated by alternative promoter and/or splicing is the isoform-specific region, and the COOH-terminus, including the DBD and LBD, is the isoform common region. The numbers on the right refer to the total number of amino acids in the ROR $\alpha$ . ROR $\alpha$ , retinoic acid receptor-related orphan receptor  $\alpha$ ; AF-1, activation function region 1; AF-2, activation function region 2; DBD, DNA-binding domain; LBD, ligand-binding domain.

each ROR gene generates several isoforms that contain a common DBD and LBD, which differ only in their amino termini.<sup>10</sup> To date, four ROR $\alpha$  isoforms, ROR $\alpha$ 1–4, have been identified in humans, whereas only two isoforms,  $\alpha$ 1 and  $\alpha$ 4, have been reported in mice.<sup>17,18</sup> The human ROR $\beta$  gene generates only the ROR $\beta$ 1 isoform, whereas that of mice appears to express two isoforms,  $\beta$ 1 and  $\beta$ 2.<sup>15</sup> Both human and mouse ROR $\gamma$  genes generate the  $\gamma$ 1 and  $\gamma$ 2 (also  $\gamma$ t) isoforms.<sup>19,20</sup>

Most isoforms have a distinct tissue-specific expression pattern, regulate different physiological processes, and affect the expression of different target genes. For example, human ROR $\alpha$ 2 is located in the testis and skin, whereas human ROR $\alpha$ 3 is found exclusively in the testis.<sup>21,22</sup> ROR $\alpha$ 1 and ROR $\alpha$ 4 are prominently expressed in the mouse cerebellum, whereas other mouse tissues predominantly express ROR $\alpha$ 4.<sup>17,18</sup> Moreover, the expression of ROR $\alpha$ 4, but not ROR $\alpha$ 1, is highly induced by hypoxia-inducible factor (HIF)-1 under hypoxic conditions in various cells.<sup>23,24</sup> Conditions that mimic the effects of hypoxia, including exposure to cobalt chloride and the iron chelator deferoxamine, also enhance ROR $\alpha$ 4 expression.<sup>25</sup> In addition, ROR $\alpha$ 1 and ROR $\alpha$ 4 have unique intracellular localization; ROR $\alpha$ 1 is translocated to the nucleus and although ROR $\alpha$ 4 is primarily translocated to the nucleus, it is also partially localized in the cytoplasm.<sup>26</sup> Furthermore, the transcriptional regulation mechanism of Wnt/ $\beta$ -catenin signaling, depending on differences in the N-terminal domain of the ROR $\alpha$ 1 and ROR $\alpha$ 4 isoforms, has been clarified.<sup>27,28</sup> The N-terminal domain of ROR $\alpha$ 1 specifically recognizes and binds to  $\beta$ -catenin, resulting in transcriptional activation *via* binding to the response elements of target genes by ROR $\alpha$ 1 through  $\beta$ -catenin as a coactivator.<sup>27</sup> Meanwhile, transcriptional regulation by  $\beta$ -catenin with T cell factor (TCF)/lymphoid enhancer factor (LEF) complexes is suppressed *via* binding of ROR $\alpha$ 1 to  $\beta$ -catenin. In contrast, the N-terminal domain of ROR $\alpha$ 4 has no effect on  $\beta$ -catenin with TCF/LEF.<sup>28</sup> Although the DBD and LBD are common, the transcriptional control function differs greatly depending on the difference in the N-terminal, and the role of each isoform

differs.<sup>28</sup> Although most ROR $\alpha$  isoforms are under the control of different promoters, little is known about the transcriptional regulation of their tissue-specific expression (Fig. 1B).

