Transcriptional Activation of a Cartilaginous Fish (Elephant Shark, *Callorhinchus milii*) Mineralocorticoid Receptor by Corticosteroids, Progestins and Spironolactone

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Abstract. A distinct mineralocorticoid receptor (MR) first appears in cartilaginous fishes (Chondrichthyes), the oldest group of extant jawed vertebrates. To investigate steroid specificity of cartilaginous fish MR, we studied transcriptional activation of full-length elephant shark (*Callorhinchus milii*) MR by aldosterone, cortisol, 11-deoxycorticosterone, corticosterone, 11-deoxcortisol, progesterone and 19-norprogesterone. All investigated corticosteroids and progestins showed half-maximal responses (EC50s) below 1 nM for elephant shark MR, and hence are potential physiological mineralocorticoids. Progesterone and 19-norprogesterone are antagonists for human, Xenopus and alligator MRs, but agonists for ray-finned fish and chicken MRs, indicating that MR activation by progestins is an ancestral response, conserved in ray-finned fish, lost in Xenopus, alligator and human, and distinct from chicken MR activation, which arose independently. RNA-seq analysis finds strong MR expression in elephant shark ovary and testis, in which progesterone-activated MR may

have novel functions.

Running title: Steroid specificity of elephant shark mineralocorticoid receptor

Key words: elephant shark MR, MR evolution, corticosteroids, mineralocorticoids, progesterone

Introduction

The mineralocorticoid receptor (MR) belongs to the nuclear receptor family, a large and diverse group of transcription factors that also includes receptors for glucocorticoids (GR), progestins (PR) androgens (AR) and estrogens (ER) (Baker, Nelson, & Studer, 2015; Evans, 1988). Sequence analysis revealed that the MR and GR are closely related (Arriza et al., 1987); phylogenetic analysis indicates that MR and GR evolved from a corticosteroid receptor (CR) that was present in jawless vertebrates, such as lamprey and hagfish (Baker, Funder, & Kattoula, 2013; Baker & Katsu, 2017; Rossier, Baker, & Studer, 2015; Thornton, 2001). A distinct mineralocorticoid receptor (MR) first appears in cartilaginous fishes (Chondrichthyes), the oldest group of extant jawed vertebrates (gnathostomes) that diverged from bony vertebrates about 450 million years and are a crucial group in understanding the origin and evolution of jawed vertebrate morphology and physiology (Inoue et al., 2010; Venkatesh et al., 2014). Like mammals, cartilaginous fishes contain the full complement of adrenal and sex steroid receptors: AR, ER, GR, MR and PR (Baker et al., 2013; Baker et al., 2015; Bridgham, Carroll, & Thornton, 2006; Evans, 1988).

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 (D) and a C-terminal ligand-binding domain (LBD) (domain E) (Evans, 1988; Huang, Chandra, & Rastinejad, 2010; Rastinejad, Huang, Chandra, & Khorasanizadeh, 2013; Sugimoto et al., 2016) (Figure 1). The E domain alone is competent to bind steroids (Baker et al., 2013; Bledsoe et al., 2005; Fagart et al., 2005; Huang et al., 2010; Y. Li, Suino, Daugherty, & Xu, 2005). Interactions between the NTD (A/B domains) and the LBD and coactivators are important regulators of transcriptional activation of mammalian MR (Faresse, 2014; Fischer, Kelly, Watt, Price, & McEwan, 2010; Fuller, 2015; Katsu, Oka, & Baker, 2018; Kumar & Litwack, 2009; Pippal, Yao, Rogerson, & Fuller, 2009; Rogerson & Fuller, 2003; Rupprecht, Arriza, et al., 1993) and fish MR (Fuller, 2015; Katsu et al., 2018; Pippal, Cheung, Yao, Brennan, & Fuller, 2011).

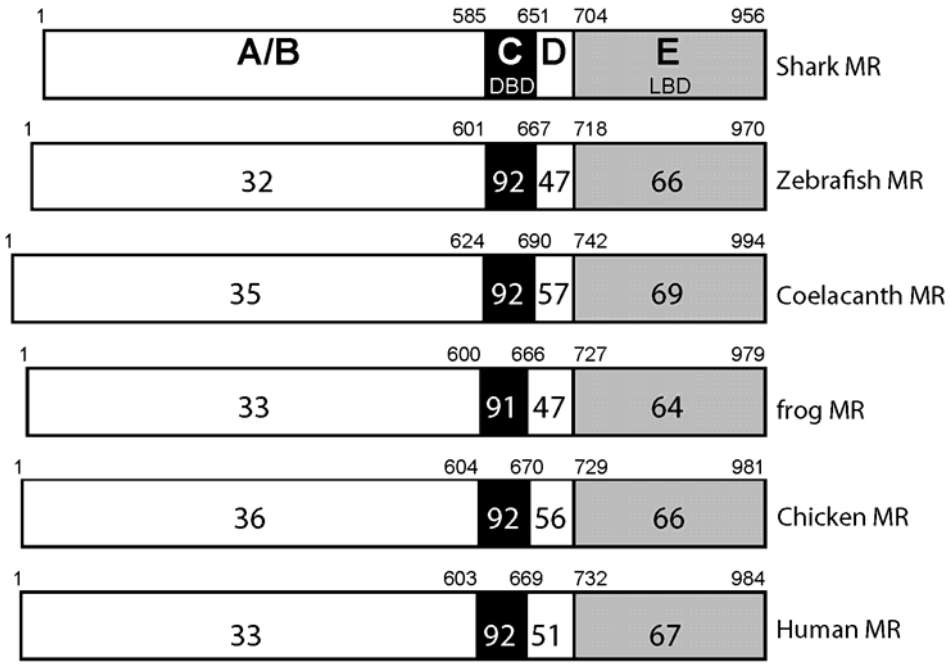

Figure 1. Comparison of domains in elephant shark MR with vertebrate MRs. MRs from elephant shark (shark), zebrafish, coelacanth, Xenopus (frog), chicken and human are compared. The functional A/B domain to E domains are schematically represented with the numbers of amino acid residues and the percentage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

