STEROID HORMONES

Transcriptional activation of elephant shark mineralocorticoid receptor by corticosteroids, progesterone, and spironolactone

Yoshinao Katsu[1,2]*, Satomi Kohno[3], Kaori Oka[1], Xiaozhi Lin[1], Sumika Otake[1], Nisha E. Pillai[4], Wataru Takagi[5], Susumu Hyodo[5], Byrappa Venkatesh[4], Michael E. Baker[6]*

The mineralocorticoid receptor (MR) is a nuclear receptor and part of a large and diverse family of transcription factors that also includes receptors for glucocorticoids, progesterone, androgens, and estrogens. The corticosteroid aldosterone is the physiological activator of the MR in humans and other terrestrial vertebrates; however, its activator is not known in cartilaginous fish, the oldest group of extant jawed vertebrates. Here, we analyzed the ability of corticosteroids and progesterone to activate the full-length MR from the elephant shark (*Callorhinchus milii*). On the basis of their measured activities, aldosterone, cortisol, 11-deoxycorticosterone, corticosterone, 11-deoxcortisol, progesterone, and 19-norprogesterone are potential physiological mineralocorticoids. However, aldosterone, the physiological mineralocorticoid in humans and other terrestrial vertebrates, is not found in cartilaginous or ray-finned fish. Although progesterone activates MRs in ray-finned fish, progesterone does not activate MRs in humans, amphibians, or alligator, suggesting that during the transition to terrestrial vertebrates, progesterone lost the ability to activate the MR. Both elephant shark MR and human MR are expressed in the brain, heart, ovary, testis, and other nonepithelial tissues, suggesting that MR expression in diverse tissues evolved in the common ancestor of jawed vertebrates. Our data suggest that 19-norprogesterone– and progesterone-activated MR may have unappreciated functions in reproductive physiology.

INTRODUCTION

The mineralocorticoid receptor (MR) belongs to the nuclear receptor family, a large and diverse group of transcription factors that also includes receptors for glucocorticoid receptors (GRs), progesterone receptors (PRs), androgen receptors (ARs), and estrogen receptors (ERs) (*1*, *2*). Sequence analysis has revealed that the MR and GR are closely related (*3*); phylogenetic analysis indicates that MR and GR evolved from a corticosteroid receptor (CR) that was present in jawless vertebrates, such as lamprey and hagfish (*4*–*7*). A distinct MR first appeared in cartilaginous fish (Chondrichthyes), the oldest group of extant jawed vertebrates (gnathostomes), which diverged from bony vertebrates about 450 million years. Hence, cartilaginous fish are a crucial group in understanding the origin and evolution of jawed vertebrate morphology and physiology (*8*, *9*). Similarly to mammals, cartilaginous fish contain the full complement of adrenal and sex steroid receptors: AR, ER, GR, MR, and PR (*1*, *2*, *4*, *10*).

Aldosterone (Aldo) is the physiological activator of transcriptional activity of human MR in epithelial tissues, such as the kidney distal collecting tubules and the colon, in which the MR regulates electrolyte homeostasis (*6*, *11*–*14*). The MR is also found in the brain, heart, aorta, lung, liver, spleen, adipose tissue, testis, breast, and ovary (*12*–*21*), tissues in which the MR is not likely to regulate electrolyte homeostasis, its classical function. The physiological function of the MR in these tissues is still being elucidated (*14*, *18*, *20*, *22*).

The MR and other steroid receptors have a characteristic modular structure consisting of an N-terminal domain (NTD) (domains A and B), a central DNA binding domain (DBD; domain C), a hinge domain (domain D), and a C-terminal ligand-binding domain (LBD) (domain E) (Fig. 1) (*2*, *4*, *23*–*25*). The LBD alone is competent to bind steroids

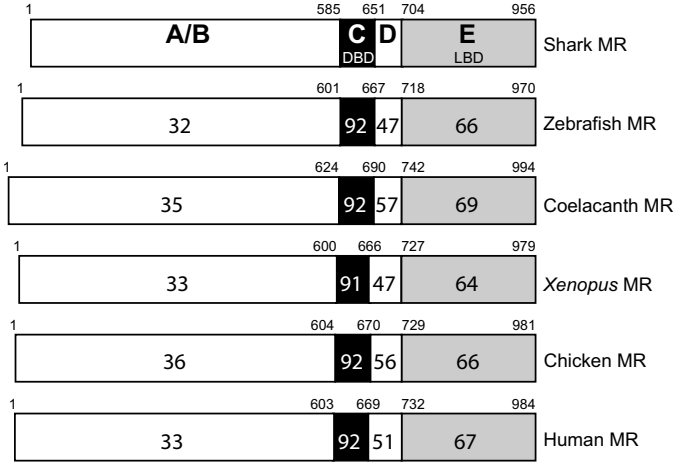

Fig. 1. Comparison of the domains in elephant shark MR with those in vertebrate MRs. MRs from elephant shark (shark), zebrafish, coelacanth, *Xenopus*, chicken, and human were compared. The functional A/B domain through to the E domain is schematically represented with the numbers of amino acid residues. The percentage of amino acid identity is depicted. GenBank accession numbers are as follows: elephant shark MR (XP_007902220), zebrafish MR (NP_001093873), coelacanth MR (XP_014348128), *Xenopus* MR (NP_001084074), chicken MR (ACO37437), and human MR (NP_000892).

[1]Graduate School of Life Science, Hokkaido University, Sapporo, Japan. [2]Faculty of Sciences, Hokkaido University, Sapporo, Japan. [3]Department of Biology, St. Cloud State University, St. Cloud, MN 56301, USA. [4]Comparative Genomics Laboratory, Institute of Molecular and Cell Biology, Agency for Science, Technology and Research (A*STAR), Biopolis, Singapore 138673. [5]Laboratory of Physiology, Atmosphere and Ocean Research Institute, University of Tokyo, Chiba, Japan. [6]Division of Nephrology, Department of Medicine, University of California, San Diego, CA 92093, USA.
*Corresponding author. Email: ykatsu@sci.hokudai.ac.jp (Y.K.); mbaker@ucsd.edu (M.E.B.)

(*4*, *23*, *26*–*28*). However, interactions between the NTD (A/B domain) and the LBD and coactivators are important regulators of the transcriptional activity of mammalian MRs (*29*–*36*) and ray-finned fish MRs (*35*–*37*). The galactose-induced gene 4 (GAL4)–DBD-hinge-LBD constructs of zebrafish MR have different responses to progesterone (Prog) and some corticosteroids than do the GAL4-DBD-hinge-LBD constructs of human, chicken, alligator, and *Xenopus* MRs (*36*).