Nuclear receptors activate or inactivate the transcription of primary target genes by binding to specific DNA sequences known as hormone-responsive elements, which are composed of 6-bp sequences from 5'-AGGTCA-3' or 5'-AGACA-3'. These sequences are referred to as consensus half-site motifs and are arranged as direct, inverted, or everted repeats.<sup>29–31</sup> As a monomer, nuclear receptor ROR $\alpha$  binds to the ROR response elements (ROREs) containing a single core half-site motif RGGTCA (R; A or G) preceded by a 6-bp A/T-rich sequence<sup>10</sup> (Fig. 2). ROR $\alpha$  is based on one half-site sequence, and the presence of dual elements that overlap with other hormone response sequences containing two half-sites is expected.<sup>13,32–34</sup> For instance, the RORE of the laminin B1 gene overlaps with that of the RA response elements (RAREs) and is transcriptionally activated by both ROR $\alpha$  and RAR.<sup>12,13</sup> In addition, the RORE of the CYP7B1 gene overlaps with liver X receptor (LXR) response elements (LXREs), resulting in the simultaneous transcriptional activation of LXR and repression of ROR $\alpha$ ; hence, the binding of ROR $\alpha$  or LXR to the response elements functions as a competitor with each of the other receptors.<sup>32</sup> The LXRE contains a direct repeat motif comprising two AGGTCA cores separated by four nucleotides.<sup>35</sup> Furthermore, owing to the similarity in the binding sequences for peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) and ROR $\alpha$ , ROR $\alpha$  can modulate PPAR signaling by competing with PPAR $\gamma$  for PPAR response elements (PPREs).<sup>33,36</sup> The PPRE contains a direct repeat motif comprising two AGGTCA cores separated by a single nucleotide.<sup>36</sup> Recent global transcriptome studies have shown that ROR $\alpha$  controls lipid homeostasis *via* the negative regulation of the transcriptional activity of PPAR $\gamma$ , which mediates hepatic lipid metabolism.<sup>33</sup> Similarly, the reverse strand of *ERBA* (REV-ERB), which has an amino acid sequence similar to that of ROR $\alpha$ , binds to ROREs.<sup>34</sup> Although REV-ERB has been identified as an inactive monomeric receptor with DNA-binding ability, it lacks the capacity to activate transcription, instead functioning as a repressor of transcription<sup>34</sup> (Fig. 2). Moreover, each nuclear receptor is dependent on specific DNA sequencing, whereas transcription is coordinated by DNA-binding affinity, expression level, and the presence or absence of a ligand.<sup>14,37</sup>

### 3. LIGANDS AND MEDIATORS ACTING ON ROR $\alpha$

ROR $\alpha$  was initially described as an orphan receptor and has long been considered a constitutive activator of transcription in the absence of exogenous ligands.<sup>12</sup> A recent study has crystallized the ROR $\alpha$  LBD and revealed the presence of cholesterol and cholesterol sulfate in the ligand-binding pocket (Protein Data Bank codes: 1N83 and 1S0X).<sup>38,39</sup> Further experiments on purified ROR $\alpha$  LBDs have shown the presence of ligands, such as cholesterol and 7-dehydrocholesterol (also provitamin D<sub>3</sub>), that represent the major ligands in the LBD.<sup>40</sup> For instance, ROR $\alpha$  LBD expressed in insect cells is in a liganded form with bound cholesterol, which stabilizes the receptor in an agonistic conformation.<sup>40</sup> Furthermore, the oxidized form of cholesterol acts as an inverse agonist.<sup>40,41</sup> One of the 7-oxygenated sterols, 7 $\alpha$ -hydroxycholesterol (7 $\alpha$ -OHC), functions as a high-affinity ligand for ROR $\alpha$  isoforms by di-

