Regulation by Progestins, Corticosteroids and RU486 of Activation of Elephant Shark and Human Progesterone Receptors: An Evolutionary Perspective

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Abstract.

We investigated progestin and corticosteroid activation of the progesterone receptor (PR) from elephant shark, a cartilaginous fish belonging to the oldest group of jawed vertebrates. Comparison with human PR provides insights into the evolution of steroid activation of human PR. At 1 nM steroid, elephant shark PR is activated by progesterone, 17-hydroxy-progesterone, 20β-hydroxy-progesterone, 11-deoxycorticosterone (21-hydroxyprogesterone) and 11-deoxycortisol. Human PR, in comparison, is activated at 1 nM steroid, only by progesterone and 11-deoxycorticosterone, indicating increased progestin and corticosteroid specificity during the evolution of human PR. RU486, an important clinical antagonist of human PR, did not inhibit progesterone activation of elephant shark PR. Cys-528 in elephant shark PR corresponds to Gly-722 in human PR, which is essential for RU486 inhibition of human PR. Confirming the importance of Cys-528 in elephant shark PR, RU486 inhibited progesterone activation of the Cys528Gly mutant PR. Compared to wild-type human PR, there was an increase in activation of human Gly722Cys PR by11-deoxycortisol and a decrease in activation by corticosterone, which may have been important

in selection for the mutation corresponding to human glycine-722 PR that first evolved in platypus PR, a basal mammal.

Key words: elephant shark PR, progesterone receptor evolution, progestins, corticosteroids, RU486

Running title: Evolution of steroid activation of elephant shark PR

Introduction

The progesterone receptor (PR) receptor belongs to the nuclear receptor family, a diverse group of transcription factors that also includes the glucocorticoid receptor (GR), mineralocorticoid receptor, androgen receptor (AR), and estrogen receptor (ER) (1–3). In humans, the progesterone receptor (PR) mediates progesterone regulation of female reproductive physiology in the uterus and mammary gland, including fertilization, maintenance of pregnancy and preparation of the endometrium for implantation and parturition (4–7). Moreover, progesterone has important physiological actions in males, including in the prostate and testes (8–11). Further, progesterone activates the PR in the brain, bone, thymus, lung and vasculature in females and males (12,13). Thus, progesterone is a steroid with diverse physiological activities in many organs in females and males.

Although activation by progesterone of the PR in chickens (4,14) , humans (15) , and zebrafish (16,17) has been examined, steroid activation of a PR in the more basal cartilaginous fish lineage has not been fully investigated. To remedy this omission, we studied the activation by a panel of progestins and corticosteroids (Figure 1) of the PR from the elephant shark (*Callorhinchus milii*), a cartilaginous fish belonging to the oldest group of jawed vertebrates, which diverged about 450 million years ago from bony vertebrates (18,19).

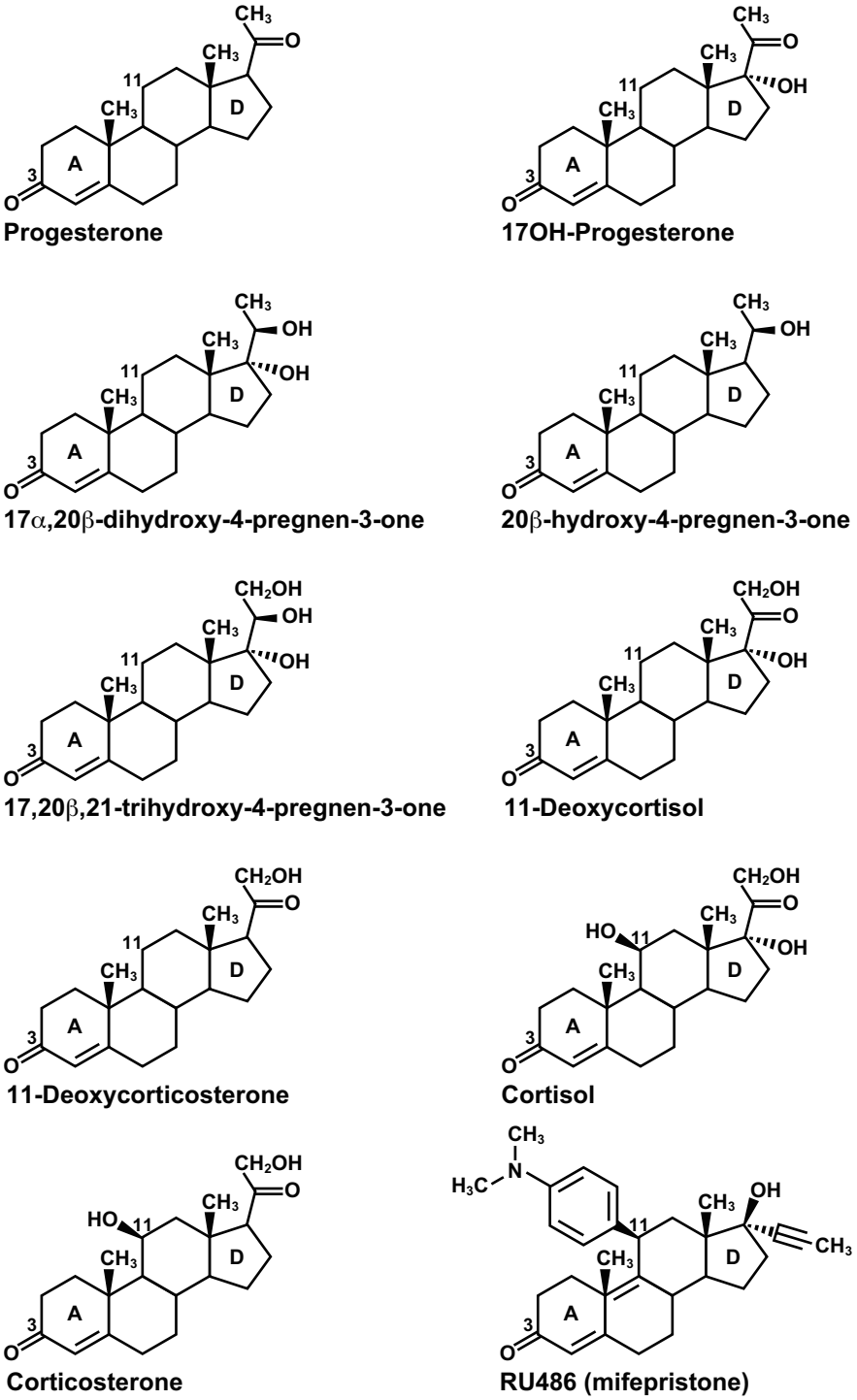

Figure 1. Structures of corticosteroids and progestins.