There is scant data on transcriptional activation of cartilaginous fish MR by corticosteroids (Carroll, Bridgham, & Thornton, 2008). Carroll et al. investigated transcriptional activation of skate MR by corticosteroids and found that aldosterone (Aldo), corticosterone (B), 11-deoxycorticosterone (DOC), and cortisol (F) (Figure 2) are strong transcriptional activators of skate MR. The half-maximal response for transcriptional activation (EC50) by corticosteroids of skate MR is 30 pM for DOC, 70 pM for Aldo, 90 pM for B, 1 nM for F and 22 nM for 11-deoxycortisol (S) (Carroll et al., 2008). Interestingly, Aldo, the physiological mineralocorticoid in terrestrial vertebrates, has not been found in cartilaginous fishes or ray-finned fishes. Aldo first appears in lobe-finned fish (Joss, Arnoldreed, & Balment, 1994). Indeed, the identity of the

physiological mineralocorticoid in cartilaginous fishes and ray-finned fishes is not established, although F and DOC have been proposed (Baker, 2003; Baker, Chandsawangbhuwana, & Ollikainen, 2007; Bury & Sturm, 2007; Sakamoto et al., 2011; Sturm et al., 2005; Takahashi & Sakamoto, 2013).

Complicating the identity of the physiological mineralocorticoid in cartilaginous fishes is evidence that progesterone (Prog) and 19-norprogesterone (19norProg), along with spironolactone (Spiron) (Figure 2), are agonists of several ray-finned fish MRs (Pippal et al., 2011; Sturm et al., 2005; Sugimoto et al., 2016) and chicken MR (Katsu et al., 2018). However, these steroids are antagonists for human MR (Bledsoe et al., 2005; Fagart et al., 2005; Geller et al., 2000), alligator MR and Xenopus MR (Katsu et al., 2018). Ray-finned fish MRs and chicken MR differ in their response to Prog, 19norProg and Spiron, raising the question of whether the response to these steroids evolved in ray-finned fish before or after the divergence of ray-finned fish from the lobe-finned fish lineage that led to tetrapods.

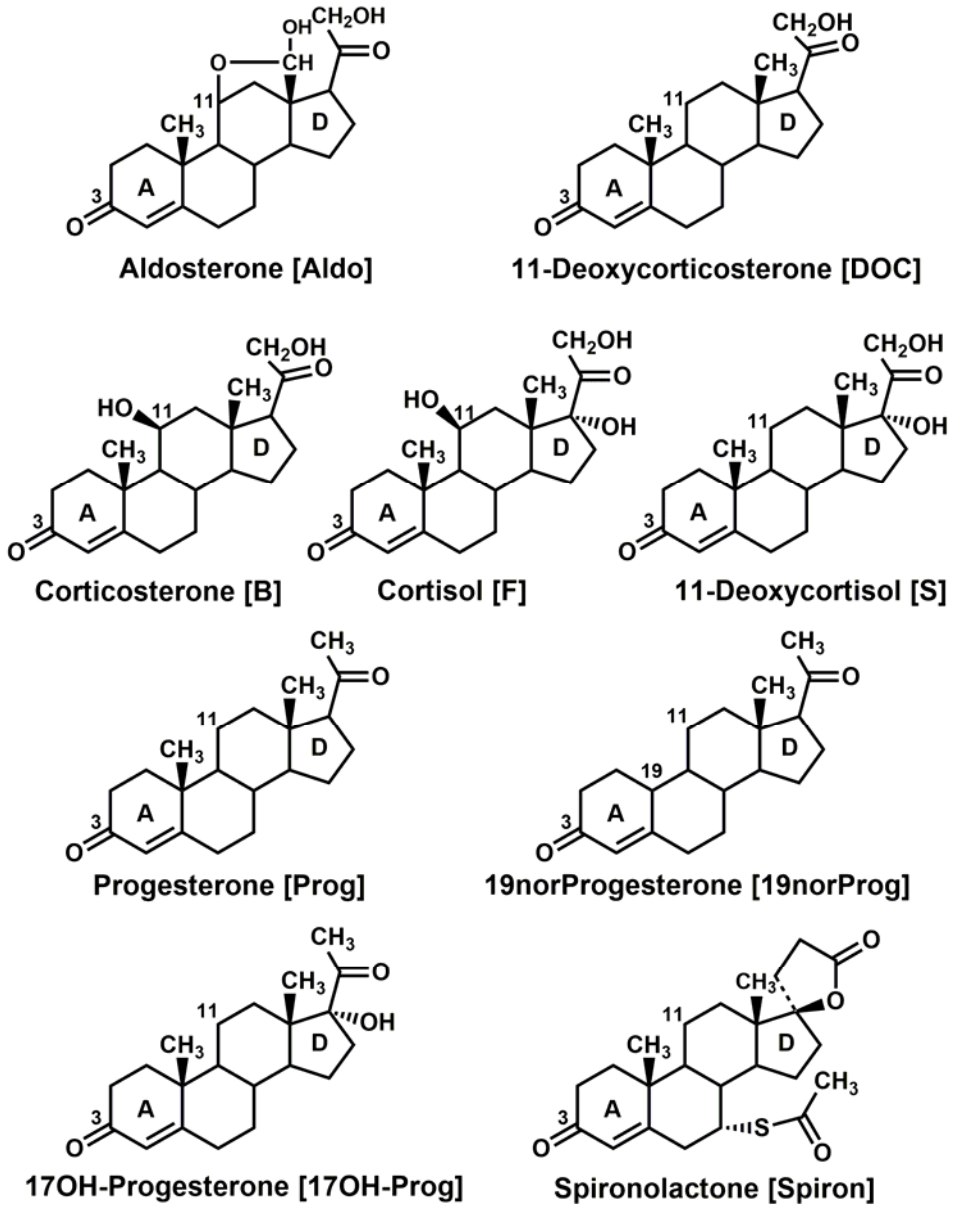

Figure 2. Structures of steroids that are ligands for the MR.