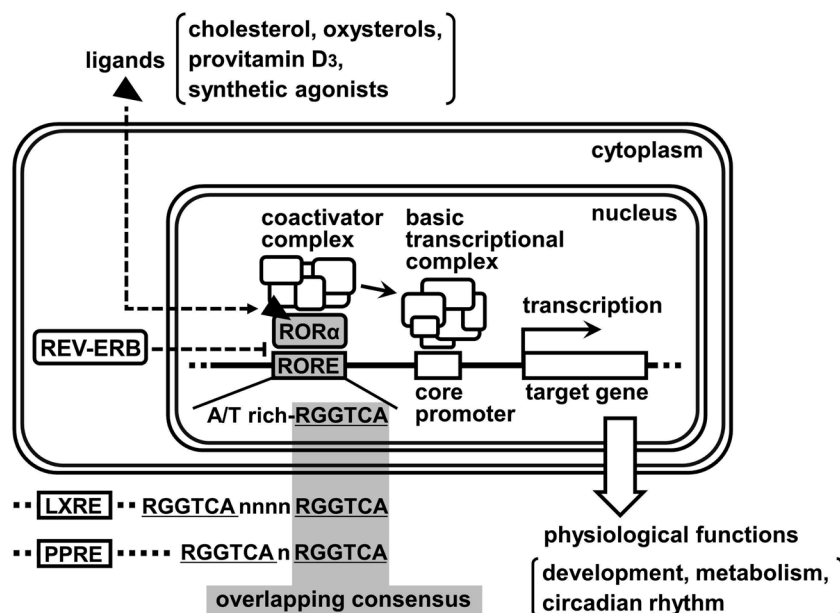


Fig. 2. ROR $\alpha$  Transcriptional Regulation System and Its Physiological Functions

ROR $\alpha$  binds as a monomer to ROREs containing the RGGTCA (R; A or G) consensus motif preceded by an A/T-rich region. REV-ERB can compete with ROR $\alpha$  for binding to ROREs. LXR and PPAR bind the LXRE and PPARE as overlaps to RORE, respectively. ROR $\alpha$  interacts with coregulators as coactivators or corepressors to positively or negatively regulate gene transcription. Certain ligands (including cholesterol, oxysterols, provitamin D<sub>3</sub>, and their derivatives) can modulate ROR $\alpha$  transcriptional activity. ROR $\alpha$  is critical in the regulation of several physiological functions and may have a role in various pathologies. ROR $\alpha$  and its regulatory networks might serve as potential novel targets for therapeutic strategies to intervene in disease processes. ROR $\alpha$ , retinoic acid receptor-related orphan receptor  $\alpha$ ; RORE, ROR response element; REV-ERB, reverse strand of *ERBA*; LXR, liver X receptor; LXRE, LXR response elements; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR response elements.

rectly binding to their LBDs, modulating coactivator binding, and suppressing the transcriptional activity of the receptors.<sup>41)</sup> 7 $\alpha$ -OHC serves as a key intermediate in bile acid metabolism, and has been shown to modulate the expression of ROR target genes.<sup>41)</sup> Additionally, 25-OHC functions as a low-affinity ligand for ROR $\alpha$ .<sup>40)</sup> Interestingly, the expression of cholesterol 25-hydroxylase, which produces 25-OHC from cholesterol, is suppressed by ROR $\alpha$ -deficient conditions.<sup>42,43)</sup> ROR $\alpha$  could sense 25-OHC and subsequently regulate the expression of cholesterol 25-hydroxylase, thereby regulating the synthesis of 25-OHC.<sup>43)</sup> Similarly, 24S-OHC also serves as a high-affinity ligand for ROR $\alpha$ .<sup>44)</sup> 24S-OHC, also known as cerebrosterol owing to its high level in the brain, plays an essential role as an intermediate in cholesterol elimination from the central nervous system<sup>45)</sup> and functions as an inverse agonist of ROR $\alpha$ , decreasing the capacity of ROR $\alpha$  to recruit coactivators when bound to the target gene promoter.<sup>44)</sup> The expression of CYP39A1, which selectively metabolizes 24S-OHC, is induced by the binding of ROR $\alpha$  to the promoter and first intron of CYP39A1.<sup>46)</sup> Thus, ROR $\alpha$  is involved in the metabolic regulation of its inverse agonist, 24S-OHC.<sup>46)</sup> Although these oxysterols act as inverse agonists of ROR $\alpha$ , they are also agonists for LXRs and are involved in gene expression regulation.<sup>35,47)</sup> In addition, vitamin D hydroxy-metabolite, made from 7-dehydrocholesterol, acts as an inverse agonist of ROR $\alpha$  and an agonist for vitamin D receptors (VDR).<sup>48,49)</sup> ROR $\alpha$  may competitively bind to and activate ligands for VDRs and LXRs, and each of these nuclear receptors senses the concentration of common endogenous metabolites<sup>47,48,50)</sup> (Fig. 2). Therefore, it is presumed that transcription is mutually controlled; however, the complete underlying mechanism remains unclear.

