

**Aldosterone postnatally, but not at birth, is required for optimal induction of renal mineralocorticoid receptor expression and sodium reabsorption.**

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1 **Aldosterone postnatally, but not at birth, is required for optimal induction**  
2 **of renal mineralocorticoid receptor expression and sodium reabsorption**

3

4 **Abbreviated Title: Aldosterone regulation of renal MR expression**

5 Laetitia Martinerie<sup>1,2,6</sup>, Say Viengchareun<sup>1,2</sup>, Geri Meduri<sup>1,3</sup>, Hyung-Suk Kim<sup>4</sup>, James M.  
6 Luther<sup>5</sup>, Marc Lombès<sup>1,2,6,7</sup>

7 <sup>1</sup> *Inserm, U693, 63 rue Gabriel Péri, Le Kremlin-Bicêtre, F-94276, France;*

8 <sup>2</sup> *Univ Paris-Sud, Faculté de Médecine Paris-Sud, UMR-S693, Le Kremlin Bicêtre, F-94276,*  
9 *France;*

10 <sup>3</sup> *Assistance Publique-Hôpitaux de Paris, Hôpital Bicêtre, Service de Génétique moléculaire,*  
11 *Pharmacogénétique et Hormonologie, Le Kremlin Bicêtre, F-94275, France;*

12 <sup>4</sup> *Department of Pathology, University of North Carolina, Chapel Hill, NC, USA;*

13 <sup>5</sup> *Division of Clinical Pharmacology, Vanderbilt University Medical Center, Nashville, TN,*  
14 *USA;*

15 <sup>6</sup> *Assistance Publique-Hôpitaux de Paris, Hôpital Bicêtre, Service d'Endocrinologie et*  
16 *Maladies de la Reproduction, Le Kremlin Bicêtre, F-94275, France;*

17 <sup>7</sup> *PremUp Foundation, Paris, F-75006, France.*

18

19 **\* Corresponding author:**

20

21 Dr Marc LOMBES, MD, PhD  
22 Inserm U693, Faculté de Médecine Paris-Sud  
23 63, rue Gabriel Péri, 94276 Le Kremlin Bicêtre Cedex France  
24 Tel 33 1 49 59 67 09  
25 Fax 33 1 49 59 67 32  
26 Email: [marc.lombes@u-psud.fr](mailto:marc.lombes@u-psud.fr)

27

28 Reprint request should be addressed to Dr Marc LOMBES

29

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31

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33

34 **Precis:** Aldosterone is dispensable at birth but rather crucial in the postnatal period for  
35 optimal induction of the mineralocorticoid signaling pathway and for sodium homeostasis.

36

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39

40 **Abstract**

41 Sodium wasting during the neonatal period is the consequence of a physiological aldosterone  
42 resistance, related to a low renal mineralocorticoid receptor (MR) expression at birth, both in humans  
43 and mice. To investigate whether aldosterone is involved in the neonatal regulation of MR expression,  
44 we compared aldosterone and corticosterone levels and renal MR expression by quantitative real time  
45 PCR, between aldosterone-synthase knock-out (ASKO), heterozygous and wild type (WT) mice, at  
46 birth (D0) and eight days postnatal (D8). Analysis of MR transcripts showed a similar expression  
47 profile in all genotypes, demonstrating that the lack of aldosterone does not modify either the low  
48 renal MR expression at birth or its postnatal induction. However, mRNA levels of the  $\alpha$  subunit of  
49 the epithelial sodium channel (ENaC), a MR target gene, were significantly higher in WT compared to  
50 ASKO mice, both at D0 and D8, despite high corticosterone levels in ASKO mice, indicating that  
51 aldosterone is required for optimal renal induction of ENaC. Using organotypic cultures of newborn  
52 WT kidneys, we confirmed that aldosterone does not regulate MR expression at birth, but is instead  
53 capable of increasing MR expression in mature kidneys, unlike dexamethasone. In sum, we  
54 demonstrate both *in vivo* and *in vitro*, that, whereas aldosterone has no significant impact on renal MR  
55 expression at birth, it is crucial for optimal MR regulation in postnatal kidneys and for appropriate  
56 hydroelectrolytic balance. Understanding of MR regulatory mechanisms could therefore lead to new  
57 therapeutic strategies for the management of sodium loss in preterms and neonates.

58

## 59 **Introduction**

60 In the neonatal period, the human kidney displays a tubular immaturity, with associated sodium  
61 wasting, negative sodium balance, and impaired water reabsorption (1). This inability to maintain  
62 homeostatic function is accentuated in preterm infants (2-4) and represents a critical problem that  
63 pediatricians have to deal with. Therefore, a better understanding of water and sodium regulation  
64 during this specific developmental period is of major importance in order to propose new therapeutic  
65 strategies for the management of preterm infants.