The timing of the evolution of this difference in transcriptional activity between full-length and truncated MRs in ray-finned fish and terrestrial vertebrates, as well as when the expression of the MR in nonepithelial tissues evolved, is not known. In addition, unresolved is the identity of the ancestral mineralocorticoid in cartilaginous fish and the current mineralocorticoid in ray-finned fish because Aldo, the physiological mineralocorticoid in terrestrial vertebrates, is not found in either cartilaginous fish or ray-finned fish. Aldo first appeared in lungfish, a lobe-finned fish (*38*), which are the forerunners of terrestrial vertebrates (*39*). Thus, the identity of the physiological mineralocorticoid in cartilaginous fish and ray-finned fish is unknown, although cortisol and 11-deoxycorticosterone have been proposed as candidates (*40*–*46*).

Complicating the identity of the physiological mineralocorticoid in cartilaginous and ray-finned fish is evidence that Prog and 19-norprogesterone (19norProg), together with spironolactone (Spiron) (Fig. 2), are transcriptional activators of several ray-finned fish MRs (*25*, *37*, *45*), including zebrafish MR (*36*, *47*) and chicken MR (*36*). In contrast, these steroids are antagonists for human MR (*26*, *28*, *48*), alligator MR, and *Xenopus* MR (*36*). Ray-finned fish MRs and chicken MR differ in their responses to Prog, 19norProg, and Spiron, raising the question of whether the response to Prog and Spiron evolved in ray-finned fish before or after the divergence of ray-finned fish from the lobe-finned fish lineage that led to tetrapods.

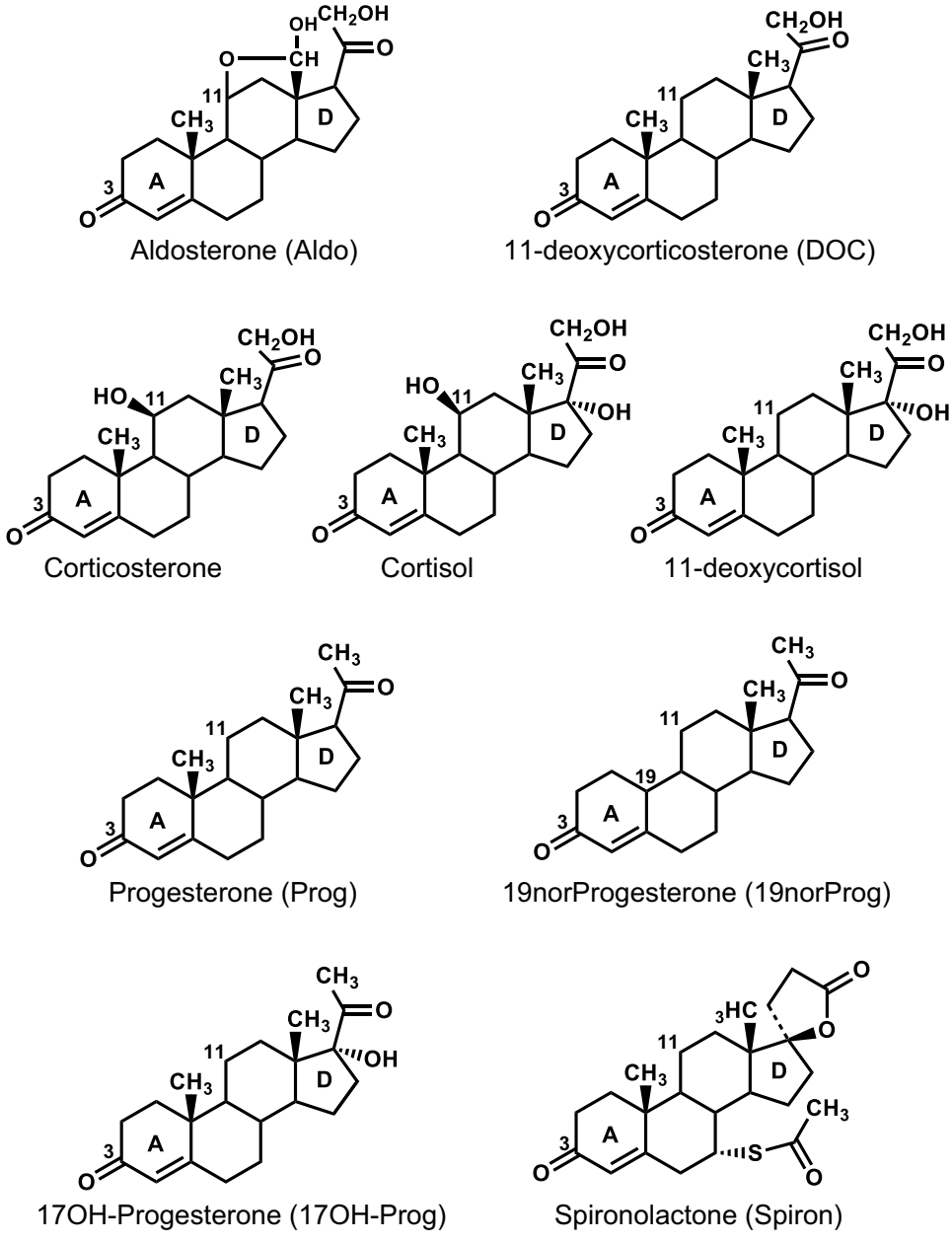

Fig. 2. Structures of steroids that are ligands for the MR. Aldo, 11-deoxycorticosterone, and 11-deoxycortisol are physiological mineralocorticoids in terrestrial vertebrates (*4*, *6*, *12*, *76*). 11-deoxycortisol is both a mineralocorticoid and a glucocorticoid in lamprey (*4*, *77*), whereas functions as a glucocorticoid in ray-finned fish (*78*). Cortisol is a physiological glucocorticoid in terrestrial vertebrates and ray-finned fish (*4*, *10*, *79*–*81*). Corticosterone is a glucocorticoid in rats and mice (*4*). Aldo, 11-deoxycorticosterone, cortisol, corticosterone, and Prog have a similar high affinity for human MR (*3*, *82*–*84*). Prog, 19norProg, 17OH-Prog, and Spiron are antagonists for human MR (*25*, *48*, *82*) and rat MR (*85*, *86*). Prog, 19norProg, and Spiron are agonists for fish MRs (*25*, *37*, *45*), whereas 19norProg is a weak agonist for rat MR (*54*, *55*). All of the ligands shown here have a ketone at C3 and, thus, are called 3-ketosteroids.