Aldo, DOC and S are physiological mineralocorticoids in terrestrial vertebrates (Baker et al., 2013; Fuller, Yao, Yang, & Young, 2012; Hawkins, Gomez-Sanchez, Gomez-Sanchez, & Gomez-Sanchez, 2012; Rossier et al., 2015). S is both a mineralocorticoid and a glucocorticoid in lamprey (Baker et al., 2013; Close, Yun, McCormick, Wildbill, & Li, 2010) and a glucocorticoid in ray-finned fish (Y. Li, Sturm, Cunningham, & Bury, 2012). F is a physiological glucocorticoid in terrestrial vertebrates and ray-finned fish (Arterbery et al., 2011; Baker et al., 2013; Bridgham et al., 2006; Bury, 2016; Sturm, Colliar, Leaver, & Bury, 2011). B is a glucocorticoid in rats and mice (Baker et al., 2013). Aldo, DOC, F, B and Prog have a similar high affinity for human MR (Arriza et al., 1987; Hellal-Levy et al., 1999; Rupprecht, Reul, et

al., 1993). Prog, 19norProg, 17OH-Prog and Spiron are antagonists for human MR (Geller et al., 2000; Rupprecht, Reul, et al., 1993; Sugimoto et al., 2016) and rat MR (Kagawa, 1958; Wambach & Higgins, 1978). Prog, 19norProg, and Spiron are agonists for fish MRs (Pippal et al., 2011; Sturm et al., 2005; Sugimoto et al., 2016). 19norProg is a weak agonist for rat MR (Funder & Adam, 1981; Hall, Gomez-Sanchez, Hungerford, & Gomez-Sanchez, 1981).

Also of interest is the role of the NTD/DBD in transcriptional activation of cartilaginous fish MR. Carroll et al. (Carroll et al., 2008) used an expression plasmid consisting of the GAL4 DBD fused to the D domain and E domain of the MR (MR-LBD). Thus, transcriptional activation by corticosteroids and progestins of full-length cartilaginous fish MR, which is the physiological MR in cartilaginous fishes, has not been investigated. The absence of data on steroid activation of full length cartilaginous fish MR is important because interactions between the NTD and LBD regulate corticosteroid activation of transcription of human MR (Katsu et al., 2018; Pippal et al., 2009; Rogerson & Fuller, 2003; Rupprecht, Arriza, et al., 1993) and zebrafish MR (Fuller, 2015; Katsu et al., 2018; Pippal et al., 2011). Moreover, GAL-DBD-hinge-LBD constructs of zebrafish MR have different responses to progestins and some corticosteroids than GAL-DBD-hinge-LBD constructs of human, chicken, alligator and Xenopus MRs (Katsu et al., 2018). The timing of the evolution of this difference between the MR in ray-finned fish and terrestrial vertebrates is not known.

Elephant shark MR is an attractive receptor to study early events in the evolution of mechanisms for regulating MR transcription because genomic analyses reveal that elephant shark genes are evolving slowly (Venkatesh et al., 2014), making their genes windows into the past. With the above in mind, we studied transcriptional activation by Aldo, DOC, B, S, F, Prog, 19norProg, 17-hydroxyprogesterone (17OH-Prog) and Spiron of full-length and truncated elephant shark MR. Interestingly, all 3-ketosteroids had EC50s of 1 nM or lower for full-length elephant shark MR. Transcriptional activation by Prog, 19norProg and Spiron of truncated elephant shark MR resembled that of zebrafish MR and not chicken MR, indicating that progestin activation of MR is an ancestral response, conserved in ray-finned fish, lost in Xenopus, alligator and human MRs, and distinct from activation of chicken MR, which arose independently. We also performed RNA-seq analysis of elephant shark MR and find widespread expression of MR in various elephant shark organs (gill, kidney, heart,

intestine, liver, spleen, brain), with strong MR expression in ovary and testis, which are likely targets for Prog, suggesting a role for Prog-MR complexes in elephant shark reproduction, as well as in some other functions in other MR-containing organs. Our data suggests that Prog or a related progestin may have been one of the ancestral mineralocorticoids.

Results

Functional domains of elephant shark MR and other vertebrate MRs.

In Figure 1, we compare the functional domains of elephant shark MR to selected vertebrate MRs. Elephant shark MR and human MR have 92% and 67% identity in DBD and LBD, respectively. Interestingly, elephant shark MR has similar conservation to the DBD (91-92%) and LBD (64-69%) of other MRs. The A, B and D domains of elephant shark MR and other MRs are much less conserved.

Transcriptional activation of full-length and truncated elephant shark MR by corticosteroids, progestins and spironolactone.

We screened a panel of steroids at 0.1 nM and 1 nM for transcriptional activation of full-length and truncated elephant shark MR. Aldo, F, B, DOC and S were strong activators of full-length elephant shark MR (Figure 3A) indicating that elephant shark MR has broad specificity for corticosteroids. Interestingly, at these low concentrations, all progestins and Spiron are transcriptional activators of full-length elephant shark MR, with 19-norProg having the strongest activity and 17OHProg having the weakest activity (Figure 3A).