Progesterone is female reproductive steroid that also is important in male physiology (4,11). 17,20β-dihydroxy-progesterone is a maturation inducing hormone of teleost fish (20–22). 17,20β,21-trihydroxy-progesterone is a major ovarian steroid produced by the teleost fish

70 (23). Cortisol, corticosterone and 11-deoxycortisol are physiological glucocorticoids in
71 terrestrial vertebrates and ray-finned fish (24,25). 11-deoxycorticosterone is a
72 mineralocorticoid (25–28). RU486 is an antagonist of human PR (29,30).
73
74 Elephant shark PR is an attractive receptor to investigate the ancestral regulation of
75 steroid-mediated PR transcription because, in addition to its phylogenetic position as a
76 member of the oldest lineage of jawed vertebrates, genomic analyses reveal that elephant
77 shark genes are evolving slowly (19), making studies of its PR useful for studying ancestral
78 proteins, including the PR, for comparison for similarities and differences with human PR to
79 elucidate the evolution of steroid specificity for the PR in terrestrial vertebrates (19,31,32).
80 In addition, we were interested in the response of elephant shark PR to RU486
81 (Mifepristone), which is an antagonist for the human PR (29,30,33) and also a potential
82 anticancer drug for treating progesterone-dependent breast cancer (34).
83 We find that elephant shark PR is activated by progesterone, 17-hydroxy-
84 progesterone, 20β-hydroxy-progesterone, 17,20β-dihydroxy-progesterone, corticosterone,
85 11-deoxycorticosterone (21-hydroxy-progesterone) and 11-deoxycortisol. In contrast
86 human PR is activated only by progesterone, 20β-hydroxy-progesterone, 11-
87 deoxycorticosterone and corticosterone, indicating that human PR has increased specificity
88 for progestins and corticosteroids.
89 We also find that RU486 does not inhibit progesterone activation of elephant shark
90 PR. We show that this is due to cysteine-528 in elephant shark PR, which corresponds to
91 glycine-722 on human PR, an amino acid that Benhamou et al. (35) reported was essential for
92 antagonist activity of RU486. They found that mutation of glycine-722 to cysteine
93 abolished RU486 inhibition of progesterone activation of human PR. Analyses of vertebrate
94 PRs reveals that an ancestor of human PR-Gly722 first appeared in platypus, a basal mammal
95 (31).
96 To search for functional changes in human PR that correlate with the evolution of
97 RU486 antagonist activity, we constructed the elephant shark PR-Gly528 mutant and the
98 human PR-Cys722 mutant and studied their activation by several steroids. Elephant shark
99 PR-Gly528 had a weaker response to 11-deoxycortisol and 17-hydroxy-progesterone.
100 Human PR-Cys722 displayed increased activation by 11-deoxycortisol and decreased
101 activation by corticosterone. An altered response to one or more of these steroids may have
102 been selective for the evolution of an ancestor of glycine-722 in a PR in an ancestral platypus
103 at the base of the mammalian line.
104

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                                528
Elephant shark PR 520 LLTSLNRLCEKQLVSVVKWAKS 541
Human PR          714 --------G-R--L-----S-- 735
Mouse PR          707 ------Q-G-R--L-----S-- 728
Platypus PR       458 ------Q-G-R--L-----S-- 479
Chicken PR        567 ------H-C-R--LC----S-L 588
Xenopus PR        513 --S---Q-C-R--VC----S-- 534
Zebrafish PR      398 --N-----C-R--LWI-R-S-- 419
Coelacanth PR     544 ------Q-C-R--L-----S-- 565
Little skate PR    81 --------C-R--VA------- 102
Lamprey PR        345 --------C-R--VP------V 366
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Figure 2. Alignment of α helix-3, containing a key amino acid necessary for RU486 inhibition of human PR and activation of elephant shark PR.
Alignment of α-helix-3 in human PR, containing Gly-722 that is essential for RU486 inhibition of progesterone activation of human PR (35), the PR in elephant shark and other selected vertebrates. RU486 activates elephant shark PR, which contains cysteine-528 corresponding to human PR Gly-722. A glycine first appears in this position in platypus PR, a basal mammal. Amino acids that are identical to amino acids in elephant shark PR are denoted by (-).

Lastly, our studies provide an insight into the evolution of steroid activation of fish PRs. In fish, instead of progesterone, it is 17,20β-dihydroxy-progesterone that is the physiological ligand for the PR in zebrafish (16,17) and other teleosts (20–22,36,37). We find that for elephant shark PR, the half-maximal response (EC50) of progesterone and 17,20β-dihydroxy-progesterone are 0.18 nM and 2.6 nM, respectively. This ten-fold higher EC50 of elephant shark PR for 17,20β-dihydroxy-progesterone compared to progesterone, indicates that the during the evolution of ray-finned fish, there was a reversal between progesterone and 17,20β-dihydroxy-progesterone in their selectivity for elephant shark PR and for zebrafish PR and other fish PRs, and that this role for 17,20β-dihydroxy-progesterone, instead of progesterone, as a ligand for fish PR evolved after the divergence of ray-finned fish from cartilaginous fish (18,19).

128

129 **Materials and Methods**

130 **Chemical reagents**

131 Cortisol, corticosterone, 11-deoxycorticosterone, 11-deoxycortisol, progesterone,
132 17α-hydroxy-progesterone, 17,20β,21-tri-hydroxy-progesterone, 20β-hydroxy-progesterone,
133 and 17,20β-dihydroxy-progesterone were purchased from Sigma-Aldrich. RU486 was
134 purchased from Cayman Chemical. For reporter gene assays, all hormones were dissolved
135 in dimethyl-sulfoxide (DMSO); the final DMSO concentration in the culture medium did not
136 exceed 0.1%.