Transcriptional activation by corticosteroids and other 3-ketosteroids of a full-length cartilaginous fish MR has not been investigated. Only truncated skate MR (*49*), consisting of the GAL4-DBD fused to D and E domains of the MR (MR-LBD), has been studied for its response to corticosteroids. In those studies, Carroll *et al.* found that Aldo, corticosterone, 11-deoxycorticosterone, and cortisol (Fig. 2) activate the transcriptional activity of truncated skate MR. Elephant shark MR is an attractive receptor to study early events in the evolution of mechanisms for regulating MR activity because, in addition to its phylogenetic position as a sister group of bony vertebrates, genomic analyses reveal that elephant shark genes are evolving slowly (*9*), making their genes a window into the past. We therefore investigated the transcriptional activity of full-length and truncated elephant shark MR by Aldo, 11-deoxycorticosterone,

corticosterone, 11-deoxycortisol, cortisol, Prog, 19norProg, 17-hydroxyprogesterone (17OH-Prog), and Spiron. We found that all 3-ketosteroids, including Prog, had a half-maximal response (EC_{50}) of 1 nM or less for full-length elephant shark MR. Activation by Prog, 19norProg, and Spiron of truncated elephant shark MR resembled that of zebrafish MR, but not chicken MR, indicating that the activation of MR by Prog is an ancestral response, conserved in cartilaginous fish and ray-finned fish, but lost in *Xenopus*, alligator, and human MRs, and distinct from the activation of chicken MR, which arose independently. We investigated the relative expression of elephant shark MR by using RNA-seq data and found widespread expression of MR in various elephant shark tissues (gill, kidney, heart, intestine, liver, spleen, brain, ovary, and testis). This suggests that the widespread expression of human MR in tissues, such as the brain, heart, liver, spleen, ovary, and testis, in which the MR does not regulate electrolyte homeostasis, evolved early in vertebrate evolution, in a common ancestor of jawed vertebrates. The abundant MR expression in ovary and testis suggests a role for 19norProg-MR and Prog-MR complexes in elephant shark reproduction, as well as other unappreciated functions in some other MR-containing tissues. Last, our data suggest that several 3-ketosteroids, including Prog, may have been ancestral mineralocorticoids.

RESULTS

Functional domains of elephant shark MR and other vertebrate MRs

We first compared the functional domains of elephant shark MR to those of selected vertebrate MRs (Fig. 1). Elephant shark MR and human MR have 92 and 67% identity in their DBDs and LBDs, respectively. Furthermore, elephant shark MR has similar conservation to the DBDs (91 to 92%) and LBDs (64 to 69%) of other MRs. The A, B, and D domains of elephant shark MR and those of other MRs are much less conserved (Fig. 1).

Transcriptional activation of full-length and truncated elephant shark MR by corticosteroids, Prog, and Spiron

We screened a panel of steroids at two different concentrations (0.1 and 1 nM) for their ability to stimulate the transcriptional activity of full-length and truncated elephant shark MR. At 1 nM, Aldo, cortisol, corticosterone, 11-deoxycorticosterone, and 11-deoxycortisol activated full-length elephant shark MR (Fig. 3A), indicating that elephant shark MR has broad specificity for corticosteroids. Furthermore, at 1 nM, 19norProg had activity comparable to that of the five corticosteroids, whereas Prog and Spiron had intermediate activity and 17OH-Prog had little activity (Fig. 3A). In parallel experiments, truncated elephant shark MR, lacking the A/B domain and containing a GAL4-DBD instead of the MR DBD, retained responsiveness to all corticosteroids and to 19norProg (Fig. 3B). However, Prog and Spiron had reduced activity, and 17OH-Prog had little activity for truncated elephant shark MR.

EC_{50} values for steroid activation of elephant shark MR

We next determined the concentration dependence of transcriptional activation of full-length elephant shark MR by corticosteroids (Aldo, cortisol, corticosterone, 11-deoxycorticosterone, and 11-deoxycortisol; Fig. 4A) and by Prog, 19norProg, 17OH-Prog, and Spiron (Fig. 4C). We also determined the corresponding concentration-dependent curves for activation of truncated elephant shark MR (Fig. 4, B and D). We then calculated the EC_{50} values of corticosteroids for full-length and truncated elephant shark MR (Table 1). The five corticosteroids, Aldo, corticosterone, 11-deoxycorticosterone, cortisol, and 11-deoxycortisol, had similar EC_{50} values and fold activation of full-length elephant shark MR (Fig. 4A). The EC_{50} values varied from 0.063 nM for 11-deoxycorticosterone to 0.46 nM for cortisol (Table 1). EC_{50} values of <1 nM are consistent with each corticosteroid being a physiological activator of elephant shark MR. Fold activation compared to that of Aldo (which was set at 100%) varied from 83% for 11-deoxycorticosterone and 11-deoxycortisol to 114% for cortisol (Fig. 4A and Table 1), indicating that all five corticosteroids are activators of elephant shark MR.

Truncated elephant shark MR was also substantially activated by corticosteroids, which had EC_{50} values ranging from 0.024 nM for 11-deoxycorticosterone to 0.19 nM for cortisol (Table 1). Fold activation compared to that of Aldo (100%) decreased to 79% for cortisol, 81% for 11-deoxycorticosterone, and 77% for 11-deoxycortisol.