66 Renal sodium reabsorption is mainly controlled by aldosterone, a steroid hormone synthesized in the  
67 adrenal gland zona glomerulosa, secondary to renin stimulation *via* angiotensin II and to potassium  
68 stimulation (5). In the distal nephron, aldosterone, by binding to its receptor, the mineralocorticoid  
69 receptor (MR), a transcription factor (6), tightly regulates the expression and activity of several  
70 transporting proteins implicated in sodium homeostasis, including the alpha subunit of the epithelial  
71 sodium channel ( $\alpha$ ENaC) (7). The selectivity of the mineralocorticoid signaling pathway in the  
72 epithelial cells is controlled at a prereceptor level by the 11 $\beta$ -hydroxysteroid dehydrogenase type 2  
73 enzyme (11 $\beta$ HSD2) which metabolizes cortisol (or corticosterone in rodents) into inactive  
74 compounds, incapable of MR binding (8, 9). We have previously demonstrated that the neonatal  
75 sodium wasting is related to a physiological transient renal aldosterone resistance (10). We have also  
76 established that this physiological aldosterone resistance is associated in both mice and humans with a  
77 low renal MR expression at birth, both at the mRNA and protein level (11). This low renal MR  
78 expression in newborns is followed by a significant increase in the postnatal period, with a complete  
79 renal tubular expression developed at 8 postnatal days in mice and during the first year of life in  
80 humans, paralleling renal maturation. The underlying mechanisms, responsible for the low renal MR  
81 expression at birth and its postnatal increase, are currently unknown. Since aldosterone levels are very  
82 high at birth and have a tendency to decrease in the postnatal period, mirroring MR expression (11),  
83 we hypothesized that these high hormonal levels could contribute to the low neonatal renal MR  
84 expression. To investigate the role of aldosterone on neonatal MR regulation, we utilized the  
85 aldosterone-synthase knock-out mouse model ( $AS^{-/-}$ ). These transgenic mice were originally generated

86 by Hyung-Suk Kim, using standard gene targeting methods, with a final construct lacking exons 1, 2,  
87 part of exon 3 and introns 1 and 2 of the AS gene (12), and have undetectable levels of plasma  
88 aldosterone. AS<sup>-/-</sup> genotype is compatible with fetal development but newborns fail to thrive  
89 postnatally and about 30% die between day 7 and 28 (12). Adult AS<sup>-/-</sup> mice are small, weigh 25% less  
90 than wild type animals, have low blood pressure, abnormal electrolyte homeostasis (increased plasma  
91 concentrations of K<sup>+</sup>, Ca<sup>2+</sup> and Mg<sup>2+</sup>, decreased concentrations of HCO<sub>3</sub><sup>-</sup> and Cl<sup>-</sup> but no difference of  
92 plasma Na<sup>+</sup> level under normal diet) and altered water metabolism (higher urine output, decreased  
93 urine osmolality, and impaired urine concentrating and diluting ability). Higher levels of plasma  
94 corticosterone and strong activation of the renin-angiotensin system are observed. In contrast, AS<sup>+/-</sup>  
95 mice have normal plasma aldosterone concentrations, normal blood pressure and no electrolyte  
96 disturbances (13). To evaluate whether renal MR expression in the neonatal period is dependent on  
97 aldosterone, we have quantified and compared MR mRNA steady state levels in the kidneys of AS<sup>-/-</sup>,  
98 AS<sup>+/-</sup> and wild type littermates, using quantitative real-time PCR. To determine whether maternally-  
99 derived aldosterone exposure *in utero* modifies MR expression at birth, we conducted two breeding  
100 strategies, utilizing pups derived from either AS<sup>-/-</sup> or AS<sup>+/-</sup> mice. We have also analyzed the expression  
101 of other actors of the mineralocorticoid (11βHSD2, αENaC) and glucocorticoid (GR) signaling  
102 pathways, and measured hormonal status. Our results were subsequently confirmed *in vitro*, using  
103 organotypic cultures of wild type newborn murine kidneys. We demonstrate that, whereas aldosterone  
104 is dispensable for MR regulation at birth, it is pivotal for optimal induction of renal MR expression  
105 and sodium homeostasis in the postnatal mature kidney, effects that cannot be compensated by the  
106 glucocorticoid signaling pathway.

107

## 108 **Materials and Methods**

109

### 110 *Animals and Breeding strategy*

111 All experiments were reviewed and approved by the Vanderbilt University Institutional Animal Care  
112 and Use Committee. AS<sup>-/-</sup> mice, previously described (12), were generated on a 129 background and  
113 backcrossed over  $\geq 15$  generations onto the C57Bl6/J strain (Jackson Laboratory, Bar Harbor, ME).  
114 Mice were genotyped by real-time PCR (Applied Biosystems 7900HT, Foster City, CA) using  
115 Taqman probes for a sequence in the *Cyp11b2* gene and for a portion of the gene contained in the  
116 neomycin resistance cassette, as previously described (14). Animals were housed in a temperature-  
117 controlled facility with a 12-hour light/dark cycle. AS<sup>-/-</sup> female mice were mated with AS<sup>-/-</sup> male mice  
118 in order to obtain AS<sup>-/-</sup> littermates. AS<sup>+/-</sup> female mice were mated with AS<sup>+/-</sup> male mice in order to  
119 obtain AS<sup>-/-</sup>, AS<sup>+/-</sup> and wild type littermates. Mice were sacrificed at birth and at post-natal day 8 by  
120 decapitation. Kidneys were immediately collected, snap-frozen in liquid nitrogen and stored at -80°C  
121 until analysis.