137

138 **Construction of plasmid vectors**

139 The full-length PRs were amplified by PCR with KOD DNA polymerase. The PCR
140 products were gel-purified and ligated into pcDNA3.1 vector (Invitrogen). Site-directed
141 mutagenesis was performed using KOD-Plus-mutagenesis kit (TOYOBO). All cloned DNA
142 sequences were verified by sequencing.

143

144 **Transactivation assay and statistical methods**

145 Transfection and reporter assays were carried out in HEK293 cells, as described
146 previously (38,39). All experiments were performed in triplicate. The values shown are
147 mean ± SEM from three separate experiments, and dose-response data, which were used to
148 calculate the half maximal response (EC50) for each steroid, were analyzed using GraphPad
149 Prism. Comparisons between two groups were performed using paired *t*-test. $P < 0.05$
150 was considered statistically significant. The use of HEK293 cells and an assay temperature
151 of 37C does not replicate the physiological environment of elephant sharks. Nevertheless,
152 studies with HEK293 cells and other mammalian cell lines have proven useful for other
153 studies of transcriptional activation by steroids of steroid hormone receptors from non-
154 mammalian species (39–41).

155

156 **Results**

157 **Transcriptional activation of full-length elephant shark PR by progestins and**
158 **corticosteroids.**

159 We screened a panel of steroids for transcriptional activation of full-length elephant
160 shark and human PRs using HEK293 cells. At 10 nM, progesterone, 17-hydroxy-
161 progesterone, 17, 20β-dihydroxy-progesterone, a fish maturation hormone, and 20β-hydroxy-
162 progesterone activated elephant shark PR (Figure 3A). At 10 nM, 11-deoxycorticosterone

163 (21-hydroxyprogesterone), 11-deoxycortisol and corticosterone activated elephant shark PR
164 (Figure 3C) indicating that elephant shark PR responds to corticosteroids.
165 At 10 nM steroid, human PR responded only to progesterone and 20β-hydroxy-
166 progesterone and not to the other progestins (Figure 3B). At 10 nM, 11-deoxycorticosterone
167 and corticosterone activated human PR (Figure 3D).
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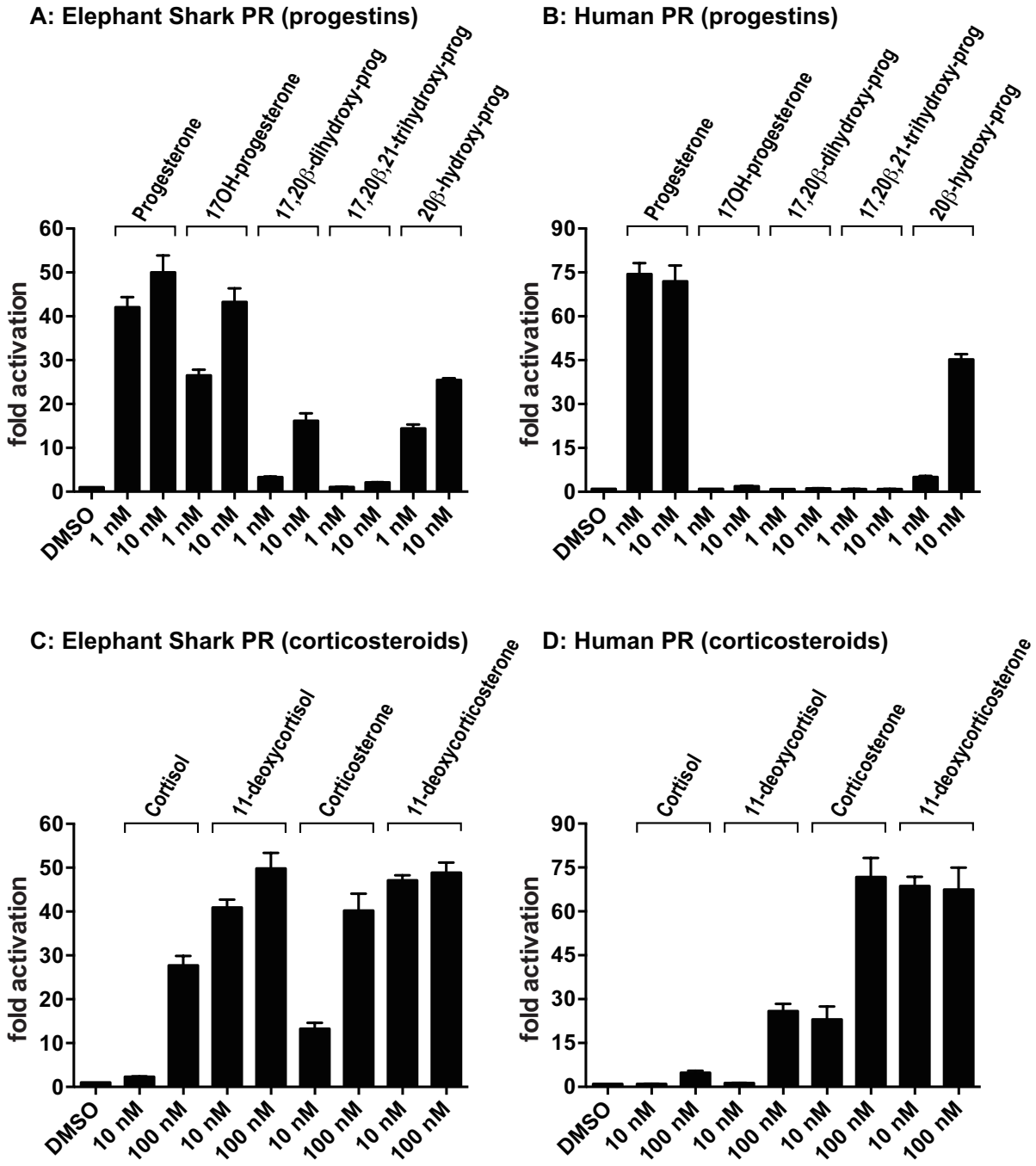

