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The Mineralocorticoid Receptor: Insights in its Molecular and (Patho)Physiological Biology

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Abbreviations

11βHSD2: 11 beta HydroxySteroid Dehydrogenase 2; ACE: Angiotensin Converting Enzyme; ACTH: AdrenoCorticoTrophic Hormone; ADAMTS1: A Disintegrin And Metalloproteinase with Thrombospondin-like motifs 1; adPHA1: autosomal dominant Pseudohypoaldosteronism type 1; AF1: Activation Function 1; AF2: Activation Function 2; ANF: Atrial Natriuretic Factor; AR: Androgen Receptor; ASC2: Activating Signal Cointegrator 2; BMP2: Bone Morphogenetic Protein 2; CBP: CREB Binding Protein; CHIF: Channel-Inducing Factor; CNS: Central Nervous System; DAXX: Death-Associated protein 6; DBD: DNA Binding Domain; EGF-R: Epidermal Growth Factor Receptor; Egr-1: Early growth response gene-1; ELL: Eleven-nineteen Lysine-rich Leukemia; ENaC: Epithelial Sodium Channel; ERK: Extracellular signal-Regulated Kinase; ET-1: Endothelin-1; FAF1: Fas Associated Factor 1; FLASH: FLICE Associated Huge; GILZ: Glucocorticoid-Induced Leucine Zipper protein; G6PD: Glucose-6-Phosphate Dehydrogenase; GR: Glucocorticoid Receptor; GRE: Glucocorticoid Responsive Element; HAS2: Hyaluronic Acid Synthase 2; HDAC: Histone Deacetylase; hMR: human Mineralocorticoid Receptor; HRE: Hormone Responsive Element; hsp: heat shock protein; KS-WNK1: Kidney Specific With No lysine [K] Kinase 1; LBD: Ligand Binding Domain; LXRβ: Liver X Receptor beta; MAPK: Mitogen-Activated Protein Kinase; MDM2: Murine Double Minute gene 2; MR: Mineralocorticoid Receptor; MRE: Mineralocorticoid Responsive Element; NAD: Nicotinamide Adenine Dinucleotide; NCoR: Nuclear Receptor Corepressor; NDRG2: N-Myc Downstream Regulated Gene 2; Nedd: Neuronal precursor cell-expressed, developmentally down-regulated gene; NES: Nuclear Export Signal; NLS: Nuclear Localization Signal; NR: Nuclear Receptor; NTD: N-Terminal Domain; Orm: Orosomucoid; p/CAF: p300/CBP-Associated protein; PAI-1: Plasminogen Activator Inhibitor-1; PDK1: 3-Phosphoinositide-Dependent Kinase 1; PGC-1α: Peroxisome proliferator-activated receptors Gamma (PPARgamma) Coactivator-1 alpha; PGC-1β: PPAR Gamma Coactivator-1 beta; PIAS: Protein Inhibitor of Activated Signal transducer and activator of transcription; PR: Progesterone Receptor; RGS2: Regulator of G-protein Signaling 2; RHA: RNA Helicase A; RIP140: Receptor-Interacting Protein 140; RXRβ: Retinoid X Receptor beta; SGK1: Serum and Glucocorticoid-regulated Kinase 1; SMRT: Silencing Mediator of Retinoid and Thyroid hormone receptors; SR: Steroid Receptor; SRC-1: Steroid Receptor Coactivator-1; STAT: Signal Transducer and Activator of Transcription; SUMO: Small Ubiquitin-like Modifier; TIF: Transcription-Intermediary-Factor; TM: Transcriptional Machinery; TNX: Tenascin-X; Ubc9: Ubiquitin-like protein SUMO-1 E2-conjugating enzyme 9; UCP-1: Uncoupling Protein-1; UPAR: Urokinase-type Plasminogen Activator Receptor; Usp2-45: Ubiquitin-specific protease 2-45
Abstract
The last decade has witnessed tremendous progress in the understanding of Mineralocorticoid Receptors (MR), their molecular mechanism of action, and their implication for physiology and pathophysiology. After the initial cloning of MR, and identification of its gene structure and promoters, it now appears as a major actor in protein-protein interaction networks. The role of transcriptional coregulators and the determinants of mineralocorticoid selectivity have been elucidated. Targeted oncogenesis and transgenic mouse models have identified unexpected sites of MR expression and novel roles for MR in non-epithelial tissues. These experimental approaches have contributed to the generation of new cell lines for the characterization of aldosterone signaling pathways, and have also facilitated a better understanding of MR physiology in the heart, vasculature, brain and adipose tissues. This review describes the structure, molecular mechanism of action and transcriptional regulation mediated by MR, emphasizing the most recent developments at the cellular and molecular level. Finally, through insights obtained from mouse models and human disease, its role in physiology and pathophysiology will be reviewed. Future investigations of MR biology should lead to new therapeutic strategies, modulating cell-specific actions in the management of cardiovascular disease, neuroprotection, mineralocorticoid resistance, and metabolic disorders.
A brief history

In the late 1960’s, evidence for the presence of specific receptors mediating corticosteroid action in the toad bladder was initially proposed by the group of Edelman [Porter and Edelman, 1964]. Subsequently, Type I and Type II corticosteroid receptors were described and identified as mineralocorticoid (MR) and glucocorticoid receptors (GR) [Marver et al., 1974]. MR was characterized as a high affinity (Kd~1 nM), low capacity (20-50 fmol/mg protein) receptor and demonstrated to be a major regulator of sodium reabsorption in the kidney [Funder et al., 1972]. Fifteen years later, the human MR (hMR) cDNA was cloned by the Evans’ laboratory by screening a human kidney cDNA library at low stringency with a probe encompassing the DNA binding domain of the GR [Arriza et al., 1987]. MR were subsequently cloned and characterized in many species including xenopus, fish (zebra fish, teleost fish [Greenwood et al., 2003], rainbow trout [Sturm et al., 2005]), bird [Hodgson et al., 2007; Porter et al., 2007] and mammals (mouse, rat [Patel et al., 1989], mole, pig, cow, monkey [Patel et al., 2000; Pryce et al., 2005]). In the late 1990’s came the identification of multiple transcription coregulators that mediate MR transcriptional potency at aldosterone-target genes as reviewed by [O’Malley, 2007]. MR are now recognized as crucial transcription factors involved in many physiological processes and pathological disorders.