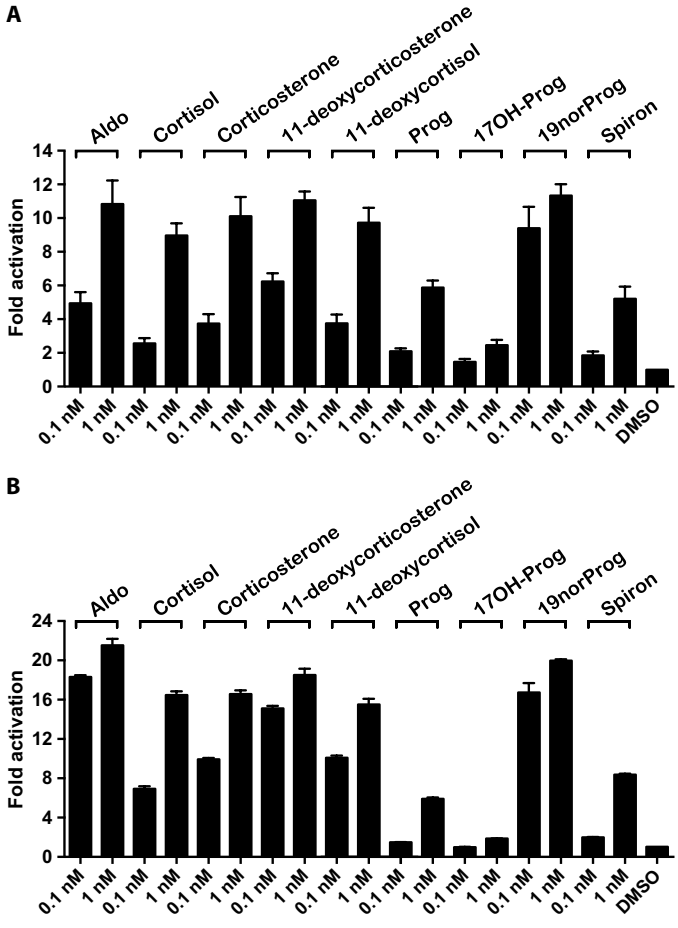

Fig. 3. Transcriptional activation of elephant shark MR by 3-ketosteroids. (**A** and **B**) Full-length (A) and truncated (B) elephant shark MR were expressed in human embryonic kidney (HEK) 293 cells with an MMTV-luciferase reporter for full-length MR or a GAL4-binding site (GAL4-BS)–luciferase reporter for truncated MR. Cells were treated with dimethyl sulfoxide (DMSO) as a negative control or with the indicated ligands at either 0.1 or 1.0 nM. The *y* axis indicates the fold activation of luciferase compared to the luciferase activity of cell expressing the control vector that were treated with vehicle (DMSO) alone, which was set at 1. Results are means ± SEM of three independent experiments (*36*, *73*).

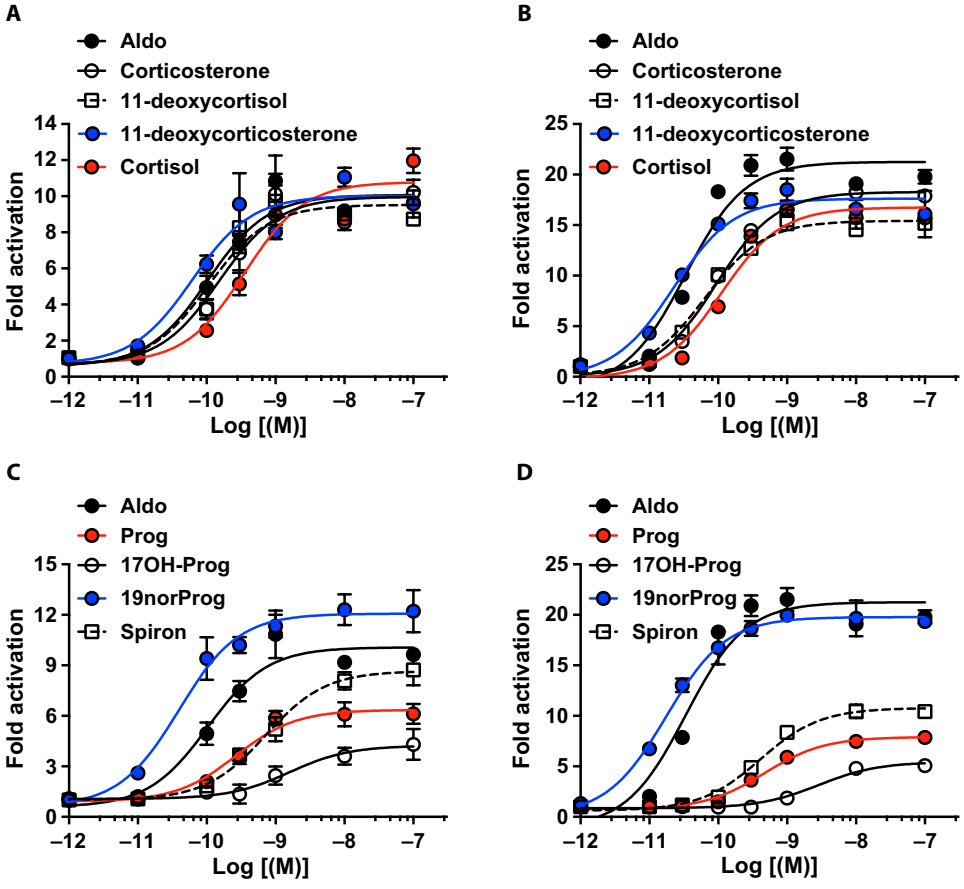

Fig. 4. Concentration-dependent transcriptional activation of full-length and truncated elephant shark MR by 3-ketosteroids. (**A** to **D**) Full-length (A and C) and truncated (B and D) elephant shark MR were expressed in HEK 293 cells with an MMTV-luciferase reporter for full-length MR or a GAL4-BS–luciferase reporter for truncated MR. (A and B) Cells were treated with DMSO (vehicle control) or with the indicated concentrations of Aldo, cortisol, corticosterone, 11-deoxycorticosterone, or 11-deoxycortisol. (C and D) Cells were treated with DMSO (vehicle control) or with the indicated concentrations of Aldo, Prog, 17OH-Prog, 19norProg, or Spiron. The *y* axis indicates the fold activation of the luciferase activity compared to the luciferase activity of cells expressing the control vector and treated with vehicle (DMSO) alone, which was set at 1. Results are means ± SEM of three independent experiments (*36*, *73*).

We also compared these EC_{50} values to the previously determined EC_{50} values of corticosteroids for full-length and truncated human, chicken, alligator, *Xenopus*, and zebrafish MRs (*36*) and for skate MR (*49*). Unlike elephant shark MR, there are differences among terrestrial vertebrate MRs in their EC_{50} values and in their fold activation for the five glucocorticoids. Whereas Aldo and corticosterone had similar nanomolar or lower EC_{50} values for the transcriptional activation of full-length and truncated terrestrial vertebrate MRs, 11-deoxycorticosterone and 11-deoxycortisol had higher EC_{50} values for the activation of truncated human, alligator, and *Xenopus* MRs, compared to their EC_{50} values for the activation of the corresponding full-length MRs (Table 1) (*36*). Furthermore, all corticosteroids had low EC_{50} values and resulted in substantial fold activation of full-length and truncated zebrafish MR (Table 1) (*36*), suggesting that zebrafish MR retains responses to corticosteroids found in elephant shark MR.