Cholesterol depletion in osteosarcoma cells by statins,

which are inhibitors of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase, in low-density lipoprotein (LDL)-free serum significantly decreases the transcriptional activity of ROR $\alpha$ , implying that cholesterol is an endogenous ligand rather than merely a structural cofactor.<sup>38)</sup> In addition, the statin-induced reduction in cholesterol levels in hepatocytes with active cholesterol synthesis causes ROR $\alpha$  target gene expression to decrease owing to the attenuation of the ROR $\alpha$  transcriptional activation in the ligand form.<sup>51)</sup> Moreover, the ability of ROR $\alpha$  to activate transcription is attenuated following the suppression of cholesterol synthesis by azoles acting as inhibitors of lanosterol 14 $\alpha$ -demethylase (CYP51).<sup>52)</sup> Mice with hepatocyte-specific knockout of Cyp51 are characterized by the progressive onset of liver injury with fibrosis.<sup>53)</sup> Endogenous ligands of ROR $\gamma$ , a subtype of ROR $\alpha$ , are situated downstream of lanosterol and upstream of zymosterol in the cholesterol synthesis pathway; the levels of these ligands are reduced by Cyp51 knockout.<sup>53)</sup> The reduction in ROR $\alpha$  and ROR $\gamma$  transcriptional activity is greater in Cyp51 knockout mice and is correlated with their downregulated amino and fatty acid metabolism *via* the transcriptional attenuation of ROR $\alpha$  target genes.<sup>53)</sup> Therefore, ROR $\alpha$  may also impact transcriptional regulatory ability through the action of cholesterol derivatives as ligands. However, how nuclear receptors sense differences in the concentrations of multiple metabolites and interact with each other to activate or suppress the expression of target genes *in vivo* remains to be determined.

The conformational changes that occur following agonist binding cause receptors to enter an activated state, which induces chromatin remodeling *via* histone acetylase activity and, in turn, facilitates the recruitment of coactivator complexes that increase the transcription of the target gene.<sup>54)</sup> The recruitment of the corepressor complex induces chromatin

compression as well as the suppression of gene expression through histone deacetylase activity.<sup>55)</sup> RORs interact with coactivators and corepressors, suggesting that they function as activators and repressors of gene transcription.<sup>14)</sup> During ROR $\alpha$ -mediated transcriptional regulation, RORs recruit different coactivator complexes when bound to ROREs in the promoter region of different genes,<sup>56)</sup> which suggests that their promoter functions play important roles in determining which coactivators are recruited by RORs.<sup>57)</sup> The coregulators found within ROR protein complexes include the coactivators steroid receptor coactivator-1 (SRC-1, NCOA1), SRC-2 (NCOA2, TIF2, GRIP1), SRC-3 (NCOA3), PPARG coactivator 1 alpha (PGC-1 $\alpha$ , PPARGC1A), cAMP response element-binding protein (CBP), p300, human immunodeficiency virus type 1 (HIV-1) Tat interactive protein (HTATIP, TIP60), and  $\beta$ -catenin with the following corepressors: nuclear receptor corepressor 1 (NCOR1, N-CoR), NCOR2 (SMRT), receptor interacting protein 140 (RIP140), Hairless (HR), cadherin 4 (CDH4), and neuronal interacting factor X (NIX1)<sup>14,57-60)</sup> (Fig. 2). Cell-specific interactions with specific coregulators may contribute to the molecular mechanism underlying the distinct physiological functions of ROR $\alpha$ .<sup>57)</sup> Members of the p160 family containing SRCs were among the first coregulators to be cloned, based on their ligand-dependent recruitment to the nuclear receptor LBD through three-helical LXXLL (X, any amino acid) motifs located in their N-terminal region, and conserved leucine-rich motifs in their C-terminal activation domain that mediate interactions with additional coregulators.<sup>61)</sup> Moreover, ligands that affect the interaction between these nuclear receptors and coregulators are attracting attention as drug discovery targets for the treatment of various diseases.<sup>61)</sup> SRCs associate with nuclear receptors in a ligand-dependent manner to enhance transcriptional activity *via* the stabilization of the basic transcriptional complex by binding indirectly to DNA and play important roles in a variety of physiological processes.<sup>61)</sup> The role of RORs in the regulation of glucose metabolism is characterized by the loss of SRC-2 in mice, leading to a phenotype similar to von Gierke's disease, which is associated with severe hypoglycemia and abnormal glycogen accumulation in the liver.<sup>62)</sup> Furthermore, the phenotypes observed in global or liver-specific SRC-2 knockout mice include hypoglycemia in the fasted state and reduced expression of hepatic glucose 6-phosphatase (G6PC) in both fed and fasted states.<sup>62)</sup> SRC-2 regulates G6PC expression by coactivating ROR $\alpha$  at an evolutionarily conserved RORE sequence of the proximal G6PC promoter.<sup>62,63)</sup> Thus, the interaction of ROR $\alpha$  with mediators and transcription factors is important in regulating the degree of gene expression.