Structure

Structure of the gene

The gene NR3C2 encoding the hMR is located on chromosome 4 in the q31.1 region and spans approximately 450 kb [Morrison et al., 1990; Zennaro et al., 1995]. As illustrated in Figure 1, the gene is composed of ten exons; the two first exons 1α and 1β are untranslated, and the following eight exons encode the entire MR protein of 984 amino acids (aa). The rat MR gene is located on chromosome 19q11 and differs slightly in having three untranslated exons (1α, 1β and 1γ) and encoding a 981 aa protein [Kwak et al., 1993]; a similar genomic structure is found for mouse MR gene, which encodes a 978 aa protein. In addition, it now appears that the MR gene does not encode only one protein but gives rise to multiple mRNA isoforms and protein variants [Pascual-Le Tallec and Lombes, 2005], thus allowing combinatorial patterns of receptor expression potentially responsible for distinct cellular and physiological responses in a tissue-specific manner.

Structure of the protein

Like all members of the nuclear receptor superfamily, MR has three major functional domains; a N-terminal domain (NTD) followed by a central DNA-binding domain (DBD), and a hinge region linking them to a C-terminal ligand-binding domain (LBD). Exon 2 encodes
most of the NTD, small exons 3 and 4 for each of the two zinc fingers of the DBD, and the last five exons of the LBD (Figure 1).

The MR NTD is the longest among all the steroid receptors (SR) (602 aa). The NTD is highly variable among SR, showing less than 15% identity, but for a given receptor, highly conserved between species (more than 50% homology), strongly suggesting a crucial functional importance. The NTD possesses several functional domains responsible for ligand-independent transactivation or transrepression, as shown schematically in Figure 1. Two distinct activation function 1 domains (AF1), referred to as AF1a (residues 1-167) and AF1b (residues 445-602), have been demonstrated in both rat [Fuse et al., 2000] and human MR [Pascual-Le Tallec et al., 2003]. A central inhibitory domain (residues 163-437) has also been characterized and seems to be sufficient to attenuate the overall transactivation strength of the NTD fused either to AF-1a or AF-1b [Pascual-Le Tallec et al., 2003]. These different domains of the NTD recruit various coregulators responsible for modulating the transcriptional activity of MR in a highly selective manner compared with other SR, and are now considered to be important determinants of mineralocorticoid selectivity [Pascual-Le Tallec and Lombes, 2005].

The DBD has the ability to recognize specific target DNA sequences or hormone response elements (HRE). The MR DBD is 94% identical with that of GR, and more than 90% compared with the progesterone receptor (PR) and the androgen receptor (AR). The MR DBD is a 66 aa domain encoded by exons 3 and 4: by analogy with the crystal structure obtained of the GR DBD, it contains two perpendicular \( \alpha \) helices, structurally coordinated by a zinc ion that interacts with four cysteine residues and thus responsible for the zinc finger’s structure. The first zinc finger contains the “P box” (defined by the three residues Gly\( _{621} \)Ser-Val\( _{625} \)) responsible for tight binding to the minor groove of the DNA double helix. The second zinc finger facilitates receptor dimerization through the so-called “D box” (Ala\( _{640} \)GlyArgAsnAsp\( _{645} \)), located in the N-terminal part of the DBD. In this context, it is interesting to note that MR is able to heterodimerize with other members of the SR subgroup, most notably GR and AR [Liu et al., 1995], consistent with the possibility that heterodimerization might play a role in some physiological responses at the level of transcriptional regulation.

The MR LBD is a complex and multifunctional domain that spans 251 aa. It is relatively conserved among SR (~55% homology) and highly conserved across species (80-97% homology), and allows selective hormone binding thus transducing endocrine messages into specific transcriptional responses. The MR LBD crystal structure has recently been solved, thus confirming the remarkable similarity in structure among all SR [Bledsoe et al., 2005; Fagart et al., 2005; Li et al., 2005]. Basically, the MR LBD consists of 11 \( \alpha \) helices and four small antiparallel \( \beta \) strands that fold into a three layer helical sandwich. Solving the crystal
structure allowed identification of the crucial amino acid residues interacting with the functional group of steroid ligands. For instance, Gln\textsubscript{776} of helix H3 and Arg\textsubscript{817} at the end of helix 5 directly contact the 3-ketone group of aldosterone, and Asn\textsubscript{770} of helix H3 stabilizes aldosterone 18-hydroxyl group. Other residues in helices 6 and 7, as well as Thr\textsubscript{945} on helix 10, also directly contact the steroid ligand. A single residue at position 848 in helix H7 switches hormone specificity between MR and GR [Li et al., 2005], and amino acids 820-844 (which are not part of the ligand binding pocket) are also critical for aldosterone binding and ligand binding selectivity [Rogerson et al., 2007]. The role of Met\textsubscript{852} in accommodating the C7 substituents of antimineralocorticoid spirolactones has very recently been described [Huyet et al., 2007]. Finally, on the basis of the high similarity between the LBD of MR and GR, and considering the evolutionary tree of this receptor subgroup, it has been proposed that MR was closer to the primordial ancestral corticosteroid receptor [Hu and Funder, 2006], which has been proposed as having high affinity for aldosterone, well before the hormone appeared [Bridgham et al., 2006]. In this context, it is also interesting to note that the Ser\textsubscript{949} in human MR is deleted in almost all GR and that the His\textsubscript{950} in human MR, conserved in MR in Old World monkeys, is a glutamine in all teleost and land vertebrate MR and may thus represent a breaking point during evolution [Baker et al., 2007].

The MR LBD possesses a ligand-dependent AF-2 constituted by the helices H3, H4, H5 and H12. Upon ligand binding, a rearrangement of the LBD occurs: H12 closes over the ligand pocket which, in combination with the bending of helices H3, H5 and H11, forms a hydrophobic cleft on the surface of the LBD. This groove serves as a docking surface for transcriptional coactivators possessing a NR box defined by the LXXLL motif, an interaction essential for activation of MR transcriptional activity. Given the high level of homology between their LBD it is not surprising that GR and MR recruit almost identical coactivators through their AF2 domains. A recent study, using an isolated MR LBD as bait to screen interaction with a LXXLL peptide library showed that MR interacts with a restricted number of coactivator peptides including SRC-1, ASC2, PGC-1\(\alpha\) and PGC-1\(\beta\) [Hultman et al., 2005].