122

### 123 *Hormonal Analyses*

124 Trunk blood was collected into dipotassium-EDTA tubes (Microvette CB K2E, Sarstedt AG & Co),  
125 centrifuged at 6,000 rpm for 5 min, and plasma was stored at -80°C until assay. Aldosterone was  
126 determined as previously described (14) using a radioimmunoassay utilizing <sup>125</sup>I-aldosterone (MP  
127 Biomedicals, Irvine CA), a primary antibody to aldosterone (NIDDK National Hormone & Peptide  
128 Program, Torrance CA), and a secondary anti-rabbit gamma globulin antibody (Linco Research, St.  
129 Charles, MO). Corticosterone was measured using a commercially available radioimmunoassay kit  
130 (ImmuChem Double Antibody Corticosterone Kit, MP Biomedicals, Irvine, CA) as described in (14).

131

### 132 *Organotypic cultures*

133 Kidneys were collected from wild type mice on the day of birth (D0) or at postnatal day 8 (D8). Each  
134 D0 kidney was incubated for 15 min in 150  $\mu$ l of accutase (PAA Laboratories, Les Mureaux, France)  
135 and D8 kidneys were incubated for 1 h in 300  $\mu$ l of trypsin (Invitrogen, Cergy-Pontoise, France).

136 Then, kidneys were manually dissected with a needle. Homogenates were pooled and centrifuged for 3  
137 min at 300 g, supernatant was withdrawn and cellular pellet was resuspended in a specific epithelial  
138 medium described below. Cellular suspensions were then seeded on collagen I-coated 12-well plates  
139 (Collagen I from Institut Jacques Boy, France), and routinely cultured for seven days at 37°C in a  
140 humidified incubator gassed with 5% CO<sub>2</sub> within an epithelial medium composed of DMEM/HAM's  
141 F12 (1:1), 2 mM glutamine, 50 nM dexamethasone, 50 nM sodium selenite, 5 µg/ml transferrin, 5  
142 µg/ml insulin, 10 ng/ml EGF, 2 nM T3, 100 U/ml penicillin/streptomycin, 20 mM HEPES, pH 7.4, 5%  
143 dextran charcoal-treated serum and 1% amphotericin B (the latter added to the medium for the first  
144 48 h of culture only).

145 To investigate aldosterone and corticosteroid action, the epithelial medium was replaced at day 7 of  
146 culture by a minimal medium (MM), which has the same composition as the epithelial medium but  
147 lacks dexamethasone and dextran charcoal-treated serum. After 24 h in MM, either ethanol or  
148 aldosterone or dexamethasone, as well as spironolactone, were added to the medium for 24 h as  
149 indicated.

150

#### 151 *RT-PCR and Quantitative real-time PCR*

152 Total RNA was extracted from tissues or cells with the TRIZOL reagent (Invitrogen) according to the  
153 manufacturer's recommendations, and RNA was thereafter processed for RT-PCR, as previously  
154 described (11). One µg of total RNA, isolated from frozen samples, was subjected to DNase I  
155 Amplification Grade treatment (Invitrogen) and then reverse-transcribed by use of High-Capacity  
156 cDNA RT kit from Applied Biosystems (Courtaboeuf, France). Samples were diluted 10-fold then  
157 1/20 of the reverse transcription reaction was used for qRT-PCR using the Fast SYBR<sup>®</sup> Green Master  
158 Mix (ABI, Applied Biosystems) containing 300 nM of specific primers (supplemental Table). qPCR  
159 was carried out on a StepOnePlus<sup>™</sup> Real-Time PCR System (Applied Biosystems, Foster City, CA).  
160 Reaction parameters were as follows: 95°C for 20 s, then 40 cycles at 95°C for 1 s and 60°C for 20 s.  
161 For standards preparation, amplicons were purified from agarose gel and subcloned into pGEMT-easy  
162 plasmid (Promega, Charbonnières-les-Bains, France) and sequenced to confirm the identity of each  
163 sequence. Standard curves were generated using serial dilutions of linearized standard plasmids,



164 spanning 6 orders of magnitude. Standard and sample values were amplified in duplicate and analyzed  
165 from three independent experiments. Ribosomal 18S was used as an internal control for data  
166 normalization. Relative expression of a given gene is expressed as the ratio attomoles of specific  
167 gene/femtomoles of 18S. Results are mean  $\pm$  SEM and represent the relative expression compared  
168 with that obtained in AS<sup>-/-</sup> mice kidneys at D0, which was arbitrarily set at 1. Supplemental Table  
169 indicates primer sequences of genes analyzed by qRT-PCR.

170

### 171 *Immunocytochemistry*

172 Cells were fixed in 10% buffered-formol in PBS (pH 7.3) for 15 min and then washed 3 times in PBS  
173 before processing for immunocytochemistry, as previously described (11). For immunocytochemical  
174 analyses, we used the monoclonal anti-MR antibody clone 6G1, generously provided by Dr Gomez-  
175 Sanchez (University of Mississippi, USA) (15), at the dilution of 1:40.

176

### 177 *Statistical analyses*

178 Results are expressed as mean  $\pm$  SEM of at least three independent analyses with at least 6 samples for  
179 each developmental stage. Statistical analyses were performed using a non parametric Mann Whitney  
180 test (Prism4, Graphpad Software, Inc., San Diego, CA), with significant threshold at 0.05.