Among the progestins, we found that 19norProg was the most active for full-length and truncated elephant shark MR (Fig. 4D and Table 2). 19norProg had EC_{50} values of 0.43 and 0.018 nM for full-length and truncated elephant shark MR, respectively. These values are comparable to the EC_{50} values of corticosteroids for full-length and truncated elephant shark MR. The fold activation by 19norProg of full-length and truncated elephant shark MR was 84 and 98%, respectively, compared to that of Aldo, suggesting that 19norProg could be a physiological activator of elephant shark MR. Prog and Spiron had EC_{50} values of 0.27 and 0.66 nM, respectively, for full-length elephant shark MR. However, their fold activation of full-length MR was about 45% of that of Aldo. 17OH-Prog had an EC_{50} value of 1.9 nM for full-length MR and exhibited only 25% of the fold activation induced by Aldo.

Previously, we reported that Prog, 19norProg, and Spiron are transcriptional activators of full-length and truncated chicken and zebrafish MRs (*36*). However, the EC_{50} values of Prog, 19norProg, and Spiron for these MRs were not determined. We have remedied this omission and now report their EC_{50} values, as well as the EC_{50} values for 17OH-Prog (Fig. 5 and Table 2), for full-length and truncated elephant shark MR for comparison. With respect to full-length chicken MR, Prog and 19norProg had EC_{50} values of 0.68 and 0.71 nM, respectively, which would be expected to be sufficient for the physiological activation of chicken MR (Fig. 5A and Table 2). The fold activation of full-length chicken MR by Prog and 19norProg was 62 and 68%, respectively, compared to that of Aldo. 17OH-Prog had an EC_{50} value of 29 nM, and its fold activation of the receptor was 15% of that of Aldo. The EC_{50} value of Spiron for full-length chicken MR was 5.1 nM. The extent of activation of truncated chicken MR by Prog, 19norProg, 17OH-Prog, and Spiron was too low for the calculation of EC_{50} values (Fig. 5C and Table 2), which suggests that allosteric interactions between the NTD and LBD are important in the activation of chicken MR by progestins.

With respect to zebrafish MR, 19norProg had an EC_{50} value of 0.9 nM and exhibited fold activation of the receptor that was 83% of that of Aldo, whereas Prog had an EC_{50} value of 2.4 nM and gave 77% of the fold activation induced by Aldo for full-length zebrafish MR. These responses are sufficient for both 19norProg and Prog to be physiological activators of zebrafish MR. In contrast, 17OH-Prog had an EC_{50} value of 18 nM and its fold activation of the full-length zebrafish MR was only 44% of that of Aldo. The EC_{50} values of all progestins and Spiron for truncated zebrafish MR were greater than their EC_{50} values for full-length zebrafish MR. The EC_{50} values of Prog and 19norProg for the truncated zebrafish MR were 98 and 64 nM, respectively, which suggests that they are less effective at activating the truncated receptor than they are at activating full-length

Table 1. Activation of full-length MR and truncated MRs (LBD) by corticosteroids. Analysis of luciferase activity in the presence of corticosteroids as a percentage of the luciferase activity induced by Aldo alone. Statistical analysis was performed as described previously (*36*). DOC, 11-deoxycorticosterone.

MR	Aldo	Corticosterone	Cortisol	DOC	DOC
	EC_{50} (M)	EC_{50} (M)	EC_{50} (M)	EC_{50} (M)	EC_{50} (M)
Elephant shark full	1.1×10^{-10}	1.7×10^{-10}	4.6×10^{-10}	6.3×10^{-11}	1.1×10^{-10}
	100%	101%	114%	83%	83%
Elephant shark LBD	3.7×10^{-11}	9.9×10^{-11}	1.9×10^{-10}	2.4×10^{-11}	6.8×10^{-11}
	100%	90%	79%	81%	77%
Skate LBD*	7×10^{-11}	1×10^{-10}	1×10^{-9}	3×10^{-11}	2.2×10^{-8}
Human full†	2.7×10^{-10}	1.2×10^{-9}	5.5×10^{-9}	4.2×10^{-10}	3.6×10^{-9}
	100%	119%	133%	74%	42%
Human LBD†	2.8×10^{-10}	5.9×10^{-10}	3.2×10^{-9}	1.8×10^{-9}	‡
	100%	95%	74%	44%	8%§
Chicken full†	6.2×10^{-11}	5.1×10^{-11}	2.8×10^{-10}	3.4×10^{-11}	6.7×10^{-10}
	100%	109%	128%	110%	112%
Chicken LBD†	1.3×10^{-10}	1.6×10^{-10}	6.9×10^{-10}	1.7×10^{-10}	4.7×10^{-9}
	100%	92%	75%	92%	36%
Alligator full†	2.8×10^{-10}	3.6×10^{-10}	6.9×10^{-9}	2.3×10^{-10}	2.7×10^{-9}
	100%	138%	176%	85%	45%
Alligator LBD†	3.5×10^{-10}	3.8×10^{-10}	2.3×10^{-9}	5.2×10^{-10}	‡
	100%	88%	68%	51%	8%§
Xenopus full†	4.6×10^{-10}	6.2×10^{-10}	1.1×10^{-8}	7.6×10^{-10}	9.1×10^{-9}
	100%	105%	126%	59%	31%
Xenopus LBD†	1.5×10^{-9}	1.9×10^{-9}	1.2×10^{-8}	‡	‡
	100%	74%	37%	10%§	6%§
Zebrafish Full†	8.2×10^{-11}	3.0×10^{-10}	4.4×10^{-10}	6.3×10^{-11}	4.0×10^{-10}
	100%	112%	123%	103%	94%
Zebrafish LBD†	2.7×10^{-11}	1.5×10^{-10}	3.1×10^{-10}	1.0×10^{-10}	9.1×10^{-10}
	100%	96%	77%	99%	67%

*Values obtained from Carroll *et al.* (*49*). †Values obtained from Katsu *et al.* (*36*). ‡Curve did not saturate. §Relative induction at 1 μM compared to Aldo.

zebrafish MR. The extent of activation of truncated zebrafish MR by 17OH-Prog and Spiron was too small for their EC_{50} values to be calculated. This contrasts to the more substantial activation by corticosteroids of full-length zebrafish MR (Fig. 5 and Tables 1 and 2), suggesting that allosteric interactions between the NTD/DBD and LBD contribute to the activation of the zebrafish MR by progestins.

RNA-seq analysis of elephant shark MR

We examined the relative expression of elephant shark MR (*NR3C2*) mRNA in 10 tissues based on previously published RNA-seq data (Fig. 6A) (*9*). The *NR3C2* gene was expressed widely in all tissues, including the gills and kidney, two traditional mineralocorticoid-responsive tissues. Furthermore, there was considerably higher expression in the ovary and testis, the two reproductive tissues analyzed.