Although, the relative contributions of AF1a, AF1b and the AF2 to MR transcriptional activity appears to be highly dependent on cellular and promoter contexts, the NTD appears to account for \(~\)40-50\% of total transactivation and represents a key determinant of MR specificity [Pascual-Le Tallec and Lombes, 2005].

As noted above, MR are expressed at least as two different proteins, MRA and MRB [Pascual-Le Tallec et al., 2004], resulting from strong Kozak sequences initiating alternative translation. These variants display distinct transactivation capacities \textit{in vitro}; it remains to be established whether they are differentially expressed \textit{in vivo}, and the extend to which they contribute to fine-tuning of MR transcriptional activity and the differential patterns of gene
expression in different cellular contexts, as previously described for GR [Lu and Cidlowski, 2005].

Interactions
In its non-liganded state MR interact with a large variety of proteins, most notably in the cytoplasmic compartment, thus forming part of a hetero-oligomer [Rafestin-Oblin et al., 1989]. MR contact chaperone proteins such as the heat shock protein hsp90 [Binart et al., 1995], and indirectly interact with hsp70, the p23 and p48 proteins and the FKBP-59 immunophilins or CYP40 cyclophilin [Bruner et al., 1997; Pratt and Toft, 1997]. These chaperones play a pivotal role in maintaining MR in an appropriate conformation for ligand binding. Besides the chaperone proteins, the MR also interacts with actin [Jalaguier et al., 1996] which may thus play a role in the ligand-dependent nuclear translocation. Upon hormone binding, the MR dissociates from chaperone proteins, undergoes nuclear translocation and interacts with numerous molecular partners in a coordinate and sequential manner to ensure appropriate transcriptional regulation. For over a decade, yeast two-hybrid screening, GST pull-down and co-immunoprecipitation assays have been used to identify various MR interacting nuclear proteins.

From a functional point of view, the most important are the transcriptional coregulators acting either as coactivators or corepressors of MR transactivation. Since the initial description of coactivators in the mid 1990’s, our knowledge of the complexity of transcriptional regulation has considerably increased. SR are now considered as platforms recruiting in an ordered and cyclical manner different coregulators [Metivier et al., 2003] which exhibit various enzymatic activities to play the role of transcriptional master switches [O’Malley, 2007]. The first identified member of the large coactivator family was steroid receptor coactivator-1 (SRC-1) [Onate et al., 1995], known to initiate transcription by recruiting a series of proteins involved in chromatin remodeling, histone acetylation and methylation [Freiman and Tjian, 2003; Rosenfeld et al., 2006]. Since then, a dozen coregulators have been demonstrated to interact with MR and modulate its activity (for an exhaustive review on this topic see [Pascual-Le Tallec and Lombes, 2005]). As summarized and referenced in Table 1, the most important coactivators for MR transactivation appeared to be the histone acetylase CBP/p300; the helicase RHA; the transcriptional coactivators SRC-1, SRC-1e, and PGC-1; and finally the Pol II elongation factor ELL that constitutes the first example of a selective transcriptional coregulator of MR [Pascual-Le Tallec et al., 2005]. Corepressors able to bind MR and repress its transcriptional function include the widely repressive SMRT and NCoR; the apoptosis regulator DAXX; and the specific SUMO-ligase PIAS proteins. Of interest, sumoylation now emerges as an important post-translational modification for many nuclear receptors and coregulators. SUMO-E3 ligase PIAS proteins repress MR, potentially in
collaboration with DAXX protein [Lin et al., 2006]; it has recently been shown, however, that the SUMO-E2 activating enzyme Ubc9 interacts with the MR NTD/DBD (1-670 aa) to potentiate aldosterone-dependent MR transactivation [Yokota et al., 2007], further increasing the complexity of sumoylation-mediated regulation. The MR interaction network per se appears not to determine functional MR activity; rather, the coordination and sequential interaction of molecular partners, directly or indirectly with MR, controls activation and function of cooperative transcriptional complexes, probably in a cell- and promoter-dependent manner. It should be mentioned that MR also heterodimerizes with other SR, notably GR and AR [Liu et al., 1995; Savory et al., 2001] thus providing an additional support for MR functional diversity of action.

**MR expression**

Until the late 1970's MR expression measured by binding assays was considered restricted to polarized tight epithelia which show aldosterone-dependent transepithelial sodium transport [Marver et al., 1974]. MR expression was located by immunohistochemistry in the kidney, most notably in the distal convoluted tubules and cortical collecting ducts [Krozowski et al., 1989; Lombes et al., 1990]. MR seem also to be expressed at the messenger and the protein levels in glomeruli, especially in mesangial cells [Miyata et al., 2005; Nishiyama et al., 2005] and podocytes [Shibata et al., 2007], where aldosterone has been reported to modulate podocyte function, possibly through the induction of oxidative stress and of the serum and glucocorticoid-regulated kinase 1 (SGK1). MR expression was also detected, by specific binding of $[^3$H]-aldosterone, in the distal colon of rat [Pressley and Funder, 1975], human [Lombes et al., 1984] and chick [Rafestin-Oblin et al., 1989]. The lung may represent another aldosterone target tissue, in that MR binding sites were demonstrated in airway epithelia from bronchiole to trachea [Krozowski and Funder, 1981]. MR expression (transcript and/or protein) was clearly revealed in the salivary [Funder et al., 1972] and sweat glands [Kenouch et al., 1994], in the liver [Duval and Funder, 1974] and in the inner ear [Furuta et al., 1994; Pitovski et al., 1993; Teixeira et al., 2006]. Importantly, epithelial expression of MR was always associated with expression of the 11-beta hydroxysteroid dehydrogenase 2 (11-$\beta$HSD2), the enzyme that allows aldosterone to selectively activate MR, by converting glucocorticoid hormones to their 11-keto analogs, unable to bind MR [Edwards et al., 1988; Funder et al., 1988].