181

182

183 **Results**

184 *Breeding strategy*

185 We conducted a specific breeding strategy in order to obtain four different newborn mice with various  
186 genotypes and distinct developmental endocrine patterns. We obtained AS<sup>-/-</sup> pups that had never been  
187 exposed to aldosterone, by mating female AS<sup>-/-</sup> mice with AS<sup>-/-</sup> male mice. We obtained AS<sup>-/-</sup> pups  
188 that had been exposed to maternal aldosterone, through transplacental crossing (16), by mating AS<sup>+/-</sup>  
189 female mice with AS<sup>+/-</sup> male mice, which also generated AS<sup>+/-</sup> littermates and wild type newborn mice.  
190 No difference was observed between genotypes regarding weight at birth ( $1.23 \pm 0.07$ ,  $1.39 \pm 0.02$ ,  
191  $1.38 \pm 0.04$ ,  $1.42 \pm 0.11$  g, respectively), and at postnatal day eight ( $3.51 \pm 0.23$ ,  $3.54 \pm 0.19$ ,  $4.11 \pm$   
192  $0.17$ ,  $4.02 \pm 0.27$  g, respectively). Kidneys were collected at birth (D0) and at postnatal-day eight  
193 (D8). No renal structural abnormality was observed.

194

195 *Aldosterone and corticosterone levels*

196 Aldosterone and corticosterone levels were measured in AS<sup>-/-</sup> and wild type (WT) newborn mice at the  
197 time of kidney retrieval (Fig 1). As expected, aldosterone was undetectable in AS<sup>-/-</sup> mice both at D0  
198 and D8. In wild type mice, mean aldosterone level at birth was  $466 \pm 245$  pg/ml and decreased at D8  
199 ( $94 \pm 38$  pg/ml) although the difference did not reach significance ( $p=0.18$ ). Interestingly, we found, in  
200 AS<sup>-/-</sup> animals both at birth and at D8, very high levels of corticosterone ( $374 \pm 317$  and  $681 \pm 376$   
201 ng/ml, respectively) were detected in comparison to wild type mice of the same developmental stage,  
202 particularly at D8 ( $p=0.04$ ). Analogous high levels of corticosterone have also been reported in AS<sup>-/-</sup>  
203 adult mice on normal sodium diet (13). One could hypothesize that these high corticosterone levels are  
204 secreted to compensate for aldosterone deficiency.

205

206 *Mineralocorticoid receptor expression*

207 MR transcript levels were measured by quantitative RT-PCR in the kidneys of all four groups of  
208 animals at D0 and D8 (Fig 2). No difference was evident at birth between genotypes, therefore  
209 suggesting that the low renal MR expression during the neonatal period is independent of aldosterone  
210 exposure. In all conditions, a significant increase in renal MR mRNA expression level was observed in

211 all animals at D8, demonstrating that aldosterone does not intervene into the postnatal upregulation of  
212 renal MR expression. However, we found a significant difference ( $p<0.05$ ) in D8 state levels of MR  
213 transcripts between  $AS^{-/-}$  and WT mice, suggesting that aldosterone may regulate MR expression only  
214 in postnatal developed kidneys, when complete tubular maturation is achieved.

215

#### 216 *11 $\beta$ HSD2 and $\alpha$ ENaC expression*

217 We have previously demonstrated in WT mice (11), a parallel evolution throughout renal development  
218 between MR, and both the 11 $\beta$ HSD2 enzyme, which confers mineralocorticoid selectivity, and  
219  $\alpha$ ENaC, a prototypal MR target gene. Since corticosterone levels were very high in  $AS^{-/-}$  mice, we  
220 wondered whether the expression profiles of 11 $\beta$ HSD2 and  $\alpha$ ENaC would be modified in the four  
221 different genotypes. It has indeed been suggested that the glucocorticoid pathway could be implicated  
222 in the regulation of these two genes (17-19). The question raised was whether corticosterone could  
223 compensate for aldosterone deficiency in  $AS^{-/-}$  mice by inducing  $\alpha$ ENaC expression *via* binding to the  
224 glucocorticoid receptor (GR). The latter is a transcription factor closely related to MR and also known  
225 to activate gene expression implicated in sodium transport (18, 19). 11 $\beta$ HSD2 and  $\alpha$ ENaC mRNA  
226 expression were therefore analyzed at D0 and D8 in all genotypes. 11 $\beta$ HSD2 displayed an identical  
227 expression profile to that of the MR, with a low expression at birth and a significant increase in the  
228 postnatal period, independent of aldosterone status (Fig 3A). On the contrary,  $\alpha$ ENaC mRNA  
229 expression profile appeared strikingly different (Fig 3B). Indeed,  $\alpha$ ENaC mRNA levels were very low  
230 in  $AS^{-/-}$  mice both at D0 and D8, with no significant postnatal increase.  $\alpha$ ENaC expression evolved in  
231 parallel with aldosterone levels, with a 1.5-fold and a 2-fold increase at birth in  $AS^{+/+}$  and WT mice,  
232 respectively, and a 2.5-fold and 3-fold induction at D8 in these same genotypes, compared to levels in  
233  $AS^{-/-}$  mice at D0. Of interest, at D0, levels of  $\alpha$ ENaC transcripts in  $AS^{-/-}$  newborns, originating from  
234  $AS^{+/+}$  x  $AS^{+/+}$  mice, and thus prenatally exposed to aldosterone, were significantly higher than in non-  
235 exposed  $AS^{-/-}$  x  $AS^{-/-}$  littermates, whereas  $\alpha$ ENaC mRNA levels were identical at D8 in these animals.  
236 These results strongly suggest that maternally biosynthesized aldosterone may have a significant  
237 impact on fetal kidney  $\alpha$ ENaC expression. Indeed, at D8, when circulating aldosterone is no longer

238 detectable in  $AS^{-/-}$  mice born from  $AS^{+/-}$  mothers,  $\alpha$ ENaC mRNA levels do not exhibit postnatal  
239 induction as happens in  $AS^{+/-}$  and WT animals. Therefore, it appears that aldosterone is instrumental  
240 for optimal renal induction of  $\alpha$ ENaC and subsequently for sodium tubular reabsorption in the  
241 neonatal period.