In parallel experiments, truncated elephant shark MR, lacking the A/B domain and containing a GAL4-DBD instead of the MR DBD, retained a strong response to all corticosteroids and 19nor Prog (Figure 3B). Prog and Spiron had significant, but reduced activity, while 17OH-Prog had little activity for truncated elephant shark MR.

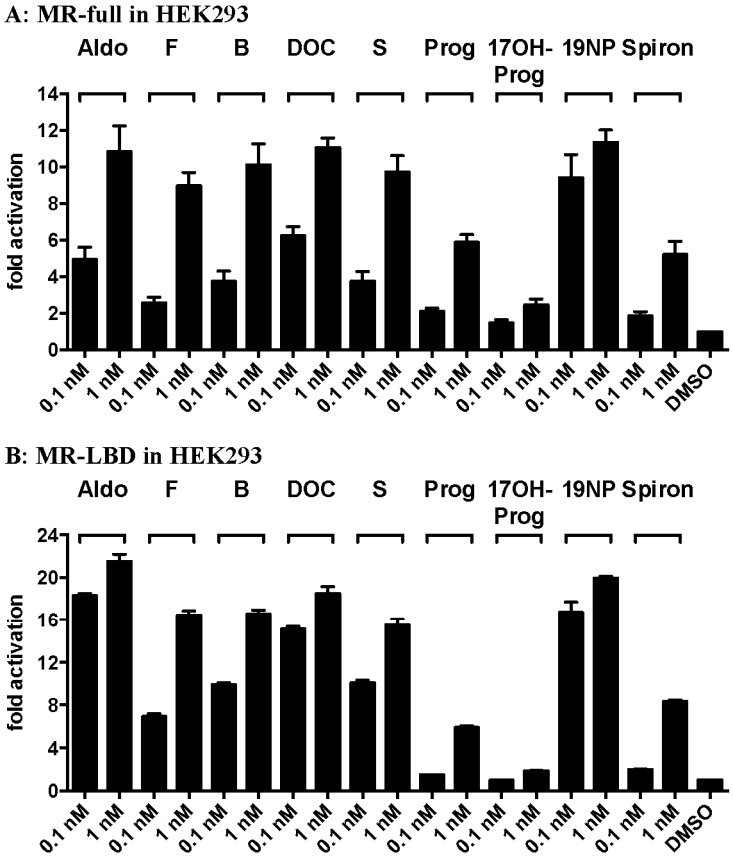

Figure 3. Transcriptional activation of elephant shark MR by 3-ketosteroids.
Full length and truncated elephant shark MR were expressed in HEK293 cells with an MMTV-luciferase reporter.

A. Full length elephant shark MR. Cells were treated with 0.1 nM or 1.0 nM Aldo, F, B, DOC, S, Prog, 19norProg (19NP), 17OH-Prog, Spiron or vehicle alone (DMSO).

B. Truncated elephant shark MR. Cells were treated with 0.1 nM or 1.0 nM Aldo, F, B, DOC, S, Prog, 19norProg (19NP), 17OH-Prog or Spiron or vehicle alone (DMSO).

Results are expressed as means ± SEM, n=3. Y-axis indicates fold-activation compared to the activity of control vector with vehicle (DMSO) alone as 1.

EC50 values for steroid activation of elephant shark MR

Concentration-dependence of transcriptional activation of full length elephant shark MR by corticosteroids (Aldo, F, B, DOC, S) is shown in Figure 4A and by

progestins (Prog, 19norProg, 17OH-Prog) and Spiron in Figure 4B. The corresponding concentration-dependent curves for truncated elephant shark MR are shown in Figures 4C and 4D, respectively. Table 1 summarizes the EC50s of corticosteroids for full-length and truncated elephant shark MR. Table 1 also contains, for comparison, previously determined EC50s of corticosteroids for full-length and truncated human, chicken, alligator, Xenopus and zebrafish MRs (Katsu et al., 2018) and skate MR (Carroll et al., 2008).