Subsequently, MR expression was detected in non-epithelial tissues, in which the expression of 11$\beta$-HSD2 was absent or extremely low. For instance, specific binding sites for aldosterone were identified in mononuclear leucocytes [Armanini et al., 1985] and in the heart [Barnett and Pritchett, 1988; Pearce and Funder, 1987] and MR transcripts were detected in specific structures of the hippocampus (dentate gyrus and CA1, 2, and 3 nuclei).
and in the hypothalamus [Han et al., 2005; Herman et al., 1989; Van Eekelen et al., 1988]. In 1992, MR were localized at the cellular level by immunohistochemistry in cardiomycocytes, endothelial cells and large vessels [Lombes et al., 1992]. Some years later, MR expression was confirmed, at the messenger and protein level in the skin, not restricted to sweat and sebaceous glands but also in keratinocytes constituting the stratified epithelium [Kenouch et al., 1994]. Recent studies have shown MR to be expressed at the transcript and protein level in adipose tissues, both in white [Caprio et al., 2007; Fu et al., 2005; Rondinone et al., 1993] and brown adipocytes [Penfornis et al., 2000; Viengchareun et al., 2001; Zennaro et al., 1998]. This is of particular interest considering the functional interaction between MR and PGC-1 [Hultman et al., 2005] and the central role of this coactivator for brown adipocyte differentiation [Lin et al., 2005]. Of note, MR is also expressed at the protein level in ocular tissues, such as retina [Mirshahi et al., 1997] and iris-ciliary body [Schwartz and Wysocki, 1997], in placenta [Hirasawa et al., 2000], and at the messenger level in uterus, ovaries and testis [Le Menuet et al., 2000], with no clear roles reported to date.

This widespread expression of MR suggests novel functions for this receptor in these target tissues. It also questions about the role played by glucocorticoid hormones (cortisol or corticosterone) in MR activation, considering the absence of 11β−HSD2 in non-epithelial tissues except in certain brain areas such as the nucleus of the solitary tract as recently reported [Geerling et al., 2006; Naray-Fejes-Toth and Fejes-Toth, 2007].

MR are now considered an ubiquitous transcription factor, and real-time PCR quantification of MR and GR transcripts reveals interesting anatomical expression patterns [Bookout et al., 2006]. MR and GR expression are equivalent and high in the gastrointestinal system, moderate in the endocrine, reproductive, metabolic and cardiovascular systems. MR expression is higher than that of GR in the central nervous system (CNS) and the structural system (skeleton), whereas GR expression is, as expected, more pronounced than that of MR in the immune system. According to the classification based on hierarchical clustering of gene expression, MR seems to belong to the same cluster than LXRβ and RXRβ, expression of what is most abundant in the CNS and crucial to the global basal metabolism, linked to circadian clocks, metabolism and cardiovascular control [Bookout et al., 2006]. Thus, distinct MR and GR expression patterns strongly support that MR and GR differentially affect transcriptional programs governing distinct physiological processes and pathophysiological disorders.

**Regulation of MR expression**

A key step in the mineralocorticoid response is the regulation of the MR expression level. Indeed, in non-epithelial tissues such as the hippocampus, MR exerts its action in balance with the GR [de Kloet, 2003]. In the brain, some compounds such as serotonin [Lai et al.,
2003] and progesterone [Castren et al., 1995] have been reported to modulate MR mRNA expression. In other tissues, MR expression levels positively correlate with the severity of heart [Yoshida et al., 2005] and kidney failure [Quinkler et al., 2005]. Of major interest, a recent report showed that lowering renal expression of MR by a RNA interference strategy in cold-induced hypertensive rats both prevented the progression of hypertension and attenuated renal damage [Wang et al., 2006], emphasizing the potential importance of regulation of MR expression. The hMR gene has two 5' untranslated exons (1α and 1β) alternatively spliced onto exon 2. Their 5'-flanking regions were identified as functional promoters, referred to as P1 and P2 respectively, and were studied in transient transfection assays [Zennaro et al., 1996]. The basal transcriptional activity of the P1 (proximal) promoter is stronger than that of the P2 (distal) promoter. In vitro, while both promoters are stimulated by glucocorticoids, only P2 is activated by aldosterone. Transgenic mouse models were established by targeted oncogenesis with each promoter fused to the simian virus 40 large T antigen as a reporter gene, providing a unique opportunity to examine tissue specific utilization of these promoters in vivo [Le Menuet et al., 2000] and to establish novel mineralocorticoid-sensitive cell lines [Le Menuet et al., 2004]. The P1 promoter was shown to be active in vivo in all MR expressing tissues whereas the P2 promoter activity was much lower and appeared to be restricted to development, questioning the role of the P2 promoter and the molecular events and transcription factors involved in regulation of MR promoter in vivo.

Cellular mechanisms & cellular biology

Subcellular distribution

In the absence of ligand, MR is located mainly in the cytoplasm [Binart et al., 1991; Lombes et al., 1994a], associated with chaperone proteins. Upon ligand binding and dissociation of receptor–associated proteins, activated MR translocate into the nuclear compartment, in response to nuclear localization signals (NLS) present in the receptor protein sequence. Three functional NLS have been described so far [Walther et al., 2005] and are depicted in Figure 1. The first one (NLS0) is located at the end of the NTD, between amino acids 590 and 602. It possesses five serine and one threonine residues that play a crucial role in the MR nuclear import dependent upon Ser601 phosphorylation. The NLS2 is located in the receptor LBD, as a sequence without any basic amino acids, a feature of NLS sequences in other SR. Nuclear translocation mediated by NLS2 seems to depend on the nature of the ligand; only MR agonists induce rapid translocation of the receptor, with antagonists less effective [Lombes et al., 1994a]. The third sequence (NLS1) is located in the C-terminal part of the DBD; NLS1 acts cooperatively with NLS0 and NLS2 to facilitate nuclear translocation of the unbound MR [Walther et al., 2005]. In addition, a nuclear export signal (NES) is
located between the two zinc fingers of the DBD near the NLS1 [Black et al., 2001].

Altogether, these data indicate that MR is a receptor, distribution of which is an active process under the control of functional NLS.

Although, it has been suggested that a membrane receptor for corticosteroids might mediate their rapid effects on cortical collecting duct cells [Le Moellic et al., 2004] and on CA1 pyramidal neurons [Karst et al., 2005] through a nongenomic signaling pathway, evidences for a membrane-bound MR is lacking.