#### 4. PHENOTYPE OF ROR $\alpha$ -DEFICIENT MICE

The staggerer (sg) mutation arose spontaneously in a stock of obese mice and is recognized by phenotypes characteristic of cerebellar lesions; the sg cerebellum is significantly smaller in obese mice than in control mice, containing fewer of each principal cell type.<sup>64)</sup> After 35 years of analysis, the sg mutation has been identified by positional cloning as a putative null allele of ROR $\alpha$ .<sup>17)</sup> Mutant mice contain a 6.4-kb intragenic deletion that removes the fifth exon, encoding the start of the LBD; loss of this exon predicts a frameshift in the mRNA, resulting in a premature stop codon. Reverse genetics analysis

of ROR $\alpha$ -null mice created by gene targeting produced phenotypes essentially identical to sg.<sup>65,66)</sup> ROR $\alpha$  expression in the brains of adult mice is restricted to a few specific neuron classes, including the Purkinje cells of the cerebellum, and is temporally regulated during postnatal development.<sup>13,67)</sup> Moreover, the ROR $\alpha$  gene is disrupted in sg mice, which show a cell autonomous defect in the development of Purkinje cells.<sup>17)</sup> Interestingly, further indications for a link between ROR $\alpha$  and autism arose from a recent study demonstrating that microRNA-137, which has been implicated in autism and schizophrenia, targets the 3'-untranslated region (UTR) of ROR $\alpha$ .<sup>68)</sup>

In other pathophysiological conditions, sg mice have enhanced susceptibility to atherosclerosis and hypoalbuminemia, displaying accelerated vascular lesion development in response to a proatherogenic diet.<sup>69)</sup> Sg mice also exhibit decreased fasting blood glucose levels, mildly improved glucose tolerance, increased insulin sensitivity, and ROR $\alpha$  involvement in metabolic diseases.<sup>70)</sup> In addition to increased ischemia-induced angiogenesis in sg mice, ROR $\alpha$  expression is observed in vascular cells, whereas ROR $\alpha$  expression is decreased in atherosclerotic plaques.<sup>21,71,72)</sup> ROR $\alpha$  also regulates gene expression in obesity-associated inflammation; sg mice fed a high-fat diet exhibit reduced adiposity and hepatic triglyceride levels compared with wild-type littermates and are resistant to the development of hepatic steatosis, adipose-associated inflammation, and insulin resistance.<sup>73)</sup> Further, ROR $\alpha$  regulates key physiological pathways, is involved in pathogenic processes, regulates lipid and glucose metabolism, and is believed to play a protective role against the development of atherosclerosis.<sup>14)</sup> Indeed, cerebral ischemia/reperfusion (I/R) injury is associated with greater cerebral infarct size, brain edema, and cerebral apoptosis in sg mice compared with those in wild-type mice. In contrast, this effect is reduced in transgenic mice with brain-specific ROR $\alpha$  overexpression, compared to nontransgenic controls.<sup>74)</sup> Furthermore, sg mice subjected to myocardial I/R injury show a significantly increased myocardial infarct size, myocardial apoptosis, and exacerbated contractile dysfunction compared with wild-type mice.<sup>75)</sup> Moreover, mice with cardiomyocyte-specific ROR $\alpha$  overexpression are less vulnerable to myocardial I/R injury than the control mice.<sup>75)</sup> In a recent study on human-induced pluripotent stem cell-derived cardiomyocytes infected *in vitro* with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), ROR $\alpha$  expression was reported to be upregulated, which was speculated to be a cardioprotective response to direct viral invasion.<sup>76)</sup>