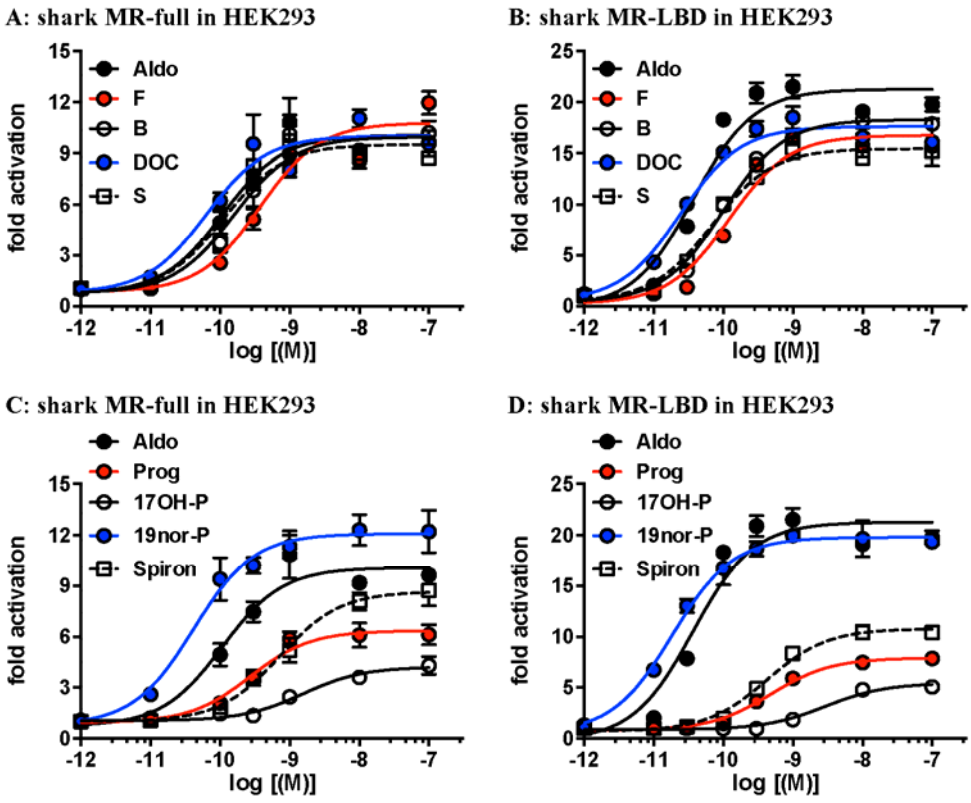

Figure 4. Concentration-dependent transcriptional activation of full-length and truncated elephant shark MR (shark MR) by 3-ketosteroids.

Full length and truncated elephant shark MR (shark MR) were expressed in HEK293 cells with an MMTV-luciferase reporter. Full-length elephant shark MR (shark MR) (A) and (C) and truncated elephant shark MR (shark MR) (B) and (D).

(A), (B) Aldo, F, B, DOC or S.

(C), (D) Aldo, Prog, 19norProg (19nor-P), 17OH-Prog (17OH-P) or Spiron.

Cells transfected with elephant shark MR were treated with increasing concentrations of

F, B, Aldo, DOC, S, Prog, 19norProg, 17OH-Prog, Spiron or vehicle alone (DMSO).

Y-axis indicates fold-activation compared to the activity of control vector with vehicle (DMSO) alone as 1.

Table 1. Corticosteroid activation of full-length MR and GAL4-DBD-MR-LBD

MR	Aldo	B	F	DOC	S
	EC50 (M)	EC50 (M)	EC50 (M)	EC50 (M)	EC50 (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%
[1]Skate LBD	7×10^{-11}	1×10^{-10}	1×10^{-9}	3×10^{-11}	2.2×10^{-8}
[2]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%
[2]Human LBD	2.8×10^{-10}	5.9×10^{-10}	3.2×10^{-9}	1.8×10^{-9}	#-
	100%	95%	74%	44%	*8%
[2]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%
[2]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%
[2]Alligator-Full	$2.8x\ 10^{-10}$	3.6×10^{-10}	$6.9x\ 10^{-9}$	2.3×10^{-10}	2.7×10^{-9}
	100%	138%	176%	85%	45%
[2]Alligator LBD	3.5×10^{-10}	3.8×10^{-10}	2.3×10^{-9}	5.2×10^{-10}	#-
	100%	88%	68%	51%	*8%
[2]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%
[2]Xenopus-LBD	1.5×10^{-9}	1.9×10^{-9}	1.2×10^{-8}	#-	#-
	100%	74%	37%	*10%	*6%
[2]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%
[2]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%

1. (Carroll et al., 2008)

2. (Katsu et al., 2018)

Curve did not saturate.

(%) Relative induction to Aldosterone induced activation.

* Relative induction at 1 μM compared to Aldo.

Previously we reported that progestins and Spiron are transcriptional activators of full-length chicken and zebrafish MRs and truncated zebrafish MR (Katsu et al., 2018). However, EC50 values of progestins and Spiron for these MR were not determined. We have remedied this omission and report their EC50 values in Table 2 and Figure 5, for comparison with full-length and truncated elephant shark MR.