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170 **Figure 3. Transcriptional activation of elephant shark PR by progestins and**
171 **corticosteroids.**
172 Elephant shark PR (A and C) and human PR (B and D) were expressed in HEK293 cells with
173 an MMTV-luciferase reporter. Cells were treated with 1 and 10 nM progestins
174 (progesterone, 17OH-progesterone, 17,20β-dihydroxy-progesterone, 17,20β,21-trihydroxy-
175 progesterone and 20β-OH-progesterone), and 10 and 100 nM corticosteroids (cortisol, 11-
176 deoxycortisol, corticosterone, 11-deoxycorticosterone), or vehicle alone (DMSO). Results
177 are expressed as means ± SEM, n=3. Y-axis indicates fold-activation compared to the
178 activity by vehicle (DMSO) alone as 1.
179
180
181 **Concentration-dependent activation by progestins and corticosteroids of elephant shark**
182 **PR and human PR**.
183 To gain a quantitative measure of progestin and corticosteroid activation of elephant shark
184 PR and human PR, we determined the concentration dependence of transcriptional activation
185 by progestins and corticosteroids of elephant shark PR (Figure 4A, C) and for comparison
186 activation of human PR (Figure 4B, D). Progesterone, 17OH-progesterone, 17,20β-
187 dihydroxy-progesterone, 20β-OH-progesterone, 11-deoxycortisol, corticosterone and 11-
188 deoxycorticosterone activated elephant shark PR, while human PR was stimulated only by
189 progesterone, 20β-OH-progesterone, corticosterone and 11-deoxycorticosterone, a more
190 limited number of steroids.

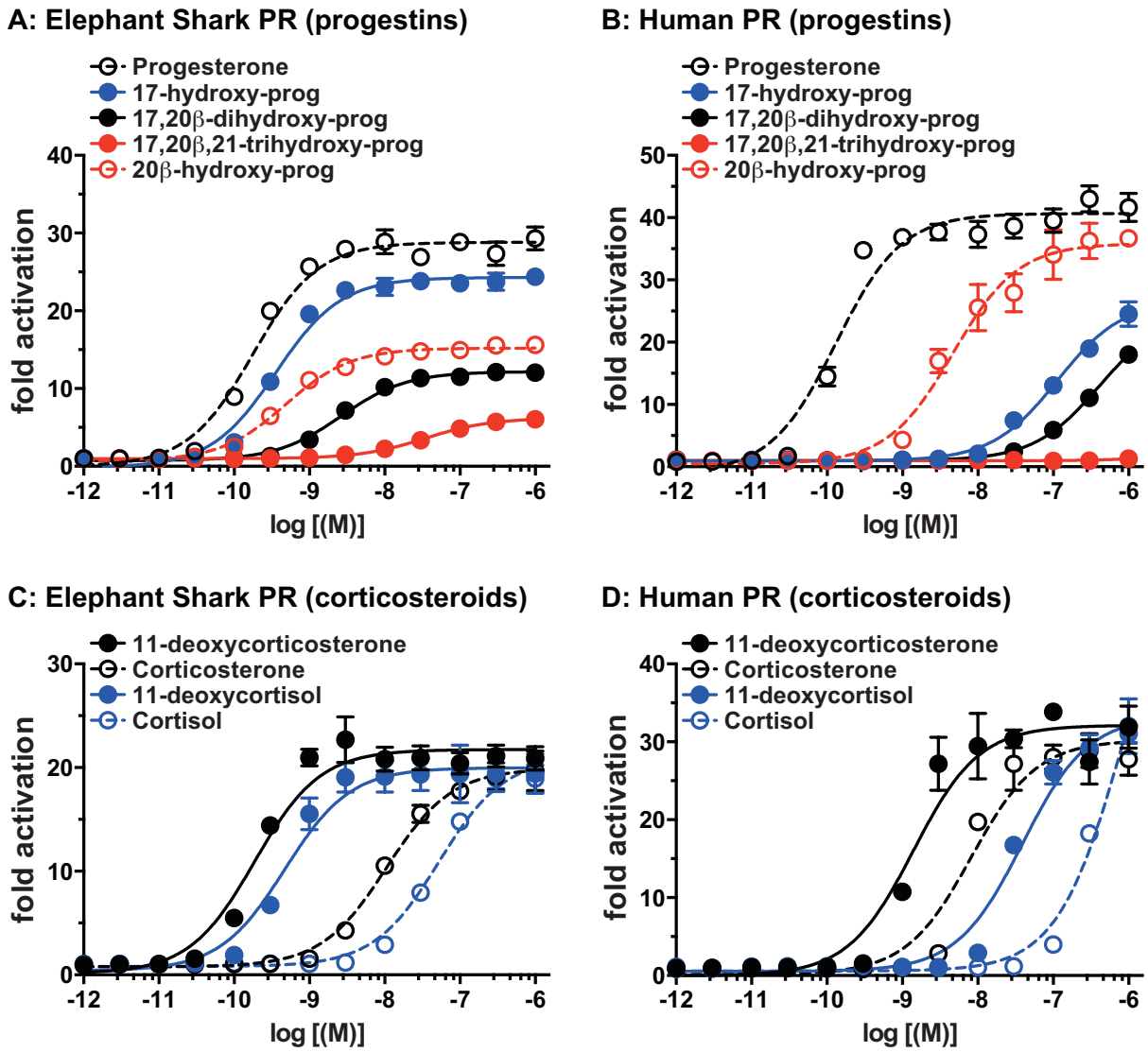

Figure 4. Concentration-dependent transcriptional activation of elephant shark and human PRs by corticosteroids and progestins.

Elephant shark PR (A and C) and human PR (B and D) were expressed in HEK293 cells with an MMTV-luciferase reporter. Cells were treated with increasing concentrations of corticosteroids (A and B), progestins (C and D) or vehicle alone (DMSO). Y-axis indicates fold-activation compared to the activity by vehicle (DMSO) alone as 1.

Table 1 summarizes the EC50s of progestins and corticosteroids for elephant shark PR and human PR. We find that elephant shark PR has low EC50s for progesterone (0.18 nM), 17-OH-progesterone (0.36 nM), 20β-OH-progesterone (048 nM), 17α,20β-OH-progesterone (2.6 nM), 11-deoxycortisol (0.47 nM) and 11-deoxycorticosterone (0.19 nM).