242 Furthermore, it becomes evident that despite the high levels of corticosterone observed in  $AS^{-/-}$  mice  
243 and their substantial levels of GR expression (see Supplemental Figure), glucocorticoid signaling  
244 cannot functionally compensate the lack of mineralocorticoid signaling.

245

#### 246 *Organotypic cultures*

247 We next decided to establish a reliable cell-based model to further investigate the hormonal regulatory  
248 mechanisms of renal MR expression in the neonatal period. We thus chose to grow organotypic  
249 cultures of WT newborn (OCD0) and postnatal-day eight (OCD8) mouse kidneys. As presented in Fig  
250 4A, after manual kidney dissection, the nephronic tubules and glomeruli can be clearly visualized in  
251 the epithelial medium. Thereafter, epithelial-like tubular cells start to proliferate from nephronic  
252 fragments attached to the collagen I-coated plates. After seven days of culture, a full layer of renal  
253 tubular cells is observed with the typical features of epithelial cells, most notably the capacity to form  
254 domes. Moreover, as shown in Fig 4B, we were unable to immunodetect MR protein in OCD0 after 7  
255 days of culture, while a specific immunolabeling was readily detected in the epithelial-like tubular  
256 cells in OCD8, just like that observed in wild type kidneys (11). The cell immunolabelling was  
257 intracytoplasmic in the absence of aldosterone (control), or in the presence of a MR antagonist,  
258 spironolactone (A-7 + Spiro-6), whereas aldosterone alone (A-7) induced a clear nuclear translocation  
259 of the activated MR. These results suggest that primary cultures of renal cells maintain the capacity to  
260 express MR protein, and therefore constitute a valuable and reliable experimental cell-based model to  
261 investigate the impact of aldosterone (or corticosteroid hormone) exposure on neonatal (D0 and D8)  
262 control of renal MR expression.

263

#### 264 *Impact of Aldosterone on gene expression in organotypic cultures*

265 To explore the impact of mineralocorticoid and glucocorticoid hormones on neonatal renal mRNA  
266 expression, we incubated organotypic cultures of wild type D0 and D8 mouse kidneys with various  
267 steroids (aldosterone or dexamethasone). No variation in MR mRNA levels was observed in OCD0  
268 after 24 h treatment with either hormone (Fig 4C), providing additional support for the lack of  
269 corticosteroid hormone effects on MR expression when comparing D0 AS<sup>-/-</sup> and WT mice (see Fig 2).  
270 However, our cell model was sensitive to glucocorticoid but not aldosterone action since  
271 dexamethasone, a steroid compound that is not submitted to metabolic conversion by the 11 $\beta$ HSD2  
272 enzyme (20), was able to induce  $\alpha$ ENaC expression (data not shown), most likely through  
273 glucocorticoid receptor activation. Indeed, the absence of MR protein in OCD0 explains why  
274 aldosterone is inefficient in inducing  $\alpha$ ENaC expression. Finally, in sharp contrast, in OCD8 cultures,  
275 clearly expressing MR protein, aldosterone, but not dexamethasone, was able to induce a significant 2-  
276 fold increase of MR transcripts ( $p < 0.01$ ) (Fig 4C). This result corroborates the *in vivo* findings  
277 obtained in mouse models, revealing a significantly higher MR mRNA expression in WT than in AS<sup>-/-</sup>  
278 mice, particularly at D8.  
279

## 280 Discussion

281 In the present paper, we demonstrate both *in vivo* and *in vitro*, that, at birth, aldosterone has no impact  
282 on the renal expression of its specific MR receptor. Physiologically, renal MR expression is extremely  
283 low during the neonatal period both in mice and humans, and increases drastically during the postnatal  
284 period, in parallel with other actors of the mineralocorticoid pathway such as 11 $\beta$ HSD2, which confers  
285 mineralocorticoid selectivity, and  $\alpha$ ENaC, a prototypal MR target gene required for apical sodium  
286 entry and transepithelial reabsorption (11). Herein, we show that the lack of aldosterone biosynthesis  
287 observed in AS<sup>-/-</sup> mice does not modify MR expression profile at this specific period of development,  
288 characterized by a persistent low expression at birth and a postnatal induction at D8. Similarly,  
289 aldosterone withdrawal or administration does not induce MR mRNA and protein expression in  
290 organotypic cultures of newborn kidneys collected on the day of birth.

291 Interestingly, unlike the MR and 11 $\beta$ HSD2 mRNA expression profiles, which were independent of  
292 aldosterone status,  $\alpha$ ENaC mRNA levels positively correlated with the degree of aldosterone  
293 exposure, following the sequence AS<sup>-/-</sup> < AS<sup>+/-</sup> < WT. Indeed, in the absence of aldosterone (in AS<sup>-/-</sup>  
294 newborns from AS<sup>-/-</sup> mothers, and in AS<sup>-/-</sup> eight-day-old mice),  $\alpha$ ENaC transcripts levels were low at  
295 birth and did not increase postnatally.