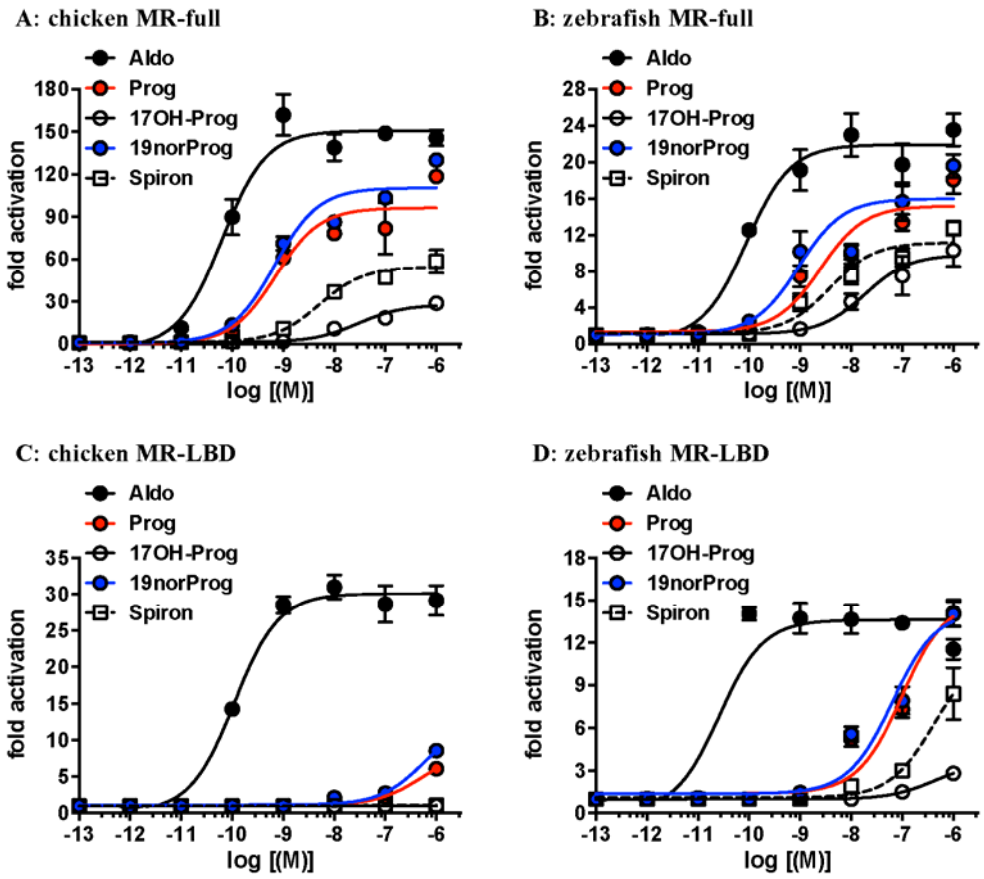

Figure 5. Concentration-dependent transcriptional activation of full-length and truncated chicken and zebrafish MR by progestins and Spironolactone.
HEK293 cells transfected with chicken and zebrafish MR were treated with increasing concentrations of Prog, 19norProg, 17OH-Prog or Spiron. Full-length chicken MR (A) and zebrafish MR (B) and truncated chicken MR (C) and zebrafish MR (D). Y-axis indicates fold-activation compared to the activity of control vector with vehicle (DMSO) alone as 1.

Table 2. EC50 values for progestin and spironolactone activation of full-length and GAL4-DBD-MR-LBD constructs of elephant shark, zebrafish and chicken MR

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%**
[2]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%**
[2]Zebrafish LBD	**2.7×10^{-11}**	**9.8×10^{-8}**	#-	**6.4×10^{-8}**	#-
	100%	**122%**	*24%	**122%**	*73%
[2]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%**
[2]Chicken LBD	**1.3×10^{-10}**	#-	#-	#-	#-
	100%	***21%**	**-**	***29%**	**-**

2. (Katsu et al., 2018)

Curve did not saturate.

(%) Relative induction to Aldosterone induced activation.

* Relative induction at 1 µM compared to Aldo.

RNA-seq analysis of elephant shark MR

We examined expression of level of elephant shark MR gene in 10 tissues based on RNA-seq data (Figure 6). The MR gene was found to express widely in all tissues, including gills and kidney, two traditional mineralocorticoid-responsive tissues. Interestingly there was considerably higher expression in the ovary and testis, the two reproductive tissues analyzed.

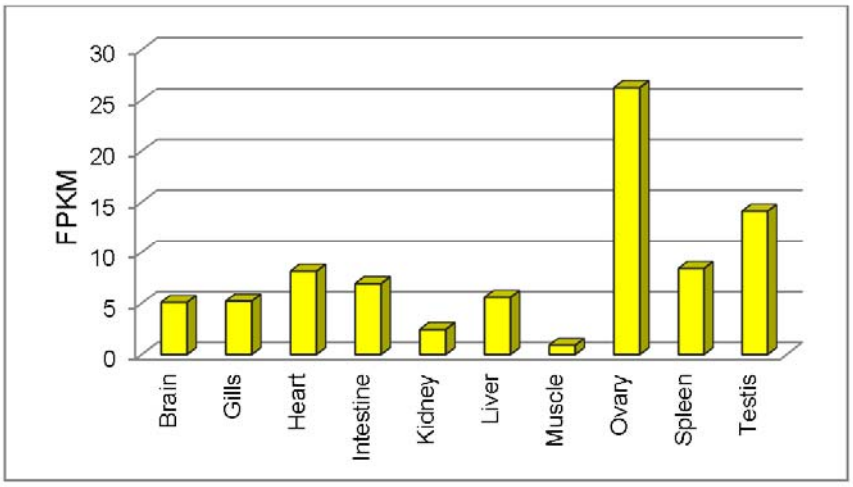

Figure 6. Expression level of MR gene in 10 tissues of elephant shark estimated based on RNA-seq data.

Transcript abundances are shown in terms of normalized counts called Fragments per kilobase of exon per million fragments mapped (FPKM) (B. Li & Dewey, 2011). FPKM is estimated by normalizing gene length followed by normalizing for sequencing depth.

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. Importantly, the elephant shark genome is evolving slowly (Venkatesh et al., 2014), making it attractive for studying ancestral proteins, including the MR, which first appears as a distinct MR ortholog in cartilaginous fish (Baker & Katsu, 2017; Bridgham et al., 2006; Carroll et al., 2008; Rossier et al., 2015).

This is the first report of transcriptional activation of a full-length cartilaginous fishes MR by a panel of five corticosteroids and Prog, 19norProg, 17OH-Prog and Spiron. Full-length elephant shark MR has EC50s below 1 nM for five corticosteroids, (Figure 4, Table 1) including Aldo, a steroid that has not been found in cartilaginous fish. Prog, 19norProg and Spiron also have sub-nM EC50s for full-length elephant shark MR. In addition to their low EC50s, all of the

corticosteroids and 19norProg have similar fold activation of transcription of full-length MR (Figure 4). Thus, several corticosteroids, as well as 19norProg and Prog, are potential physiological mineralocorticoids for elephant shark MR.

Compared to full-length elephant shark MR, the EC50s of all five corticosteroids and 19norProg are lower for its truncated MR, while the EC50 for Spiron is slightly lower, and the EC50s for Prog and 17OH-Prog are about 2 fold higher (Table 1, Table 2). Compared to fold activation by Aldo, the responses to DOC, S, Prog and 17OH-Prog are similar for full-length and truncated elephant shark MR. However, the relative responses to B and F of truncated elephant shark MR are 10% and 35% lower, respectively, (Figure 4, Table 1) and for 19norProg and Spiron, 14% and 8% higher respectively, than for full-length elephant shark MR (Figure 4, Table 2). Regarding truncated skate MR, most of the EC50s of corticosteroids (Carroll et al., 2008) are similar to that for elephant shark MR (Table 1). The exception is S, which has over 200-fold higher EC50 for skate MR than for elephant shark MR.