203 In contrast, human PR has low EC50s for progesterone (0.13 nM), 20β-OH-progesterone
204 (4.6 nM), 11-deoxycorticosterone (1.4 nM) and corticosterone (8.2 nM).
205
206 **RU486 does not inhibit transactivation of elephant shark PR**
207 Activation of human PR by progesterone is inhibited by RU486 (29,30,33). Indeed,
208 at 0.1 nM and 1 nM, RU486 inhibits of activation by 1 nM progesterone of human PR
209 (Figure 5A). Benhamou et al. (35) reported that Gly-722 in human PR, is essential for the
210 inhibition of progesterone activation of human PR by of RU486. We confirm that RU486
211 does not inhibit the human PR Cys722 mutant (Figure 5C).
212 Cys-528 of elephant shark PR corresponds to Gly-722 of human PR, which predicts
213 that RU486 would not inhibit progesterone activation of wild type elephant shark PR, and
214 indeed, as shown in Figure 5B, activation by 1 nM progesterone of elephant shark PR was
215 not inhibited by 100 nM RU486. As expected, RU486 inhibits progesterone activation of
216 elephant shark PR Gly528 (Figure 5D).
217
218

Table 1. EC50 values for steroid activation of elephant shark PR and human PR

Human Progestins

	Progesterone	17OH-Prog	20β-OH-Prog
	EC50 (M)	EC50 (M)	EC50 (M)
Elephant shark PR	0.18 nM	0.36 nM	0.48 nM
95% confidence intervals	0.14-0.22 nM	0.29-0.45 nM	0.41-0.57 nM
Human PR	0.13 nM	113.0 nM	4.6 nM
95% confidence intervals	0.094-0.18 nM	87.3-146 nM	3.2-6.6 nM

Fish Progestins

	17α,20β-DP	20β-S
	EC50 (M)	EC50 (M)
Elephant shark PR	2.6 nM	34.4 nM
95% confidence intervals	2.2-3.2 nM	29.6-40.2 nM
Human PR	408 nM	-
95% confidence intervals	342-487 nM	-

Corticosteroids

	Cortisol	11-deoxycortisol	Corticosterone	DOC
	EC50 (M)	EC50 (M)	EC50 (M)	EC50 (M)
Elephant shark PR	52.4 nM	0.47 nM	10.5 nM	0.19 nM
95% confidence intervals	44.3-62 nM	0.35-0.63 nM	9.0-12.3 nM	0.13-0.26 nM
Human PR	826 nM	39.0 nM	8.2 nM	1.4 nM
95% confidence intervals	516-1322 nM	30-51 nM	5.5-12.1 nM	0.9-2.2 nM

Human Progestins: 17OH-Prog = 17α-hydroxy-progesterone,

20β-OH-Prog = 20β-hydroxy-progesterone,

Fish Progestins: 17α,20β-DP = 17α,20β-dihydroxy-4-pregnene-3-one,

20β-S = 17α,20β,21-trihydroxy-4-pregnen-3-one,

Corticosteroids: DOC = 11-deoxycorticosterone,

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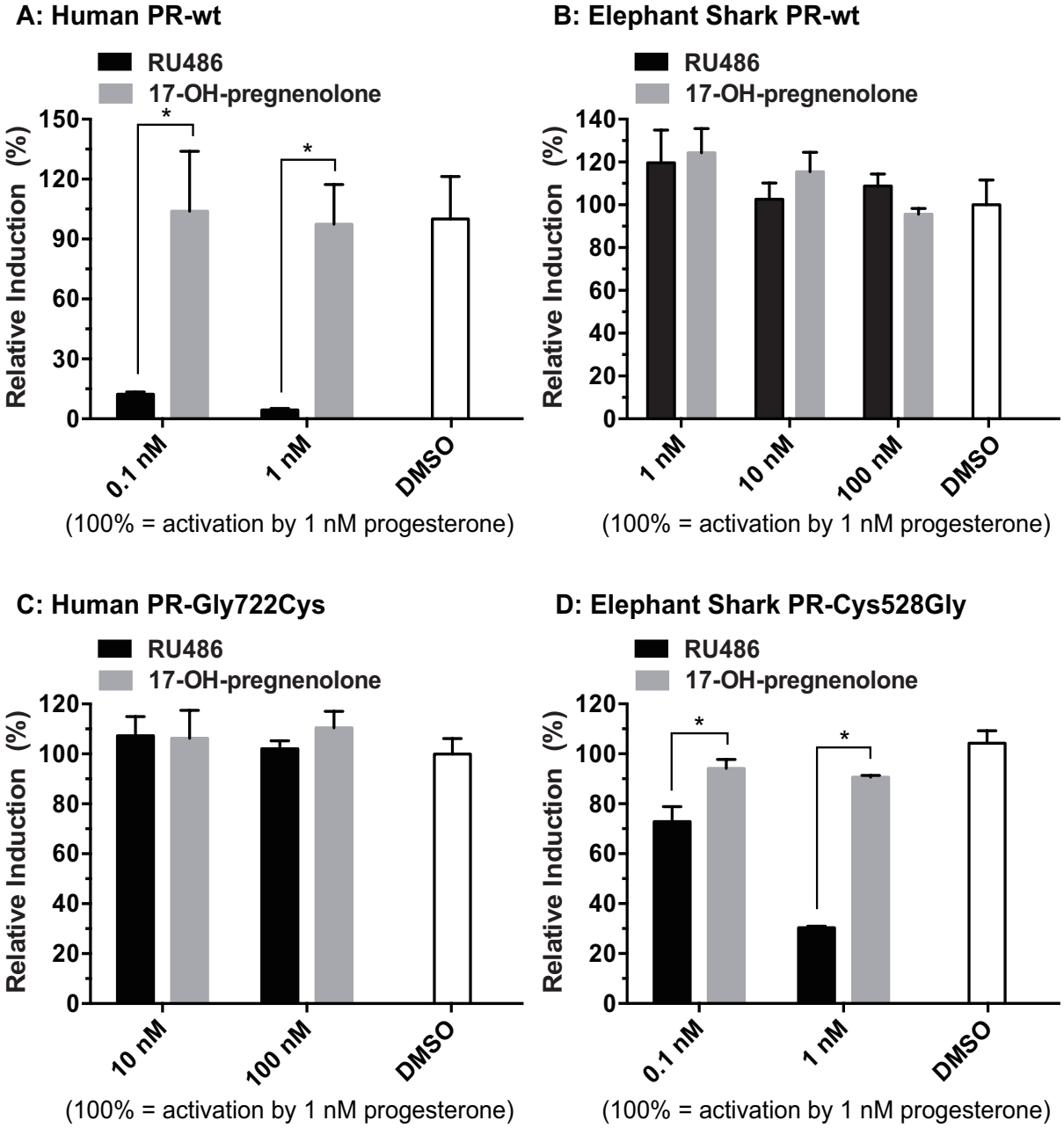

Figure 5. Effect of RU486 for Prog-induced activation of PR.