**Promoter binding and transcriptional activation**

Upon ligand binding, MR move into the nucleus acting as a transcription factor by binding to specific HRE present in target genes potentially located up to 10 kb upstream or downstream from transcriptional start sites, as recently described for GR and GREs [So et al., 2007]. This study reported that GRE sequences vary extensively around a consensus, but are strikingly conserved for a given site across species, raising the possibility of distinct sets of MREs vs GREs for MR target genes. MR when bound to response elements recruit chromatin remodeling complexes to release the nucleosome structure, and components of the transcriptional machinery to activate Pol II transcription. A complete and sequential picture of the transcriptional events remains to be clearly established for MR.

**Post-translational modifications**

Even though phosphorylation of SR has been shown to play a major role in modulating their intrinsic function, MR phosphorylation has received little attention so far despite an early report describing MR as a phosphoprotein [Alnemri et al., 1991]. Several tyrosine, serine and threonine residues are present throughout the MR protein (Figure 1). A tyrosine to cysteine substitution (Y73C), at a potential phosphorylation site was identified in the NTD of the Brown Norway rat as opposed to Fischer 344 rat, and associated with increased MR transactivation and unexpectedly, partial activation by progesterone [Marissal-Arvy et al., 2004]. This tyrosine phosphorylation site is also present in hMR and could thus be implicated in potential pathophysiological dysregulation. Phosphorylation sites at Thr735 and Ser737 were recently identified in the hMR LBD by proteomic analysis [Hirschberg et al., 2004], and may thus participate in LBD conformational change. In addition, as mentioned above, mutations in the serine/threonine-rich sequence in the MR NLS0, most notably Ser601 have highlighted their role in the receptor subcellular shuttling [Walther et al., 2005]. MR is phosphorylated within minutes after aldosterone exposure on serine and threonine residues by the protein kinase C α leading to subsequent ionic transport [Le Moellic et al., 2004], providing strong evidence for cross-talk between early (<30 min) non-genomic and late (>2h) genomic effects of MR and aldosterone (for detailed review see [Funder, 2005]).
Recently, sumoylation has emerged as an extremely important post-translational modification for regulation of transcription factors, most notably members of the nuclear receptor superfamily [Seeler and Dejean, 2003]. Sumoylation consists in covalent link with small ubiquitin-related modifier (SUMO). MR possess several sumoylation consensus motifs defined by the peptide sequence site \( \Psi'KXE \), where \( \Psi' \) is an aliphatic residue, \( K \) the target lysine for sumoylation and \( X \) any residue. Four SUMO sites have been identified in the NTD at positions \( K_{89} \), \( K_{399} \), \( K_{428} \), \( K_{494} \) and one in the LBD at position \( K_{953} \) [Pascual-Le Tallec et al., 2003] (see Figure 1). The consensus sites, also named synergy control motif [Iniguez-Lluhi and Pearce, 2000], are highly conserved throughout evolution, supporting an important functional role for sumoylation. Indeed, sumoylation is now considered as a general repressive mechanism for most of the transcription factor actions. MR-mediated transcription is repressed by sumoylation to an extent dependent on the nature of the response element bound, in that it may impair protein-protein interactions with the transcriptional initiation complex. Acetylation mediated by the p300 and p/CAF proteins, has been reported for other SR. The KXKK/RXKK acetylation motif is a perfect match with the MR NLS1, most notably the \( K_{677} \), suggesting that MR acetylation might affect not only ligand-dependent nucleo-cytoplasmic shuttling, but also protein-protein interaction and subsequent transcriptional regulation. The exact impact of acetylation on MR function, and the relative contribution of CBP/p300 and HDAC warrant further investigation.

Very recently, ubiquitylation (or ubiquitination), another post-translational modification of MR, has been reported. MR can be poly-ubiquitylated and targeted to the proteasome, a mechanism which seems to be essential for the regulation of MR-mediated transcriptional activation [Tirard et al., 2007].

### Target genes and their biological functions

**Epithelial tissues**

In classical polarized epithelial tissues (i.e., kidney, colon), MR regulates salt balance and water homeostasis by directly stimulating expression of specific ionic transporters active at the cellular membrane: the amiloride-sensitive Epithelial Na Channel (ENaC), located at the apical membrane [Canessa et al., 1994; Rossier et al., 2002], and the basolateral \( \text{Na}^+,\text{K}^+ \)-ATPase pump [Horisberger et al., 1991; Jorgensen, 1986]. These transporters are responsible for unidirectional transepithelial sodium transport from the lumen to the interstitium. Figure 2 illustrates a schematized model of aldosterone action in an epithelial renal cell and Table 2 summarizes early aldosterone-induced genes, identified in epithelial tissues. They include the ENaC subunits [Bens et al., 1999; Epple et al., 2000; Masilamani et al., 1999; Teixeira et al., 2006], the \( \text{Na}^+,\text{K}^+ \)-ATPase pump subunits [Kolla and Litwack, 2000] the channel-inducing factor (CHIF), a member of the FXDY family, which regulates pump
activity in the colon [Brennan and Fuller, 1999; Wald et al., 1996], and the K-ras2 gene, expression of which is also stimulated by aldosterone in the colon [Brennan and Fuller, 2006]. The best studied aldosterone-induced gene in epithelial tissues is the serine/threonine kinase SGK1, which plays a pivotal role in sodium homeostasis, in that aldosterone-induced expression of SGK1 strongly activates ENaC by phosphorylating the ubiquitin-ligase Nedd4-2 [Bhargava et al., 2001; Chen et al., 1999; Naray-Fejes-Toth et al., 1999]. This phosphorylation impairs the interaction of Nedd4-2 and ENaC leading to degradation of ENaC by the proteasome, thus allowing a pool of channels to remain at the apical membrane [Debonneville et al., 2001]. Several lines of evidence suggest that SGK1 is not the only mediator of aldosterone action. The glucocorticoid-induced leucine zipper protein (GILZ), initially identified as a transcription factor [Robert-Nicoud et al., 2001], was subsequently shown to stimulate ENaC-mediated Na⁺ transport in the kidney by inhibiting extracellular signal-regulated kinase (ERK) signaling [Soundararajan et al., 2005]. A recent study has identified novel early aldosterone-regulated mRNA in microdissected mouse distal nephron by microarray. Besides SGK1, the induced mRNA species include Grem2, activating transcription factor 3, and the ubiquitin-specific protease Usp2-45, which deubiquitylates ENaC and thus stimulates ENaC-mediated sodium transport [Fakitsas et al., 2007]. Of importance, several down-regulated genes have also been identified but roles in the regulation of ionic transport remain to be established. KS-WNK1 (With No lysine K) is another serine/threonine kinase which plays a crucial role in ENaC activation [Naray-Fejes-Toth et al., 2004] on aldosterone exposure, stimulating the PI-3 kinase pathway which directly activates SGK1 [Xu et al., 2005a; Xu et al., 2005b]. Interestingly, other aldosterone-induced genes have been reported so far but their biological function remains to be elucidated. For instance, NDRG2 (N-Myc downstream regulated gene 2) [Boulkroun et al., 2002; Wielputz et al., 2007], Endothelin-1 (ET-1) [Wong et al., 2007] and the Plasminogen Activator Inhibitor-1 (PAI-1) [Yuan et al., 2007] transcripts have been shown to be specifically increased upon aldosterone exposure in the kidney or distal colon.