296 The low yet detectable  $\alpha$ ENaC mRNA levels measured at birth in AS<sup>-/-</sup> mice, in the absence of  
297 aldosterone and concomitant with a very low MR expression, emphasize the redundancy of gene  
298 expression regulation. Indeed, the mineralocorticoid pathway is not the sole regulator of  $\alpha$ ENaC  
299 expression. It can also be controlled by other transcription factors, such as the GR (21). We have  
300 previously demonstrated that GR is expressed in the neonatal kidney both at the mRNA and protein  
301 levels, and is detected in the nuclei of the cortical collecting duct cells, consistent with a functional  
302 GR-mediated signaling pathway (11). GR is also detected in the kidney of AS<sup>-/-</sup> newborns. Therefore  
303 the glucocorticoid signaling pathway could account for these detectable  $\alpha$ ENaC mRNA levels at birth.

304 However, in comparison, the abundance of  $\alpha$ ENaC mRNA presented a strict positive relationship with  
305 aldosterone status, with a significant increase in  $\alpha$ ENaC mRNA levels in AS heterozygous and WT  
306 newborns as compared to AS<sup>-/-</sup> mice at D0, associated with a further increase at D8, as previously

307 demonstrated (11). Moreover,  $AS^{-/-}$  mice born from aldosterone-synthase heterozygous mothers and  
308 thus exposed to aldosterone during fetal life, presented with higher  $\alpha ENaC$  mRNA levels at birth  
309 compared to  $AS^{-/-}$  newborns from  $AS^{-/-}$  mothers. In addition, ENaC transcript levels in the kidneys of  
310 the former animals decreased during the postnatal period along with the clearance of maternal  
311 aldosterone. These results confirm the major role of aldosterone in sodium homeostasis during the  
312 neonatal period, which cannot be compensated by the activation of glucocorticoid signaling pathway,  
313 despite the high corticosterone levels measured in  $AS^{-/-}$  newborn mice and the substantial GR  
314 expression. This lack of compensation could also be the consequence of variability in GR potency at  
315 this specific period of development (22). These findings are in accordance with Lee *et al.* who have  
316 reported the death of one third of  $AS^{-/-}$  mice between 7 and 28 days (corresponding to the period of  
317 complete kidney maturation and full renal MR expression), from dehydration and failure to thrive  
318 (12). The surviving mice presented with hypotension, hyperkalemia and difficulty to concentrate  
319 urine, exacerbated under sodium-restricted diet (23), even in presence of high corticosterone levels  
320 and subnormal renal GR expression (12, 13). This is reminiscent of MR knock-out newborns which  
321 present similar symptoms and die of dehydration at 8 postnatal days (24). Both transgenic mouse  
322 models can be rescued by sodium supplementation in the neonatal period (13, 25). These observations  
323 are also reminiscent of human infants carrying heterozygous inactivating MR mutations (26) or with  
324 aldosterone-synthase deficiency (27-29), who present with failure to thrive and inability to reabsorb  
325 sodium in the postnatal period, thus requiring sodium supplementation to survive. Therefore, both  
326 aldosterone and MR appear critical for sodium reabsorption and maintenance of sodium and water  
327 homeostasis in the postnatal period, and cannot be compensated by a functional glucocorticoid  
328 signaling pathway during this specific period of renal development.

329 It seems particularly interesting that, all the components of the renal mineralocorticoid signaling  
330 pathway (MR,  $11\beta HSD2$  and  $\alpha ENaC$ ) are physiologically downregulated during the first days of life,  
331 both in mouse and human newborns. Therefore, these mechanisms concur to impair sodium  
332 reabsorption and facilitate sodium wasting, this physiological condition being of particular importance  
333 during these first days, regardless of aldosterone status. This dissociation between aldosterone and  
334 mineralocorticoid signaling pathway activation could constitute a renal protection mechanism

335 preventing inappropriate sodium retention. Alternatively, high physiological aldosterone  
336 concentrations could potentially be required for rapid non-genomic effects, as previously suggested  
337 (30-32). Nevertheless, these observations could explain why deficiency of either aldosterone  
338 biosynthesis or MR expression becomes critical after the first week of life only, both in mice and  
339 humans.

340 Interestingly, at variance with D0, we demonstrated that renal MR expression becomes regulated by  
341 aldosterone at D8, most likely once MR mRNA and protein amounts reach threshold levels in mature  
342 kidneys. Indeed, we observed a significant increase of MR mRNA levels at D8 between  $AS^{-/-}$  and WT  
343 mice. These results were corroborated by organotypic culture experiments conducted on D8 WT  
344 kidneys, where MR mRNA levels were stimulated by aldosterone but not by dexamethasone. Unlike  
345 D0, MR protein expression is readily detected in WT kidneys at D8 and is maintained after one week  
346 of organotypic culture. Therefore these results could be consistent with a positive auto-regulatory  
347 process. Other authors have already suggested an aldosterone-dependent MR auto-regulation (33, 34).  
348 The implication of MR in mediating these aldosterone regulatory effects, was established by using the  
349 MR antagonist spironolactone, (33), or by knocking down MR by small interfering RNA (34).  
350 Whether this effect is mediated by direct binding of activated MR on the regulatory region of the *MR*  
351 gene or involves intermediate transcription factors, like Sp-1 or AP-2 as suggested by Zennaro *et al.*  
352 (33), remains to be further investigated. We therefore propose that aldosterone contributes to MR  
353 regulation through a positive homologous regulatory loop, most likely *via* MR. This auto-regulation of  
354 renal MR expression is not functional at birth owing to the lack or very low abundance of MR protein  
355 in the newborn kidneys.