RNA-seq analysis of human MR

Analysis of previously published RNA-seq data for the human MR (Fig. 6B) (*50*) revealed that the *NR3C2* is expressed in the kidney, colon, brain, heart, liver, ovary, spleen, and testis. This pattern of expression of human MR in diverse tissues is similar to that of elephant shark *NR3C2*.

DISCUSSION

Cartilaginous fish, including elephant sharks, occupy a key position in the evolution of vertebrates as an out-group to ray-finned fish, the largest group of extant vertebrates, and the lobe-finned fish, which are the forerunners of terrestrial vertebrates. Furthermore, the elephant shark genome is evolving slowly (*9*), making it attractive for studying ancestral proteins, including the MR, which first appeared as a distinct MR ortholog in cartilaginous fish (*5*, *6*, *10*, *49*).

Our investigation of corticosteroid activation of elephant shark MR revealed that Aldo, corticosterone, 11-deoxycorticosterone, cortisol, and 11-deoxycortisol had EC_{50} values of <1 nM for full-length elephant shark MR (Fig. 4 and Table 1). Prog, 19norProg, and Spiron also had subnanomolar EC_{50} values for full-length elephant shark MR. In addition to their low EC_{50} values, all of these corticosteroids and 19norProg exhibited substantial fold activation of the transcriptional activity of full-length MR (Fig. 4, A and C), whereas Prog was about 43% as effective as Aldo. Thus, several corticosteroids, as well as 19norProg and Prog, are potential physiological mineralocorticoids for elephant shark MR.Compared to their EC_{50} values for full-length elephant shark MR, the EC_{50} values of all five corticosteroids and 19norProg for the truncated MR were reduced, whereas

Table 2. EC_{50} values for the activation by Prog and Spiron of full-length and truncated (LBD) constructs of elephant shark, zebrafish, and chicken MRs. Analysis of luciferase activity in the presence of Aldo, the indicated progestins, and Spiron and statistical analysis were performed as described previously (*36*). Relative induction is presented as a percentage of the luciferase activity induced by Aldo alone.

MR	Aldo	Prog	17OH-Prog	19norProg	Spiron
Elephant shark full	1.1×10^{-10}	2.7×10^{-10}	1.4×10^{-9}	4.3×10^{-11}	5.5×10^{-10}
	100%	43%	25%	84%	45%
Elephant shark LBD	3.7×10^{-11}	4.8×10^{-10}	2.9×10^{-9}	1.8×10^{-11}	4.2×10^{-10}
	100%	40%	26%	98%	53%
Zebrafish full	8.2×10^{-11}	2.4×10^{-9}	1.8×10^{-8}	9.4×10^{-10}	3.8×10^{-9}
	100%	77%	44%	83%	54%
Zebrafish LBD	2.7×10^{-11}	9.8×10^{-8}	*	6.4×10^{-8}	*
	100%	122%	24%†	122%	73%†
Chicken full	6.2×10^{-11}	7.1×10^{-10}	2.9×10^{-8}	6.8×10^{-10}	5.1×10^{-9}
	100%	62%	15%	68%	30%
Chicken LBD	1.3×10^{-10}	*	*	*	*
	100%	21%†	—	29%†	—

*Curve did not saturate. †Relative induction at 1 μM compared to Aldo.

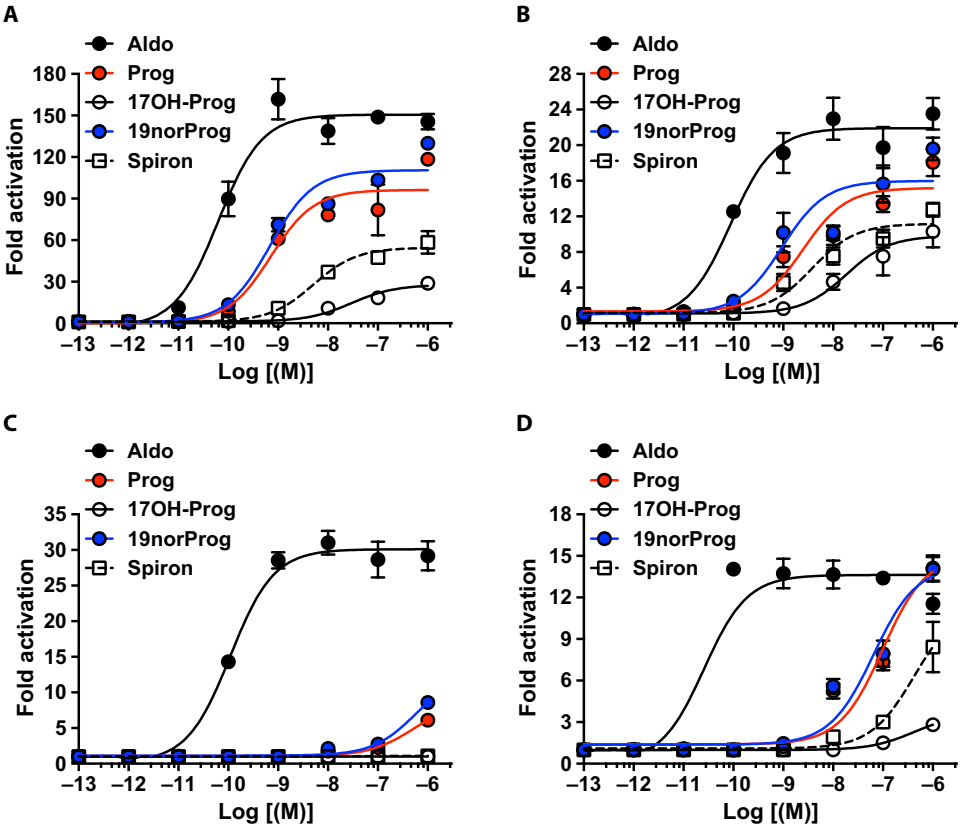

Fig. 5. Concentration-dependent transcriptional activation of full-length and truncated chicken and zebrafish MR by Prog, 19norProg, and Spiron. (**A** to **D**) HEK 293 cells were cotransfected with an MMTV-luciferase reporter or a GAL4-BS–luciferase reporter and plasmids expressing full-length chicken MR (A), full-length zebrafish MR (B), truncated chicken MR (C), or truncated zebrafish MR (D). Cells were treated with DMSO (vehicle control) or with the indicated concentrations of Aldo, Prog, 17OH-Prog, 19norProg, or Spiron. The *y* axis indicates the fold activation of luciferase compared to the luciferase activity of cells expressing the control vector and treated with vehicle (DMSO) alone, which was set at 1. Results are means ± SEM of three independent experiments.

the EC_{50} value for Spiron was slightly less, and the EC_{50} values for Prog and 17OH-Prog were about twofold greater (Tables 1 and 2). Regarding the truncated skate MR, most of the EC_{50} values of the corticosteroids (*49*) are similar to that for elephant shark MR (Table 1). The exception is 11-deoxycortisol, whose EC_{50} value was greater than 200-fold higher for skate MR than for elephant shark MR.