Wild-type of human PR (A) or elephant shark PR (B) was expressed in HEK293 cells with an MMTV-luciferase reporter. Cells with human PR were treated with 1 nM progesterone and either 0.1 nM or 1 nM RU486, 17OH- pregnenolone or DMSO. Cells with elephant shark PR were treated with 1 nM progesterone and with either 1 nM, 10 nM or 100 nM RU486, 17OH-pregnenolone or DMSO. Human PR-Gly722Cys (C) or elephant shark PR-Cys528Gly (D) was expressed in HEK293 cells with an MMTV-luciferase reporter. Cells

230 with human PR-Gly722Cys were treated with 1 nM progesterone and with either 10 nM or
231 100 nM RU486, 17OH-pregnenolone or DMSO. Cells with elephant shark PR-Cys528Gly
232 were treated with 1 nM progesterone and with either 0.1 nM or 1 nM RU486 or 17OH-
233 Pregnenolone Relative inductions were normalized between 0 and 100%, where 0 and 100
234 were defined as the bottom and tip value in vehicle-treated and 1 nM progesterone treated,
235 respectively. Results are expressed as means ± SEM, n=3. * $P < 0.05$ compared with
236 vehicle treatment (student's *t*-test).
237
238
239 **Steroid activation of human PR Gly722Cys and elephant shark PR Cys528Gly.**
240 To search for a biological basis for the functional changes in human PR due to Gly-
241 722 in human PR we constructed a human PR-Cys722 mutant and an elephant shark PR-
242 Gly528 mutant and studied their activation by various progestins and corticosteroids (Figure
243 6). Elephant shark PR-Gly528 had a weaker response to 11-deoxycortisol and 17-hydroxy-
244 progesterone. Human PR-Cys722 displayed increased activation by 11-deoxycortisol and
245 decreased activation by corticosterone.
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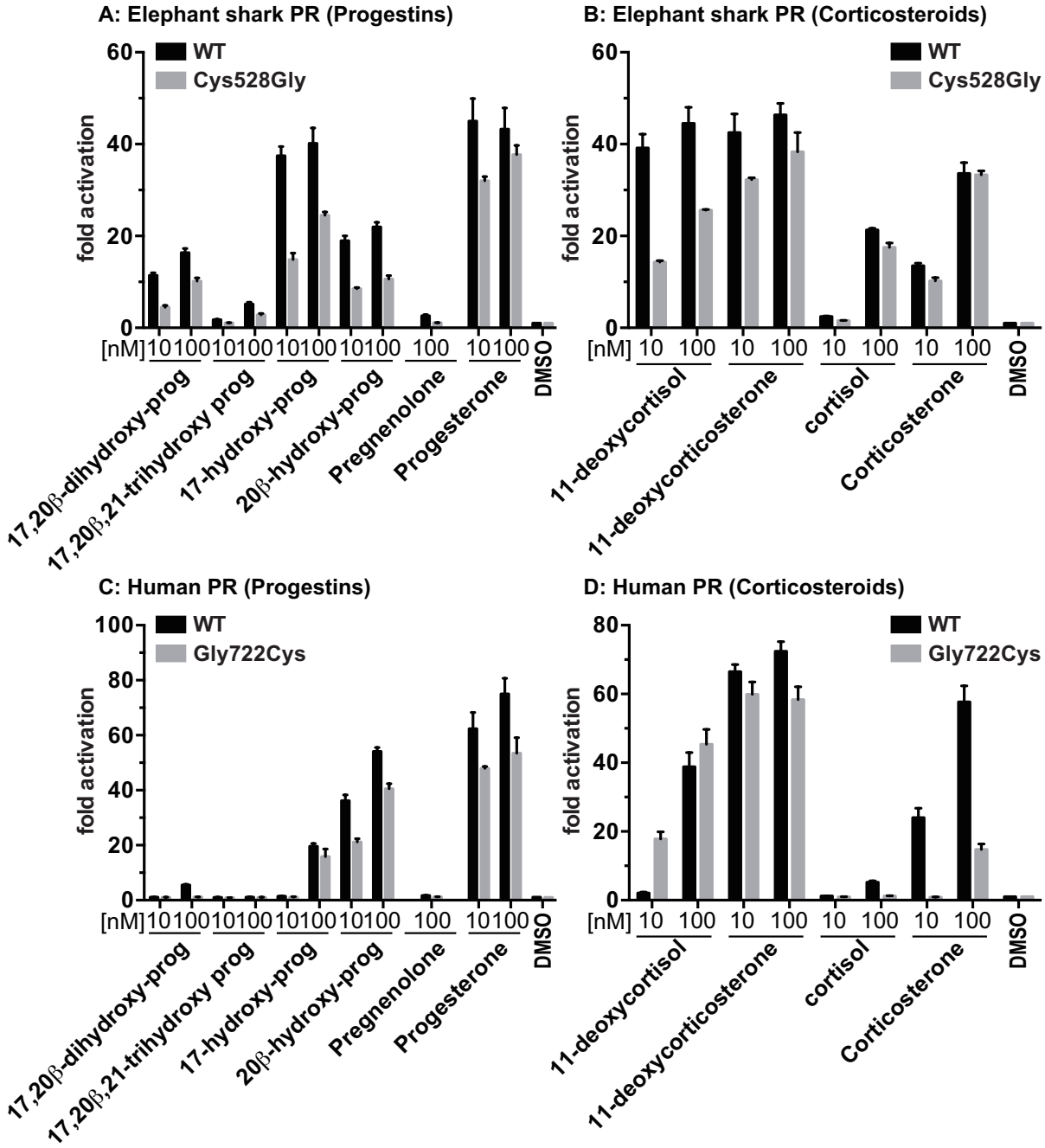

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248 **Figure 6. Progestin and corticosteroid activation of elephant shark PR-Cys528Gly and**
249 **human PR-Gly722Cys.**

250 Elephant shark PR (A and C), and human PR (B and D) were expressed in HEK293 cells
251 with an MMTV-luciferase reporter. Cells transfected with PRs were treated with increasing
252 concentrations of Prog or vehicle alone (DMSO) (A and B). Cells were treated with 10 nM
253 progestins (Prog, 17OH-Progesterone, pregnenolone 17,20β-dihydroxy-progesterone,
254 17,20β,21-trihydroxy-progesterone, 20β-OH-progesterone), corticosteroids (cortisol, 11-
255 deoxycortisol, corticosterone, 11-deoxycorticosterone), or vehicle alone (DMSO) (C and D).