**Non-epithelial tissues**

The discovery of new sites of MR expression in non-epithelial tissues such as the heart, vasculature, brain and adipocytes has led to identification of potential new aldosterone target genes in these tissues with unexpected biological functions (see Table 3). For instance, in aortic endothelial cells, aldosterone was shown to increase osteopontin and angiotensin-converting enzyme (ACE) gene expression, which may be involved in the development of endothelial dysfunction and vascular injury induced by this steroid [Sugiyama et al., 2005a; Sugiyama et al., 2005b]. Leopold et al. recently reported that aldosterone impairs vascular reactivity by decreasing endothelial glucose-6-phosphate dehydrogenase (G6PD) expression.
and activity, which results in increased oxidative stress and decreased NO levels [Leopold et al., 2007]. In vascular smooth muscle cells, MDM2 was identified as a novel mineralocorticoid-responsive gene involved in aldosterone-induced vascular remodeling [Nakamura et al., 2006]. In addition, MR activation in vitro also directly enhances EGF-R gene expression ultimately contributing to an aldosterone-induced increase in fibronectin abundance in aorta [Grossmann et al., 2007]. Several studies have demonstrated that aldosterone can stimulate collagen (I, III and IV) gene expression in cardiac [Brilla et al., 1994] or renal fibroblasts via MR-mediated ERK1/2 activation pathway [Nagai et al., 2005], which may contribute to the progression of aldosterone-induced myocardial or tubulointerstitial fibrosis. These adverse effects of aldosterone have prompted a renewed interest in the use of MR antagonists and for the identification of MR target genes, particularly in the heart. Very recently, a large number of target genes have been identified in a cardiomyocyte cell line stably expressing MR, including genes related to extracellular matrix regulation (tenascin-X, ADAMTS1, PAI-1, UPAR, and hyaluronic acid synthase-2), signaling, regulation of vascular tone (RGS2, adrenomedullin) and inflammation (orosomucoid) [Fejes-Toth and Naray-Fejes-Toth, 2007].

The role of aldosterone and MR in cell proliferation [Stockand and Meszaros, 2003] and differentiation [Caprio et al., 2007; Penfornis et al., 2000] is now well documented. Physiological studies have demonstrated the anti-apoptotic role of aldosterone and MR in hippocampus degeneration. Indeed, the balance between GR and MR seems to control limbic neuron fates [de Kloet et al., 2005] and efforts have been made to identify corticosteroid responsive genes in the hippocampus [Datson et al., 2001]. Interestingly, studies performed on hippocampal neurons confirmed the protective effects of MR on GR-induced apoptosis [Almeida et al., 2000; Crochemore et al., 2005], and are in accordance with the knockout mouse model for MR, which shows degeneration of the hippocampus granule cells in adulthood [Gass et al., 2000]. Recently, light has been shed on the molecular basis of MR-prevented apoptosis with molecular data showing that the MR NTD was able to prevent all the features (modulation of pro vs anti-apoptotic genes, and caspase inhibition) of glucocorticoid-induced apoptosis in lymphocytes [Planey et al., 2002] potentially through competition for common coregulators (like ELL, FAF, or FLASH). In brown adipocytes, it has been lately shown that aldosterone treatment induced a marked decrease in expression and function of mitochondrial uncoupling proteins UCP1 and UCP3 [Viengchareun et al., 2001], providing additional support for a role of MR in the control of energy expendititure. Finally, aldosterone-activated MR also promote osteoblastic differentiation and mineralization of vascular smooth muscle cells independently of BMP2 signaling [Jaffe et al., 2007], with the genes involved in such effects yet to be identified.
Additional studies are needed to assess the contribution of these potential target genes to the physiological and pathophysiological effects of aldosterone in non-epithelial tissues.

**MR transgenic models**

A better understanding of physiological MR actions has been achieved by its transgenic expression in various animal models. Such genetically engineered mice, including MR gene inactivation, RNA interference and MR overexpression, have been generated by several groups. MR knockout mice, in which the MR DBD has been disrupted, die around day 10 after birth from renal sodium loss [Berger et al., 1998], prevented by exogenous NaCl administration [Berger et al., 2000]. The animals so rescued show impaired neurogenesis and degeneration of the hippocampus granule cells in adulthood, as previously noted [Gass et al., 2000]. This finding emphasizes the role of MR in cell proliferation/apoptosis balance in limbic structures. At variance with the MR KO mouse model, the mouse aldosterone synthase gene knockout is not lethal. As expected, ionic homeostasis is altered in the absence of aldosterone, but as a consequence, a high level of corticosterone and angiotensin II seems to partially rescue sodium balance [Makhanova et al., 2006], underscoring in this context the importance of MR over aldosterone.

More recently, AQP2-Cre-MR mice with MR inactivation in renal principal cells exhibited normal renal sodium excretion associated with elevated aldosterone levels on a standard diet. On a low-sodium diet, however, the mice rapidly lost sodium consistent with the impaired induction of ENaC channels in principal cells of the collecting duct and late connecting tubule, in part functionally offset by the late distal convoluted tubule and early connecting tubule [Ronzaud et al., 2007]. Another MR conditional knockout mouse with forebrain specific inactivation of MR showed impaired spatial learning ability linked to behavioral stereotypy [Berger et al., 2006]. Brain-specific MR inactivation compromised the rapid effect by corticosterone providing evidence that this nongenomic effect is MR-mediated [Karst et al., 2005].