Prog, 19norProg, or both may be mineralocorticoids in cartilaginous fish

The Prog concentration in female elephant shark serum is 4.4 ng/ml (14 nM) (*51*). In draughtboard sharks (*Cephaloscyllium laticeps*), the serum concentration of Prog in females is 8 ng/ml (25.4 nM), whereas in males it is 1 ng/ml (3.2 nM) (*52*). In female zebrasharks (*Stegostoma fasciatum*), the serum concentration of Prog is 10 ng/ml (31.8 nM) (*53*). Together, these data suggest that Prog concentrations are sufficient to activate the MR in cartilaginous fish. We found that 19norProg has an EC_{50} value of 0.043 nM for elephant shark MR. Moreover, 19norProg evoked a stronger response from elephant shark MR than did Aldo (Fig. 4, A and B). C19 demethylase, which removes the C19 methyl group from steroids, has been detected in the mammalian kidney (*54*). If C19 demethylase is present in elephant shark, then 19norProg should be considered as a potential physiological mineralocorticoid.

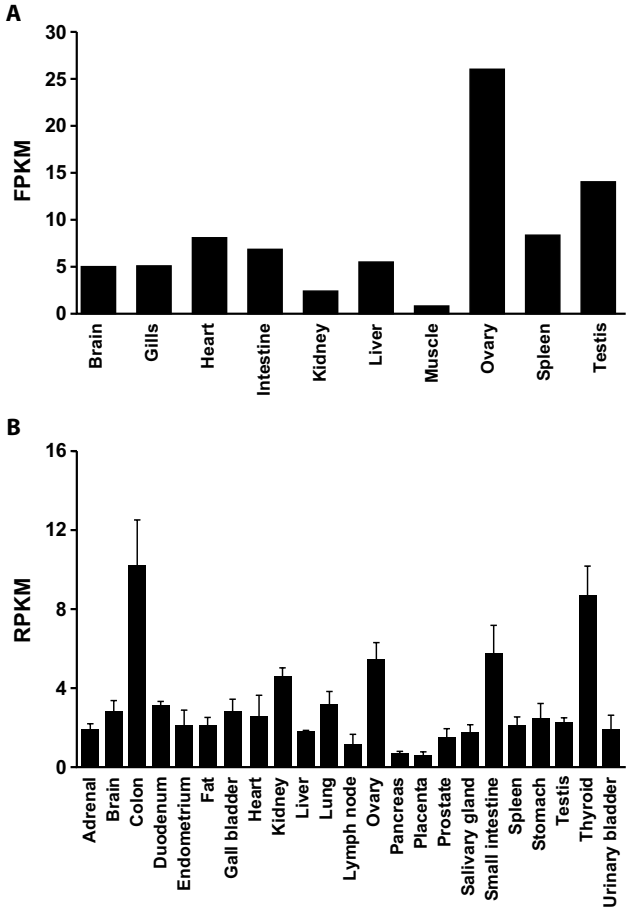

Fig. 6. Expression of elephant shark and human MRs based on RNA-seq data. (**A**) Relative expression of the elephant shark *NR3C2* based on RNA-seq data. Transcript abundances are shown in terms of normalized counts called fragments per kilobase of exon per million fragments mapped (FPKM) (*71*). FPKM values were estimated by normalizing gene length, followed by normalizing for sequencing depth. (**B**) Relative expression of the human MR based on RNA-seq data. Transcript abundances are shown in terms of normalized counts called reads per kilobase of transcript per million mapped reads (RPKM) (*50*).

Although in cell-based assays, 19norProg is an agonist for elephant shark, zebrafish, and chicken MRs (Table 2), and 19norProg at 1 nM is an antagonist for human MR (*36*, *48*). This finding contrasts to that from in vivo studies in rats, which found that 19norProg is an MR agonist with about 100-fold weaker activity than that of Aldo (*54*, *55*). A possible explanation for this difference is that in rats, 19norProg is metabolized to 19nor-11-deoxycorticosterone, which is an MR agonist (*56*–*58*). Another possibility, based on the ability of 11β-hydroxyprogesterone to activate human MR (*28*), is that 19norProg is metabolized to 11β-hydroxy19norProg.

We propose that the transcriptional activation of elephant shark MR by 19norProg, as well as by Prog and Spiron, can be explained by the discovery by Geller *et al.* (*48*) that the S810L mutant human MR is activated by 1 nM Prog, 19norProg, and Spiron, unlike wild-type human MR, for which these steroids are antagonists. On the basis of a three-dimensional model of the S810L mutant MR, Geller *et al.* (*48*) proposed that a contact between Leu^{810} and Ala^{773} was sufficient for transcriptional activation of the S810L mutant MR by Prog and 19norProg. This motivated Geller *et al.* (*48*) to construct the S810M mutant human MR, which was activated by 19norProg. Elephant shark MR and skate MR contain a methionine at the position corresponding to Ser^{810} and an alanine corresponding to Ala^{773} (Fig. 7) (*25*). On the basis of the model of Geller *et al.*, we propose that the transcriptional activation of elephant shark MR by 19norProg is due to a contact between Met^{782} (helix 5) and Ala^{745} (helix 3), which would stabilize the A ring of 19norProg, promoting transcriptional activation of the receptor.

A potential role for elephant shark MR in reproductive physiology

The EC_{50} value of Prog for elephant shark MR (0.27 nM), the physiological concentration of Prog of 14.5 nM (*51*), and the abundant expression of the MR in elephant shark ovary and testis (Fig. 6A) all suggest that a Prog-MR complex may be important in reproductive responses in elephant shark. Of course, Prog also acts as a reproductive steroid in the ovary and testis through its transcriptional activation of the PR (*59*, *60*). On the basis of evidence that Prog activates the MR in several ray-finned fish (*25*, *37*, *45*, *47*), a Prog-MR complex also may be active in reproductive tissues and other tissues in ray-finned fish, as well as in cartilaginous fish.