356 Finally, our results emphasize the multiple mechanisms (in both the mineralocorticoid and  
357 glucocorticoid signaling pathways), conserved among species, which synergistically contribute to  
358 maintain physiological sodium wasting and weigh loss in every newborn.

359 Further investigations of MR regulatory mechanisms, particularly in the neonatal period, could  
360 improve our understanding of the physiological mineralocorticoid resistance observed at birth, and  
361 might open pharmacological options for the management of sodium wasting in preterms, the most  
362 concerned by this pathology.



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370 **References**

371

- 372 1. **Holtback U, Aperia AC** 2003 Molecular determinants of sodium and water balance during  
373 early human development. *Semin Neonatol* 8:291-299
- 374 2. **Herin P, Aperia A** 1994 Neonatal kidney, fluids, and electrolytes. *Curr Opin Pediatr* 6:154-  
375 157
- 376 3. **Modi N** 1988 Development of renal function. *Br Med Bull* 44:935-956
- 377 4. **Shaffer SG, Meade VM** 1989 Sodium balance and extracellular volume regulation in very  
378 low birth weight infants. *J Pediatr* 115:285-290
- 379 5. **Bassett MH, White PC, Rainey WE** 2004 The regulation of aldosterone synthase expression.  
380 *Mol Cell Endocrinol* 217:67-74
- 381 6. **Viengchareun S, Le Menuet D, Martinerie L, Munier M, Pascual-Le Tallec L, Lombes**  
382 **M** 2007 The mineralocorticoid receptor: insights into its molecular and (patho)physiological  
383 biology. *Nucl Recept Signal* 5:e012
- 384 7. **Mick VE, Itani OA, Loftus RW, Husted RF, Schmidt TJ, Thomas CP** 2001 The alpha-  
385 subunit of the epithelial sodium channel is an aldosterone-induced transcript in mammalian  
386 collecting ducts, and this transcriptional response is mediated via distinct cis-elements in the  
387 5'-flanking region of the gene. *Mol Endocrinol* 15:575-588
- 388 8. **Edwards CR, Stewart PM, Burt D, Brett L, McIntyre MA, Sutanto WS, de Kloet ER,**  
389 **Monder C** 1988 Localisation of 11 beta-hydroxysteroid dehydrogenase--tissue specific  
390 protector of the mineralocorticoid receptor. *Lancet* 2:986-989
- 391 9. **Funder JW, Pearce PT, Smith R, Smith AI** 1988 Mineralocorticoid action: target tissue  
392 specificity is enzyme, not receptor, mediated. *Science* 242:583-585
- 393 10. **Martinerie L, Pussard E, Foix-L'Helias L, Petit F, Cosson C, Boileau P, Lombes M** 2009  
394 Physiological partial aldosterone resistance in human newborns. *Pediatr Res* 66:323-328
- 395 11. **Martinerie L, Viengchareun S, Delezoide AL, Jaubert F, Sinico M, Prevot S, Boileau P,**  
396 **Meduri G, Lombes M** 2009 Low renal mineralocorticoid receptor expression at birth  
397 contributes to partial aldosterone resistance in neonates. *Endocrinology* 150:4414-4424

- 398 12. **Lee G, Makhanova N, Caron K, Lopez ML, Gomez RA, Smithies O, Kim HS** 2005  
399 Homeostatic responses in the adrenal cortex to the absence of aldosterone in mice.  
400 *Endocrinology* 146:2650-2656
- 401 13. **Makhanova N, Lee G, Takahashi N, Sequeira Lopez ML, Gomez RA, Kim HS, Smithies**  
402 **O** 2006 Kidney function in mice lacking aldosterone. *Am J Physiol Renal Physiol* 290:F61-69
- 403 14. **Luther JM, Wang Z, Ma J, Makhanova N, Kim HS, Brown NJ** 2009 Endogenous  
404 aldosterone contributes to acute angiotensin II-stimulated plasminogen activator inhibitor-1  
405 and preproendothelin-1 expression in heart but not aorta. *Endocrinology* 150:2229-2236
- 406 15. **Gomez-Sanchez CE, de Rodriguez AF, Romero DG, Estess J, Warden MP, Gomez-**  
407 **Sanchez MT, Gomez-Sanchez EP** 2006 Development of a panel of monoclonal antibodies  
408 against the mineralocorticoid receptor. *Endocrinology* 147:1343-1348
- 409 16. **Bayard F, Ances IG, Tapper AJ, Weldon VV, Kowarski A, Migeon CJ** 1970  
410 Transplacental passage and fetal secretion of aldosterone. *J Clin Invest* 49:1389-1393
- 411 17. **van Beek JP, Guan H, Julan L, Yang K** 2004 Glucocorticoids stimulate the expression of  
412 11beta-hydroxysteroid dehydrogenase type 2 in cultured human placental trophoblast cells. *J*  
413 *Clin Endocrinol Metab* 89:5614-5621
- 414 18. **Verrey F** 2001 Sodium reabsorption in aldosterone-sensitive distal nephron: news and  
415 contributions from genetically engineered animals. *Curr Opin Nephrol Hypertens* 10:39-47
- 416 19. **Schulz-Baldes A, Berger S, Grahammer F, Warth R, Goldschmidt I, Peters J, Schutz G,**  
417 **Greger R, Bleich M** 2001 Induction of the epithelial Na<sup>+</sup> channel via glucocorticoids in  
418 mineralocorticoid receptor knockout mice. *Pflugers Arch* 443:297-305
- 419 20. **Diederich S, Eigendorff E, Burkhardt P, Quinkler M, Bumke-Vogt C, Rochel M,**  
420 **Seidelmann D, Esperling P, Oelkers W, Bahr V** 2002 11beta-hydroxysteroid  
421 dehydrogenase types 1 and 2: an important pharmacokinetic determinant for the activity of  
422 synthetic mineralo- and glucocorticoids. *J Clin Endocrinol Metab* 87:5695-5701
- 423 21. **Itani OA, Auerbach SD, Husted RF, Volk KA, Ageloff S, Knepper MA, Stokes JB,**  
424 **Thomas CP** 2002 Glucocorticoid-stimulated lung epithelial Na<sup>(+)</sup> transport is associated with  
425 regulated ENaC and sgk1 expression. *Am J Physiol Lung Cell Mol Physiol* 282:L631-641