Mouse models of MR overexpression have also been generated. The first used the human P1 proximal MR promoter to drive human MR transgene expression in most aldosterone target tissues (distal nephron, brain, heart). These animals presented with enlarged kidneys associated with renal tubular dilatation and normal blood pressure, and developed mild dilated cardiomyopathy associated with arrhythmia [Le Menuet et al., 2001]. In addition, gene expression studies of these mice revealed several alterations of cardiac and renal specific gene expression such as ANF, SGK1, egr-1 [Le Menuet et al., 2001]. Of interest, conditional cardiac MR overexpression controlled by the α-myosin heavy chain promoter led to life threatening arrhythmias [Ouvrard-Pascaud et al., 2005], consistent with the previous model. None of these MR overexpressing mice developed cardiac fibrosis, clearly different from the
cardiac phenotype previously described for salt and aldosterone excess animal models [Young et al., 1994], questioning the direct role of cardiomyocyte MR in cardiac fibrosis. MR knockdown experiments using the conditional expression of a MR antisense mRNA was reported to induce cardiac fibrosis and heart failure [Beggah et al., 2002]. This phenotype was somewhat unexpected but could be related to an imbalance between cardiac MR and GR levels which could ultimately lead to inappropriate corticosteroid responses, reminiscent of the cardiac phenotype observed with heart-specific overexpression of 11β-HSD2 [Qin et al., 2003]. Another transgenic mouse model with a targeted overexpression of MR in the forebrain, using the forebrain-specific calcium/calmodulin-dependent protein kinase II α promoter, exhibited decreased anxiety-like behavior associated with a diminution of hippocampal GR protein and increased in serotonin 5HT-1a receptor levels [Rozeboom et al., 2007]. A conditional targeted skin overexpression of MR resulted in early postnatal death when the transgene was expressed during gestation, associated with excessive keratinocyte apoptosis. Besides, postnatal transgene expression led to alopecia and hair follicle dystrophy [Sainte Marie et al., 2007].

**MR and disease**

Since the aldosterone-MR signaling pathway plays a major role not only in the control of sodium and potassium homeostasis but also in other important physiological processes, its involvement in numerous human diseases is not surprising. Here, we will focus on diseases for which a direct implication of MR has been documented. Among them, the most important genetic disorder is the autosomal dominant pseudohypoaldosteronism type 1 (adPHA1), which is caused, in most cases, by heterozygous loss-of-function mutations of MR. First described in 1958 [Cheek and Perry, 1958], this life-threatening rare condition is characterized by renal resistance to aldosterone action. Early in infancy usually during the first months of life, patients present with dehydration and failure to thrive associated with massive salt wasting, hyperkalemia and metabolic acidosis despite elevated plasma aldosterone and renin levels. They require sodium supplementation during their early years, which can be subsequently reduced, adults being usually asymptomatic in spite of high plasma aldosterone and renin levels [Geller, 2005]. To date, approximately 50 distinct mutations in the human MR gene responsible for this syndrome, have been described [Geller, 2005; Geller et al., 1998; Pujo et al., 2007] and are summarized in Figure 3. They include missense, nonsense, frameshift and splice site mutations, as well as deletions spread throughout the gene. These mutations are responsible for either an early termination of translation with MR truncation or a defect in MR activity (loss of LBD or DBD), disruption of nucleocyttoplasmic shuttling or alteration in some transcriptional coregulator recruitment. Only heterozygous mutations have been reported in humans, suggesting that the homozygous
state may be lethal in utero. The loss of one allele results in haploinsufficiency, sufficient to generate adPHA1 symptoms, underlying the importance of a substantial MR protein level most notably during the neonatal period [Geller et al., 2006].

To date, there is only one mutation known to result in a gain-of-function of MR leading to a severe inherited form of early-onset hypertension, exacerbated during pregnancy [Geller et al., 2000]. This single mutation S810L in the LBD causes a constitutive MR activation as well as illicit activation of MR by progesterone and other steroids, including cortisone, 11-dehydrocorticosterone and the mineralocorticoid antagonist spironolactone [Geller et al., 2000; Pinon et al., 2004; Rafestin-Oblin et al., 2003].

Besides pathogenic mutations of hMR gene, common MR polymorphisms have been described and seem to be mostly related to modulation of stress responsiveness. In the brain, where 11β−HSD2 is not expressed except in discrete brain areas, MR may be sensitive to cortisol level variations induced by stress. Thus, the MR allele I180V, which has a frequency of approximately 15% varying with ethnic background [Arai et al., 1999; Balsamo et al., 2007], has been found associated with enhanced endocrine and autonomic responses to a psychological stressor, without any modification of sodium homeostasis or blood pressure [DeRijk et al., 2006]. Given this finding, other polymorphisms deserve to be characterized in terms of functional consequences both for sodium handling or clinical implications.

Besides genetic diseases, the prominent role played by MR in the pathogenesis of cardiac dysfunction in humans is now well documented. Indeed, large clinical trials (RALES, EPHESUS, 4E) have demonstrated the major benefits of anti-mineralocorticoid therapy such as spironolactone and eplerenone, in reducing the mortality and improving the prognosis of heart failure patients [Pitt et al., 1999] or after post-acute myocardial infarction [Pitt et al., 2003]. However, the molecular mechanisms involved in the protective effects of these MR antagonists remain to be fully characterized. It is noteworthy that a recent report shows that MR activation is a critical factor in the early pathogenesis of renal disease in both type 1 and type 2 diabetes mellitus [Guo et al., 2006].

**Ligands**

In human, aldosterone is the physiological ligand of MR, most notably for epithelial MR. This hormone is synthesized in the zona glomerulosa of adrenal cortex under the regulation of angiotensin II, ACTH and serum potassium concentration. Another important physiologically ligand of MR is cortisol (corticosterone in rodents) which has the same affinity for MR even though the dynamic binding parameters and the functional properties of glucocorticoid-MR complexes are somehow different from that of aldosterone-MR [Kitagawa et al., 2002; Lombes et al., 1994b]. The most potent MR-protective mechanism against MR activation by
glucocorticoid hormones is $11\beta$-HSD2, the NAD-dependent enzyme which converts cortisol into its inactive metabolite cortisone, in epithelial and vascular target tissues. Deoxycorticosterone (most notably in fish) [Sturm et al., 2005] as well as other glucocorticosteroid compounds (dexamethasone, fludrocortisone) act as mineralocorticoid agonists, at least \textit{in vitro}. It is now clear that progesterone and androgens and their derivatives could also bind MR and exert partial agonist and antagonist effects depending on the cellular context [Marissal-Arvy et al., 2004; Quinkler et al., 2002; Quinkler et al., 2003; Takeda et al., 2007].