256 Results are expressed as means ± SEM, n=4. Y-axis indicates fold-activation compared to
257 the activity of control vector with vehicle (DMSO) alone as 1.
258
259 **Concentration-dependent transcriptional activation of elephant shark PR-Cys528Gly**
260 **and human PR-Gly722Cys**.
261 To gain a quantitative measure of progestin and corticosteroid activation of the
262 cysteine-528 to glycine-528 mutation in elephant shark PR and the glycine-722 to cysteine-
263 722 mutation in human PR, we determined the concentration dependence of transcriptional
264 activation by progestins and corticosteroids of Cys528Gly elephant shark PR (Figure 7A, C)
265 and for comparison activation of Gly722Cys human PR (Figure 7B, D). In a separate
266 experiment we investigated the response of Cys528Gly elephant shark PR and Gly722Cys
267 human PR to corticosterone (Figure E, F).
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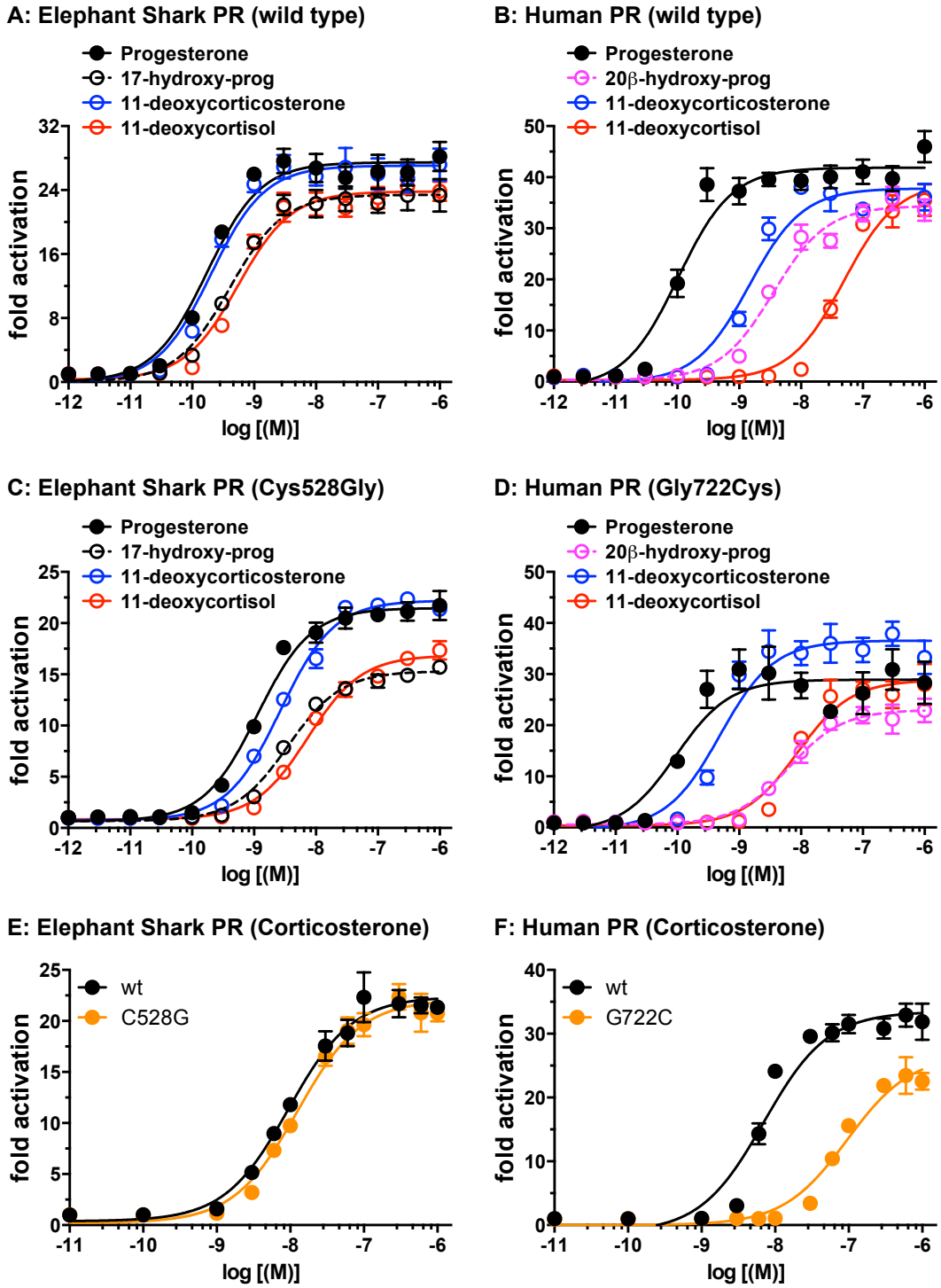
A: Elephant Shark PR (wild type)
Progesterone
17-hydroxy-prog
11-deoxycorticosterone
11-deoxycortisol
fold activation
log [(M)]
B: Human PR (wild type)
Progesterone
20β-hydroxy-prog
11-deoxycorticosterone
11-deoxycortisol
fold activation
log [(M)]
C: Elephant Shark PR (Cys528Gly)
Progesterone
17-hydroxy-prog
11-deoxycorticosterone
11-deoxycortisol
fold activation
log [(M)]
D: Human PR (Gly722Cys)
Progesterone
20β-hydroxy-prog
11-deoxycorticosterone
11-deoxycortisol
fold activation
log [(M)]
E: Elephant Shark PR (Corticosterone)
wt
C528G
fold activation
log [(M)]
F: Human PR (Corticosterone)
wt
G722C
fold activation
log [(M)]