Corticosteroid and progestin activation of full-length and truncated elephant shark MR

Comparison of transcriptional activation by corticosteroids and progestins of full-length and truncated elephant shark MR with full-length and truncated human, chicken, alligator, Xenopus and zebrafish MRs (Table 1) provides insights into the evolution of regulation of steroid-mediated transcription of these MRs by allosteric interactions between the NTD/DBD and LBD. Like elephant shark MR, truncated terrestrial vertebrate MRs and zebrafish MR have similar EC50s for Aldo, B and F as their full-length counterparts (Table 1). However, the response of truncated human, alligator and Xenopus MRs to S did not saturate at 1 μM, preventing us from determining the EC50 and explaining the low fold activation by S (Table 1). Interestingly, truncated chicken and zebrafish MRs, which are activated by progestins, have nM EC50s for S, although fold activation by S is lower than that of Aldo. The similar activity of truncated human, alligator and Xenopus MRs to Aldo, B and F indicates that allosteric interactions between the NTD and LBD are steroid-selective. Overall, it appears that corticosteroid activation, especially by S, of elephant shark MR is less sensitive to the loss of NTD/DBD than are human, chicken, alligator and Xenopus MRs.

Progestin activation of full-length and truncated chicken and zebrafish MRs

Transcriptional activation by progestins of chicken and zebrafish MRs differs from that of elephant shark MR, especially for their truncated MRs. Although Prog, 19norProg and Spiron have nM EC50s for transcriptional activation of full-length

chicken and zebrafish MRs, at 1 µM Prog 19norProg and Spiron transcriptional activation of truncated chicken MR did not saturate, and at 1 µM Spiron transcriptional activation of zebrafish MR did not saturate (Figure 5, Table 2). Fold activation compared to Aldo by all progestins and Spiron is substantially lower for truncated chicken MR and for Spiron activation of zebrafish MR. However, Prog and 19norProg have EC50s of 98 nM and 64 nM, respectively, for truncated zebrafish MR. In this respect, truncated zebrafish MR is more similar to truncated elephant shark MR than is truncated chicken MR. These data indicate that transcriptional activation by progestins of zebrafish MR is closer to that of elephant shark MR than to chicken MR, in which progesterone activation of the MR appears to have evolved independently.

Progestins may be mineralocorticoids in cartilaginous fishes

Prog is a precursor for the synthesis of the other corticosteroids (Baker & Katsu, 2017; Payne & Hales, 2004). Two parsimonious metabolites of progesterone are DOC (21-hydroxyprogesterone) and 19norProg (Figure 2), which have EC50s, 0.063 nM and 0.043 nM, respectively, for elephant shark MR. These are the two lowest EC50s among the tested steroids. Interestingly, 19norProg evokes a stronger response from elephant shark MR than Aldo (Figure 4A, B). C19 demethylase activity has been found in mammalian kidney (Funder & Adam, 1981). If C19 demethylase is present in elephant shark, then 19norProg needs to be considered as a potential physiological mineralocorticoid.

The strong response to 19norProg is interesting because *in vivo* studies in rats revealed that 19norProg is at least a 100-fold weaker MR agonist than Aldo (Funder & Adam, 1981), while in transfected cells, at 1 nM, 19norProg is an antagonist (Geller et al., 2000; Sugimoto et al., 2016). Unexpectedly, compared to Aldo, 19norAldo has less than 1% binding affinity for rat MR (Wynne, Mercer, Stockigt, & Funder, 1980) indicating that the mechanism by which C19 demethylation of Prog increases this steroid's transcriptional activity for elephant shark MR is likely to be complex.

We propose that transcriptional activation of elephant shark MR by 19norProg, as well as by Prog and Spiron, can be explained by a mechanism based on Geller et al.'s (Geller et al., 2000) discovery that S810L human MR mutant is transcriptionally activated by 1 nM Prog, 19norProg, and Spiron, unlike wild-type MR, in which these steroids are MR antagonists. Geller et al. used a 3D model of S810L MR and transcriptional analysis of a series of mutations at Ser-810 (helix 5) and Ala-773 (helix 3) to propose that a van der Waals contact between Leu-810 and Ala-773 was sufficient for transcriptional activation of S810L MR by progestins. In Figure 3C of Geller et al. (Geller et al., 2000), human S810M was activated by 19norProg. Elephant shark MR

and skate MR contain a methionine at this position and an alanine corresponding to Ala-773 (Figure7). Based on Geller et al.'s model, we propose that transcriptional activation of elephant shark MR by 19norProg is due to a van der Waals contact between Met-782 (helix 5) and Ala-745 (helix 3), which stabilizes the A ring of 19norProg, promoting transcriptional activation.

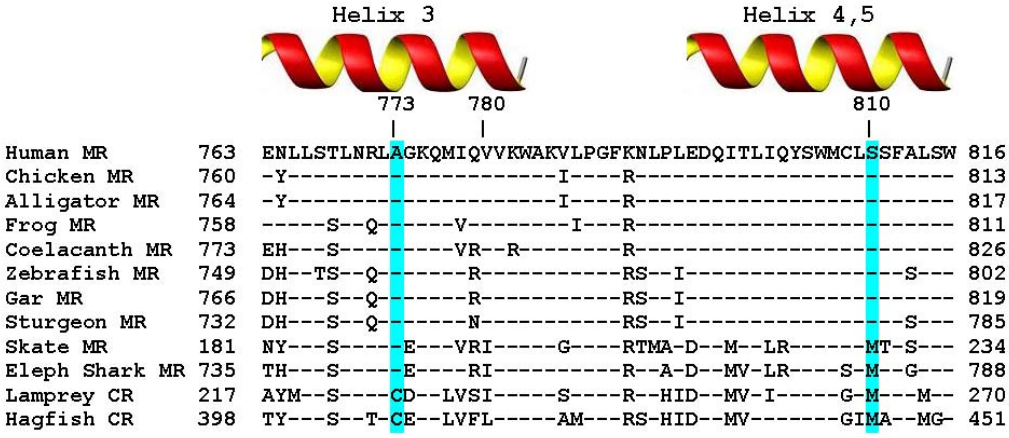

Figure 7. Alignment of elephant shark MR to Serine-810 and Alanine-773 in helices 3-5 in human MR.