Stenosis of blood vessels owing to atherosclerosis is a typical condition caused by modern unhealthy lifestyles (fat-rich, high-calorie diet, lack of exercise, and smoking). However, this condition also occurred in ancient human social groups.<sup>77)</sup> This phenomenon may be caused not only by environmental factors, such as unhealthy eating habits, but also by certain genetic factors.

#### 5. ROR $\alpha$ TARGET GENES

To identify atherosclerosis through metabolic disease-related genes, it is important to perform a forward genetics search based on the pathological conditions of atherosclerosis and reverse genetics evaluation based on the functional deletion of a specific gene. Based on transcriptional regulation analysis



using an ROR $\alpha$  functional deletion, target genes of ROR $\alpha$  have been explored.

ROR $\alpha$  regulates the circadian expression of several clock genes, including circadian locomotor output cycles kaput (CLOCK), aryl hydrocarbon receptor nuclear translocator-like protein 1 (ARNTL)/brain and muscle ARNT-like 1 (BMAL1), neuronal PAS domain protein 2 (NPAS2), and cryptochrome circadian regulator 1 (CRY1). Moreover, ROR $\alpha$  competes with REV-ERBs for binding to their shared ROREs on certain clock genes, leading to a circadian pattern.<sup>78–80</sup> Therefore, ROR $\alpha$  influences the period length and stability of the circadian rhythm.<sup>81,82</sup> Increasing evidence regarding how the cholesterol/oxysterol pathways are intertwined with the circadian clock has been reported. The identified key contact points include different forms of the RORs, REV-ERBs, and LXRs.<sup>83,84</sup> These receptors are regulated by sterols/oxysterols and the circadian clock, representing a complex interplay between sterol metabolism and the clock.<sup>83,84</sup> Moreover, ROR $\alpha$  binds to the ROREs of genes involved in lipid metabolism to regulate genes such as apolipoprotein A1 (APOA1), APOA5, and APOC3, the key regulators of sterol regulatory element-binding protein-1c (SREBP1c), and the reverse cholesterol transporters, ATP binding cassette subfamily A member 1 (ABCA1) and ABCG1.<sup>85–87</sup>

Macrophages from ROR $\alpha$ -deficient mice overexpress interleukin (IL)-1 $\beta$  following lipopolysaccharide stimulation, suggesting an anti-inflammatory role for ROR $\alpha$ .<sup>88</sup> Moreover, ROR $\alpha$  deletion in human monocytic cells causes an increase in the basal expression of a subset of nuclear factor-kappa B (NF- $\kappa$ B)-regulated anti-inflammatory genes, including IL-1 $\beta$ , IL-6, and tumor necrosis factor (TNF), both at the transcriptional and translational levels.<sup>89</sup> ROR $\alpha$  is a negative regulator of the inflammatory response, functioning *via* NF- $\kappa$ B inhibition through NF- $\kappa$ B inhibitor  $\alpha$  (I $\kappa$ B $\alpha$ ) activation by ROR $\alpha$  *via* an RORE in the I $\kappa$ B $\alpha$  promoter.<sup>90</sup> Furthermore, ROR $\alpha$  is involved in TNF and IL-6 production upon macrophage activation and plays a key role in M1/M2 polarization of murine Kupffer cells, which are liver-resident macrophages.<sup>91,92</sup> Kupffer cells with ROR $\alpha$  deletion have the proinflammatory M1 phenotype, as the shift to anti-inflammatory M2 requires ROR $\alpha$  activation.<sup>92</sup> Additionally, ROR $\alpha$  reduces lipid droplets *via* the upregulation of neutral cholesterol ester hydrolase 1 (NCEH1) in macrophages, suggesting that ROR $\alpha$  functions to protect against atherosclerosis.<sup>93,94</sup>