RNA-seq analysis found MR expression in elephant shark gills and kidneys (Fig. 6A), two classical targets for the MR-mediated regulation of electrolyte transport (*6*). Moreover, RNA-seq analysis also identified MR expression in elephant shark heart and brain, two other tissues in which corticosteroids exert important physiological actions through the mammalian MR (*16*–*18*, *20*–*22*, *61*–*64*). MR antagonists are useful in treating heart failure (*21*, *63*, *65*), although their mechanism of action is not fully understood (*21*, *64*). A study by Oakley *et al.* (*64*) provides insights into how inhibition of MR activity in the heart affects the survival of damaged cardiomyocytes in mice. Oakley *et al.* (*64*) reported that the balance between

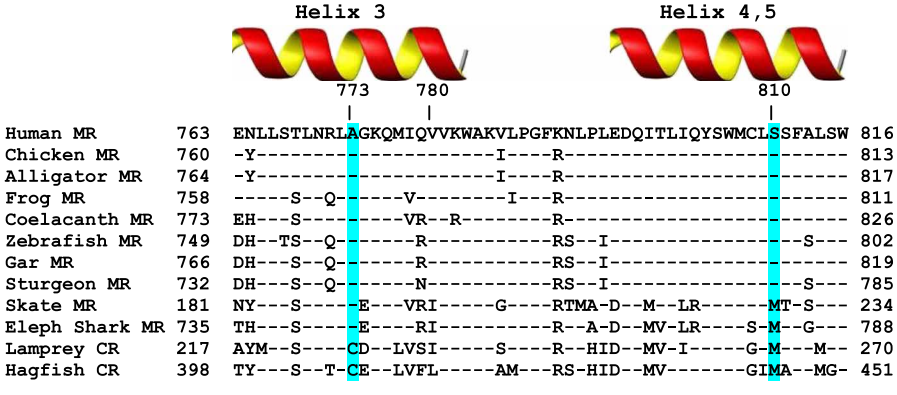

Fig. 7. Alignment of elephant shark MR to Ser^{810} and Ala^{773} in helices 3 to 5 in human MR. Elephant shark MR and skate MR each contain a methionine corresponding to Ser^{810} in the human MR and an alanine corresponding to Ala^{773}. Lamprey CR and hagfish CR also contain a corresponding methionine, as well as a cysteine corresponding to Ala^{773}. The residues Ser^{810} and Ala^{773} in the human MR are conserved in MRs from coelacanths, terrestrial vertebrates, and ray-finned fish. Amino acid residues that are identical to those in the human MR are denoted by (–).

the actions of the MR and its close relative, the GR, influences whether damaged cardiomyocytes die or survive.

RNA-seq analysis of elephant shark MR indicates that the expression of the MR in diverse tissues was conserved during the descent from cartilaginous fish to humans. Expression of shark MR in many tissues (brain, heart, liver, and ovary) in which the MR is not likely to regulate electrolyte homeostasis, the classical function of the MR, further supports evidence from the last 30 years (*3*, *17*–*19*, *21*, *61*, *63*, *65*–*67*) that mineralocorticoid activity is an incomplete functional description of this nuclear receptor. An alternative name is needed to describe more completely the functions of the MR.

MATERIALS AND METHODS

Chemical reagents

Aldo, cortisol, corticosterone, 11-deoxycorticosterone, 11-deoxcortisol, Prog, 19norProg, 17OH-Prog, and Spiron were purchased from Sigma-Aldrich. For reporter gene assays, all hormones were dissolved in DMSO; the final DMSO concentration in the culture medium did not exceed 0.1%.

Construction of plasmid vectors

The full-coding regions from elephant shark MR were amplified by polymerase chain reaction (PCR) with KOD DNA polymerase. The PCR products were gel-purified and ligated into pcDNA3.1 vector (Invitrogen) for the full-coding region or into the pBIND vector for D and E domains (*68*).

Elephant shark MR gene expression analysis

We had previously generated RNA-seq for several tissues of elephant shark as part of the elephant shark genome project (*9*) and submitted them to National Center for Biotechnology Information (accession number SRA054255). We downloaded RNA-seq reads for the brain, gills, heart, intestine, kidney, liver, muscle, ovary, spleen, and testis, and assembled each of them into transcripts using the program Trinity version r2013-08-14 (*69*). The assembled transcripts were used to determine the extent of expression of the *NR3C2* gene. To determine the relative expression of the MR gene, we performed abundance estimation of transcripts from the aforementioned 10 tissues. Trinity transcripts from all 10 tissues and the full-length complementary DNA sequence of the MR gene were combined together and clustered using CD-HITv4.6.1 at 100% identity (*70*). RNA-seq reads from each of the 10 tissues were independently aligned to the clustered transcript sequences, and the abundance of MR transcripts was estimated by RSEMv1.2.25 (*71*), which uses bowtiev2.2.6 for aligning (*72*). Transcript abundances were measured in terms of normalized counts called FPKM (*71*). FPKM is estimated by normalizing the gene length, followed by normalizing for sequencing depth.

Transactivation assay and statistical analysis

Transfection and reporter assays were performed with HEK 293 cells, as described previously (*68*, *73*). All experiments were performed in triplicate. The values shown in the figures are means ± SEM from three separate experiments, and the dose-response data and EC_{50} values were analyzed and calculated with GraphPad Prism. Comparisons between two groups were performed using the Student's *t* test, and all multigroup comparisons were performed by one-way analysis of variance (ANOVA), followed by Bonferroni test. $P < 0.05$ was considered to be statistically significant. The use of HEK 293 cells and an assay temperature of 37°C does not replicate the physiological environment of elephant sharks. Nevertheless, studies with HEK 293 cells and other mammalian cell lines have proven useful for other studies of transcriptional activation by corticosteroids of skate MR (*49*) and teleost fish (*37*, *45*, *74*, *75*) MRs.

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Transcriptional activation of elephant shark mineralocorticoid receptor by corticosteroids, progesterone, and spironolactone

Yoshinao Katsu, Satomi Kohno, Kaori Oka, Xiaozhi Lin, Sumika Otake, Nisha E. Pillai, Wataru Takagi, Susumu Hyodo, Byrappa Venkatesh and Michael E. Baker

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Expanding mineralocorticoid functions

Mineralocorticoid receptors (MRs) belong to the nuclear receptor family of transcription factors. Aldosterone is a physiological ligand for the human MR, which is best known for regulating electrolyte homeostasis. Noting that the MR first arose in cartilaginous fish, which do not have aldosterone, Katsu *et al.* examined the binding and activity profiles of a range of corticosteroids and steroid hormones for the MR of the elephant shark, a cartilaginous fish found in the oldest group of jawed vertebrates. These studies suggest that elephant shark MR is activated by progesterone, which acts as an antagonist of the human MR. Given the abundance of the MR in elephant shark ovaries and testis, these findings suggest that the MR may play an unappreciated role in reproductive physiology.