Elephant shark MR and skate MR contain a methionine corresponding to human Ser-810 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. Human Ser-810 and Ala-773 are conserved in coelacanths, terrestrial vertebrate and ray-finned fish MRs. Amino acids that are identical to amino acids in human MR are denoted by (-).

Our results indicate that progestin activation of the MR is an ancient response. The timing of evolution of Prog as an MR antagonist is not known. One necessary, but not sufficient event, is the evolution in helix 5 of a serine corresponding to serine-810 in human MR. This occurs in coelacanth MR, which contains Ser-816, corresponding to human MR Ser-810 (Figure 7) and to elephant shark MR Met-782. This mutation in coelacanth MR would be expected to eliminate the proposed van der Waals contact between helix 5 and helix 3, leading to antagonist activity of Prog in some terrestrial

vertebrate MRs (Katsu et al., 2018). However, a serine corresponding to Ser-810 in human MR also is present in chicken MR and ray-finned fish MRs (Figure 8), indicating another mechanism is involved in Prog activation of the MR in these vertebrates.

Role for elephant shark MR in reproductive physiology

The evidence from RNA-seq analysis of high MR expression in ovary and testis (Figure 6) suggests that a Prog-MR complex is important in reproductive responses in elephant shark. Of course, Prog also acts as a reproductive steroid in ovary and testis via transcriptonal activation of the PR (Aquila & De Amicis, 2014; Conneely, Mulac-Jericevic, DeMayo, Lydon, & O'Malley, 2002). Based on evidence that Prog activates the MR in several ray-finned fishes (Pippal et al., 2011; Sturm et al., 2005; Sugimoto et al., 2016), a Prog-MR complex also may activate transcription in reproductive tissues and other organs in ray-finned fish, as well as cartilaginous fish.

RNA-seq analysis also finds MR expression in elephant shark gills and kidneys (Figure 6), two classical targets for MR regulation of electrolyte transport (Rossier et al., 2015). RNA-seq analysis also identifies MR expression in elephant shark heart and brain, two other organs in which corticosteroids have important physiological actions via the MR (de Kloet et al., 2016; DuPont & Jaffe, 2017; Funder, 2010; Gomez-Sanchez, 2011; Joels & de Kloet, 2017; Young & Rickard, 2015).

Materials and Methods

Chemical reagents

Aldo, F, B, DOC, S, Prog, 19norProg, 17OH-Prog and Spiron were purchased from Sigma-Aldrich. For reporter gene assays, all hormones were dissolved in dimethylsulfoxide (DMSO) and the final concentration of DMSO in the culture medium did not exceed 0.1%.

Construction of plasmid vectors

The full-coding regions from elephant shark MR were amplified by PCR with KOD DNA polymerase. The PCR products were gel-purified and ligated into pcDNA3.1 vector (Invitrogen) for the full-coding region or pBIND vector for D-E domains (Oka et al., 2013).

RNA-sequence analysis

The RNA-seq reads from following tissues of elephant shark were down-loaded from the Sequence Read Archive database of NCBI (accession number SRA054255): brain, gills, heart, intestine, kidney, liver, muscle, ovary, spleen, and testis. For each tissue, sequences were assembled *de novo* using Trinity, version r2013-08-14

(Grabherr et al., 2011).

Gene Expression Analyses

To determine the expression level of MR genes, we performed abundance estimation of transcripts from the afore mentioned 10 tissues. Trinity transcripts from all ten tissues and full-length cDNA sequence of the MR gene were combined together and clustered using CD-HITv4.6.1 at 100% identity (W. Li & Godzik, 2006). RNA-seq reads from each of the ten tissues was independently aligned to the clustered transcript sequences and abundance of MR gene transcripts was estimated by RSEMv1.2.25 (B. Li & Dewey, 2011) which uses bowtiev2.2.6 for aligning (Langmead & Salzberg, 2012). Transcript abundances were measured in terms of normalized counts called Fragments per kilobase of exon per million fragments mapped (FPKM) (B. Li & Dewey, 2011). FPKM is estimated by normalizing the gene length followed by normalizing for sequencing depth.

Transactivation Assay and Statistical Methods

Transfection and reporter assays were carried out in HEK293 cells, as described previously (Oka et al., 2015; Oka et al., 2013). All experiments were performed in triplicate. The values shown are mean ± SEM from three separate experiments, and dose-response data and EC50 were analyzed using GraphPad Prism. Comparisons between two groups were performed using *t*-test, and all multi-group comparisons were performed using one-way ANOVA followed by Bonferroni test. $P < 0.05$ was considered statistically significant.

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Author contributions: Y.K., S.K., K.O., X.L., S.O. carried out the research. Y.K. and S.K. analyzed data. W.T. and S.H. aided in the collection of animals. NEP assembled and analyzed RNAseq data. Y.K. and M.E.B. conceived and designed the experiments. Y.K., M.E.B. and B.V. wrote the paper. All authors gave final approval for publication.

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