In brain endothelial cells, claudin domain containing 1 (CLDN1), which is involved in tight junction formation, is regulated at the transcriptional level by ROR $\alpha$  binding to cholesterol derivatives as ligands and the myeloid zinc finger 1 transcription factor, as well as at the post-transcriptional level by microRNA-124.<sup>51,95–97</sup> Moreover, decreased CLDN1 expression enhances vascular permeability, which consequently increases the risk of cerebellar hemorrhage.<sup>98,99</sup>

Within the liver, ROR $\alpha$  specifically functions as a positive modulator of genes encoding phase I and phase II proteins, which are involved in the metabolism of lipids, steroids, and xenobiotics, such as sterol 12 $\alpha$ -hydroxylase (CYP8B1), sterol 7 $\alpha$ -hydroxylase (CYP7B1 and CYP39A1), aromatase (CYP19A1), and sulfotransferase family 2A member 1 (SULT2A1); these enzymes catalyze the metabolism of oxysterols, such as cytotoxic compounds.<sup>32,46,100–103</sup> In addition, through the phosphorylation of its serine 100 residue, ROR $\alpha$

activity has been integrated into the constitutive androstane receptor (CAR) and RXR heterodimer for the phenobarbital-inducible CYP2B6 gene in the formation of the coregulator complex involved in metabolizing some xenobiotics, such as anticancer drugs.<sup>104,105</sup> Various cholesterol hydroxylases contribute to cholesterol metabolism in extrahepatic tissues by converting cellular cholesterol to circulating oxysterols, which regulate diverse physiological processes.<sup>32,46,93,106</sup> SRC-2 functions as a coactivator with ROR $\alpha$  to modulate the expression of the essential gluconeogenesis genes G6PC and phosphoenolpyruvate carboxykinase (PCK1), which are the rate-limiting enzymes that control glucose release into plasma.<sup>62,107</sup>

As above, research has been conducted with the aim of developing a new treatment for atherosclerosis by understanding the ROR $\alpha$  transcriptional network, which has an antiatherosclerotic effect. Specifically, a global search for ROREs as high-affinity ROR $\alpha$ -binding sites involved in transcriptional regulation within the promoter region of human genes and gene expression analyses involving both loss-of-function and gain-of-function have been performed, identifying target genes associated with antiatherosclerosis.<sup>46,93,95,107</sup>

## 6. DRUG TREATMENT OF DISEASES WITH ROR $\alpha$ SYNTHETIC LIGANDS

Transcriptional conjugating factors in the cell nucleus bind to nuclear receptors in a ligand binding-dependent manner.<sup>55</sup> The transcriptional conjugation inhibitor (corepressor) that controls transcription binds to the unbound state of the ligand and dissociates owing to ligand binding, after which the transcriptional conjugation activator (coactivator) is acquired.<sup>108</sup> The role of LXR agonists and cholesterol derivatives in the modulation of ROR $\alpha$  activity has been discussed in the context of its metabolic role in several pathways. For example, the LXR agonist T0901317, a benzenesulfonamide derivative, suppresses the ability of ROR $\alpha$  chimeric forms to transactivate gene expression in a heterologous reporter assay.<sup>109</sup> Moreover, the LXR agonist modulates and suppresses the activity of gluconeogenesis-associated ROR $\alpha$  target genes in an RORE-dependent manner *via* the SRC-2 coactivator in von Gierke's disease.<sup>62</sup> These studies underscore the regulatory crosstalk that may occur between LXR and ROR signaling, showing that natural products possess overlapping receptor specificity that oppositely regulates each nuclear receptor-dependent pathway.<sup>37</sup> In addition, the amide derivative SR1078, from the T0901317 scaffold, modulates ROR $\alpha$  activity and increases coactivator recruitment in a dose-dependent manner.<sup>110</sup> ROR $\alpha$  activation *via* SR1078 induces M2 polarization through Krüppel-like factor 4 (KLF4) gene regulation in macrophages, effectively protecting against nonalcoholic steatohepatitis (NASH).<sup>92</sup> This activation also promotes the removal of accumulated cholesterol, which is the cause of atherosclerosis, by regulating lipid metabolism-related genes.<sup>46,92,93</sup> SR1078 ameliorates renal dysfunction and damage in a renal I/R injury mouse model; thus, ROR $\alpha$  is a potential endogenous protector against renal I/R injury.<sup>111</sup> Moreover, treatment of an autistic mouse model with SR1078 reduces repetitive behavior.<sup>112</sup> The thiourea derivative JCI-40, which is a ROR $\alpha$  agonist, protects against oxidative stress through induction of the antioxidant enzymes, superoxide dismutase 2 (SOD2) and glutathione peroxidase 1 (GPX1) and attenuates the methio-