Several mineralocorticoid antagonists such as spironolactone and the more selective eplerenone [Pitt et al., 2003] have been developed and are currently used as antihypertensive and cardiovascular protective agents. Drospirenone is a novel progestogen with considerable MR antagonist activity [Oelkers, 2004].

Selective mineralocorticoid receptor modulators, either steroid-derived compounds or alternatively nonsteroidal scaffold ligands, should ultimately produce beneficial effects mediated by transactivation or transrepression of MR in a tissue-dependent manner. Ideally, a MR modulator would have neuronal anti-apoptotic effects and efficient protective effects on renal and cardiovascular systems.

**Conclusion**

It now appears that the roles of MR extend far beyond control of fluid and electrolyte homeostasis, being involved in (patho)physiological processes as diverse as cardiovascular remodeling, fat storage, energy balance, and mammalian behavior. MR mouse transgenic models have been and will be extremely useful in unravelling these new aspects of MR biology. MR action must be seen in the context of its functional interaction with transcriptional coregulators, for which physiological roles are not redundant as initially thought, but quite specific as exemplified by members of the SRC family. The combinatorial tissue distribution of MR and its coregulators thus provide subtle cell-dependent tuning of MR target gene regulation and should also bring new insights into the molecular mechanisms of MR selectivity. Furthermore, recent evidence supports cross-talk between SR and other signaling pathways (like MAPK, src, STAT); the implication for MR remains to be established. The regulation of MR expression in non-epithelial tissues may differ from that in epithelia. Determining the transcriptional factors orchestrating MR expression constitutes an interesting challenge in terms of better understanding of MR-mediated responses, in addition to its potential relevance to pathophysiological disorders. Altogether, the aim of continuing research is to improve the comprehension of MR-mediated signaling both at the genomic and non-genotrophic levels, in order to propose new therapeutic strategies in human diseases.
Acknowledgments

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using an anti-idiotypic antibody that is an internal image of aldosterone. *Proc Natl Acad Sci U S A* 87, 1086-1088.


Figure legends

**Figure 1. Schematic representation of human MR structure.**
MR gene, mRNA, protein, functional domains and associated post-translational modifications are depicted. The hMR gene is composed of ten exons including two untranslated first exons (1α and 1β). The AUG translational initiation start codon is located 2 bp after the beginning of exon 2 while the stop codon is located in exon 9. Multiple mRNA isoforms generated by alternative transcription or splicing events are translated into various protein variants including those generated by utilization of alternative translation initiation sites (not shown). The receptor comprises distinct functional domains (activation function AF-1a, AF-1b and AF-2) and nuclear localization signals (NLS0, NLS1 and NSL2) as well as one nuclear export signal (NES). The positioning of amino acids targeted for phosphorylation, sumoylation, acetylation and ubiquitylation is indicated for the human MR sequence.

**Figure 2. Model of MR action in a renal polarized epithelial cell.**
Aldosterone enters a target cell and binds MR which translocates into the nucleus. MR interacts with a HRE, recruits various transcriptional coregulators (Coregulators) to activate the transcriptional machinery (TM) thus altering expression of aldosterone target genes (in blue). At the apical membrane, ENaC (epithelial sodium channel) composed of three subunits (α, β and γ) constitutes the rate-limiting step of apical Na⁺ entry. Na⁺ is then extruded into the basolateral space by the Na⁺/K⁺-ATPase pump, activity of which is modulated in the colon by the regulatory protein CHIF (corticosteroid hormone-induced factor). In the absence of aldosterone, ENaC proteins interact with Nedd4-2, an ubiquitin-ligase which targets ENAC to proteosomal degradation. SGK1 (serum and glucocorticoid-regulated kinase) is a key aldosterone-regulated target gene that plays a central role in sodium reabsorption. Upon aldosterone exposure, PDK1-activated kinase SGK1 phosphorylates Nedd4-2, which in turn dissociates from ENaC increasing its apical membrane abundance. Usp2-45, a novel early aldosterone-induced mRNA, is a ubiquitin-specific protease which deubiquitylates ENaC and thereby increases ENaC-mediated sodium transport. Other aldosterone-induced genes included kidney specific KS-WNK1 (serine/threonine kinase With No K), K-Ras2, NDRG2 (N-myc down-stream regulated gene 2), GILZ (glucocorticoid-induced leucine zipper), endothelin ET-1 and plasminogen activator inhibitor-1 (PAI-1).
Figure 3. Schematic localization of MR mutations causing pseudohypoaldosteronism type 1 and polymorphisms in the human NR3C2 gene.

Nucleotide and amino acid numbering is indicated in reference to the published cDNA where +1 is the A of the translational initiation codon [Arriza et al., 1987]. Fs, frameshift (red); X, stop codon (blue); missense (green); sp alt, splice alteration (purple); polymorphism (black).
Table legends

Table 1. MR Interacting proteins.
This table presents several categories of proteins interacting with the MR (chaperones, coactivators, corepressors). Their name, functions and their putative interacting domain are shown, together with the corresponding references.

Table 2. MR target genes and their biological functions in epithelial tissues.
This table presents the name of target genes, expression of which is regulated by MR in epithelial tissues. The function of the target gene products is listed together with the corresponding references.

Table 3. MR target genes and their biological functions in non-epithelial tissues.
This table shows the target genes, expression of which is up-regulated or down-regulated by MR in non-epithelial tissues. The functions of the target gene products are listed, together with the corresponding references.
<table>
<thead>
<tr>
<th>Category</th>
<th>Name</th>
<th>Functions</th>
<th>Interacting domain</th>
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<td>AF1b</td>
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<td>αENaC subunit</td>
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<td>Na⁺ transport</td>
<td>[Epple et al., 2000]</td>
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<td>Coronary artery endothelium</td>
<td>Impairment of vascular reactivity</td>
<td>[Leopold et al., 2007]</td>
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<td>Heart</td>
<td>Cardiac remodeling and</td>
<td>[Fejes-Toth and Naray-Fejes-Toth, 2007]</td>
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<tr>
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<td>Brown adipocytes</td>
<td>Thermogenesis</td>
<td>[Viengchareun et al., 2001]</td>
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Table 3