276 **Figure 7. Concentration dependent transcriptional activation by progestins and**
277 **corticosteroids of elephant shark PR-Cys528Gly and human PR-Gly722Cys.**
278 Elephant shark PR (A, C and E), and human PR (B, D and F) were expressed in HEK293
279 cells with an MMTV-luciferase reporter. Cells transfected with PRs were treated with
280 increasing concentrations of progesterone, 20β-OH-progesterone, 11-deoxycortisol, 11-
281 deoxycorticosterone or corticosterone. Results are expressed as means ± SEM, n=4. Y-
282 axis indicates fold-activation compared to the activity of control vector with vehicle (DMSO)
283 alone as 1.
284
285 Table 2 shows the EC50s calculated from the curves in Figure 7. Progesterone, 20β-
286 OH-progesterone, 11-deoxycortisol, corticosterone and 11-deoxycorticosterone activated
287 elephant shark PR, while human PR was stimulated only by progesterone, 20β-OH-
288 progesterone, corticosterone and 11-deoxycorticosterone, a more limited number of steroids.
289 One or more of these changes may have been selective for the evolution an ancestor of
290 glycine-722 in a PR in an ancestral platypus at the base of the mammalian line.
291
292

Table 2. EC50 values for steroid activation of wild-type and Cys528Gly mutant elephant shark PR and Gly722Cys mutant human PR

Progestins

	Progesterone	17OH-Prog	20β-OH-Prog
	EC50 (M)	EC50 (M)	EC50 (M)
Elephant shark PR	0.17 nM	0.4 nM	-
95% confidence intervals	0.13-0.24 nM	0.29-0.56 nM	-
Elephant shark PR-Cys528Gly	1.1 nM	3.4 nM	-
95% confidence intervals	0.92-1.4 nM	2.8-4.0 nM	-
Human PR	0.1 nM	-	3.5 nM
95% confidence intervals	0.07-0.15 nM	-	2.6-4.7 nM
Human PR-Gly722Cys	0.095 nM	-	6.4 nM
95% confidence intervals	0.05-0.2 nM	-	4.4-9.3 nM

Corticosteroids

	DOC	11-deoxycortisol	Corticosterone
	EC50 (M)	EC50 (M)	EC50 (M)
Elephant shark PR	0.2 nM	0.55 nM	9.4 nM
95% confidence intervals	0.14-0.28 nM	0.41-0.74 nM	7.0-12.7 nM
Elephant shark PR-Cys528Gly	2.7 nM	7.4 nM	12.5 nM
95% confidence intervals	2.3-3.1 nM	6.2-8.9 nM	9.4-16.5 nM
Human PR	1.4 nM	49.2 nM	7.0 nM
95% confidence intervals	1.0-2.1 nM	36-68 nM	4.8-10.2 nM
Human PR-Gly722Cys	0.5 nM	8.7 nM	95.0 nM
95% confidence intervals	0.32-0.76 nM	5.7-13.4 nM	67-134 nM

Progestins: 17OH-Prog = 17α-hydroxy-progesterone, 20β-OH-Prog = 20β-hydroxy-progesterone.

293 **Corticosteroids: DOC = 11-deoxycorticosterone.**

294

295

296 **Discussion**

297 An ortholog of human PR along with the corticoid receptor (CR), the ancestor of the
298 MR and GR, first appears in the more ancient cyclostomes (jawless fish), which has
299 descendants in modern lamprey and hagfish (3,27,42). It is in cartilaginous fish that distinct
300 orthologs of human MR and GR first appear (43–45) along with the beginning of the
301 evolution of differences in the responses of the PR, MR and GR for progestins and
302 corticosteroids that appear in terrestrial vertebrates and ray-finned fish (16,17,24,40,41,43–
303 52).

304 Here we report that elephant shark PR has a strong response to progesterone (EC50
305 0.18 nM) and 17-OH-progesterone (EC50 0.36 nM), as well as to 11-deoxycorticosterone
306 (EC50 0.19 nM) (Figure 4, Table 1), a corticosteroid with close structural similarity to
307 progesterone (Figure 1). Elephant shark PR also is activated by 20β-OH-progesterone
308 (EC50 0.48 nM) and 17,20β-dihydroxy-progesterone (EC50 2.6 nM) and 11-deoxycortisol
309 (EC50 0.47 nM). This broad response to steroids contrasts with the selectivity of human
310 PR, which has a strong response to progesterone (EC50 0.13 nM) and 11-
311 deoxycorticosterone, (EC50 1.4 nM) and a weaker response to corticosterone, (EC50 8.2
312 nM). The advantage this selectivity of human PR is not known.

313 The evolution of the response of human PR to RU486 is intriguing because RU486 is
314 not a physiological ligand for the PR. The glycine-722 in human PR (Figure 2) that confers
315 antagonist activity for RU486 towards human PR first appears in platypus, a basal mammal
316 (Figure 2). Human PR with Cys722, to mimic an ancestral PR, had increased activation by
317 11-deoxycortisol and decreased activation by corticosterone.

318 The lower activation of elephant shark PR by 17,20β-dihydroxy-progesterone
319 compared to progesterone is intriguing because in zebrafish (16,17), this is reversed with
320 higher activity for 17,20β-dihydroxy-progesterone compared to progesterone indicating that
321 during the evolution of ray-finned fish the response of the PR to progesterone diminished and
322 the response to 17,20β-dihydroxy-progesterone increased (21,22,36,37). The absence of
323 progesterone as a ligand for ray-finned fish PR may be relevant for progesterone functioning
324 as a ligand for fish MR (39,41,45,51,53).

325

326 **Funding, Contributions and Competing Interests.**

327 **Funding:** This work was supported by Grants-in-Aid for Scientific Research from the
328 Ministry of Education, Culture, Sports, Science and Technology of Japan (19K067309 to
329 Y.K.), and the Takeda Science Foundation (to Y.K.). M.E.B. was supported by Research
330 fund #3096.

Author contributions: X.L. and Y.K. carried out the research and analyzed data. W.T. and S.H. aided in the collection of animals. S.I. provided steroids used in this study. Y.K. and M.E.B. conceived and designed the experiments. X.L., Y.K. and M.E.B. wrote the paper. All authors gave final approval for publication.

Competing Interests: We have no competing interests.

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