nine-choline deficient diet-induced NASH in mice.<sup>113)</sup> The selective and specific ROR $\alpha$  inverse agonist, SR3335, suppresses gluconeogenesis-associated ROR $\alpha$  target gene expression and decreases blood glucose levels in a diet-induced obesity mouse model.<sup>114)</sup> The inverse agonist SR1001 suppresses ROR $\alpha$  and ROR $\gamma$ t, attenuates Th17 differentiation and insulinitis by inhibiting IL-17A production in a mouse model of type 1 diabetes. SR1001 also attenuates ROR target genes involved in hepatic gluconeogenesis and prevents hyperglycemia.<sup>107,115,116)</sup> Finally, RS-2982 is an ROR $\alpha$  agonist that increases the expression of microRNA-122 as an ROR $\alpha$  target in mouse livers. Mice treated with RS-2982 and fed a high-fat or atherogenic diet showed reduced hepatic lipotoxicity, liver fibrosis, and body weight compared to mice administered the vehicle.<sup>117)</sup> Hence, the regulation of ROR $\alpha$  activity may be a therapeutic strategy for treating several conditions, including atherosclerosis, NASH, type 2 diabetes, autoimmune disorders, and autism. In addition, gene regulation and polymorphisms of human ROR $\alpha$  are associated with the development of many conditions, including type 2 diabetes,<sup>118)</sup> multiple sclerosis,<sup>119)</sup> cerebellar ataxia,<sup>120)</sup> autism,<sup>121)</sup> bipolar disorder,<sup>122)</sup> Alzheimer's disease,<sup>123)</sup> and the severity of coronavirus disease 2019.<sup>76)</sup> Changes in the function, RNA stability, and target gene expression of ROR $\alpha$  have been reported owing to various polymorphisms in the structure, 3'-UTR, and response sequences of ROR $\alpha$ , respectively. Mutations in these nucleotide sequences may also affect individual differences in human diseases.

## 7. CONCLUSION

In summary, ROR $\alpha$  is involved in the regulation of several physiological processes, including development, metabolism, and circadian rhythm. Furthermore, ROR $\alpha$  is an important regulator of plasma cholesterol levels and is involved in lipid homeostasis. For example, apolipoprotein expression increases high-density lipoprotein (HDL) levels and CYP expression activates oxysterol metabolism by ROR $\alpha$  activation. ROR $\alpha$ -deficient mice are more likely to develop atherosclerosis through lowered HDL levels, increased inflammatory cytokine expression, and I/R-induced damage. The transcriptional activity of ROR $\alpha$  is promoted by the endogenous ligand cholesterol derivatives, which promote the formation of transcription initiation complexes. However, ROR $\alpha$  transcriptional activation is attenuated when intracellular cholesterol is reduced by lipid-lowering drugs that inhibit cholesterol synthesis, such as statins. Research has been conducted with the aim of developing a new treatment for atherosclerosis by understanding the ROR $\alpha$  transcriptional network, which has an anti-atherosclerotic effect. Thus, the recent characterization of endogenous ligands, mediators, and initiation complexes associated with the transcriptional regulation of these nuclear receptors has facilitated the development of synthetic ligands and highlighted the potential of application of ROR $\alpha$  as a therapeutic target for several diseases. However, how nuclear receptors sense differences in the concentrations of multiple metabolite ligands and interact with each other to activate or suppress target gene expression *in vivo* remains to be determined. This review sheds light on the current literature related to the structure and function of ROR $\alpha$ , its genetic inter-individual differences, and its potential as a therapeutic target in atherosclerosis.

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**Conflict of Interest** The authors declare no conflict of interest.

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