- 426 22. **Reddy TE, Pauli F, Sprouse RO, Neff NF, Newberry KM, Garabedian MJ, Myers RM**  
427 2009 Genomic determination of the glucocorticoid response reveals unexpected mechanisms  
428 of gene regulation. *Genome Res* 19:2163-2171
- 429 23. **Makhanova N, Sequeira-Lopez ML, Gomez RA, Kim HS, Smithies O** 2006 Disturbed  
430 homeostasis in sodium-restricted mice heterozygous and homozygous for aldosterone  
431 synthase gene disruption. *Hypertension* 48:1151-1159
- 432 24. **Berger S, Bleich M, Schmid W, Cole TJ, Peters J, Watanabe H, Kriz W, Warth R,**  
433 **Greger R, Schutz G** 1998 Mineralocorticoid receptor knockout mice: pathophysiology of  
434 Na<sup>+</sup> metabolism. *Proc Natl Acad Sci U S A* 95:9424-9429
- 435 25. **Bleich M, Warth R, Schmidt-Hieber M, Schulz-Baldes A, Hasselblatt P, Fisch D, Berger**  
436 **S, Kunzelmann K, Kriz W, Schutz G, Greger R** 1999 Rescue of the mineralocorticoid  
437 receptor knock-out mouse. *Pflugers Arch* 438:245-254
- 438 26. **Geller DS** 2005 Mineralocorticoid resistance. *Clin Endocrinol (Oxf)* 62:513-520
- 439 27. **Pascoe L, Curnow KM, Slutsker L, Rosler A, White PC** 1992 Mutations in the human  
440 CYP11B2 (aldosterone synthase) gene causing corticosterone methyl oxidase II deficiency.  
441 *Proc Natl Acad Sci U S A* 89:4996-5000
- 442 28. **White PC** 1994 Disorders of aldosterone biosynthesis and action. *N Engl J Med* 331:250-258
- 443 29. **White PC** 2009 Neonatal screening for congenital adrenal hyperplasia. *Nat Rev Endocrinol*  
444 5:490-498
- 445 30. **Grossmann C, Gekle M** 2009 New aspects of rapid aldosterone signaling. *Mol Cell*  
446 *Endocrinol* 308:53-62
- 447 31. **Schmidt BM, Georgens AC, Martin N, Tillmann HC, Feuring M, Christ M, Wehling M**  
448 2001 Interaction of rapid nongenomic cardiovascular aldosterone effects with the adrenergic  
449 system. *J Clin Endocrinol Metab* 86:761-767
- 450 32. **Funder JW** 2005 The nongenomic actions of aldosterone. *Endocr Rev* 26:313-321
- 451 33. **Zennaro MC, Le Menuet D, Lombes M** 1996 Characterization of the human  
452 mineralocorticoid receptor gene 5'-regulatory region: evidence for differential hormonal

453 regulation of two alternative promoters via nonclassical mechanisms. *Mol Endocrinol*  
454 10:1549-1560

455 34. **Munier M, Meduri G, Viengchareun S, Leclerc P, Le Menuet D, Lombes M** 2010  
456 Regulation of mineralocorticoid receptor expression during neuronal differentiation of murine  
457 embryonic stem cells. *Endocrinology* 151:2244-2254

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461 **Figure legends:**

462

463 **Figure 1: Aldosterone and corticosterone levels:** Aldosterone and corticosterone levels were  
464 measured in  $AS^{-/-}$  (KO) and WT mice (WT) at the day of birth (D0) and at postnatal day eight (D8).  
465 Results are expressed as the mean  $\pm$  SEM of 6 different samples. \*,  $p < 0.05$ .

466

467 **Figure 2: Mineralocorticoid receptor expression:** MR mRNA expression was measured in the  
468 kidneys of  $AS^{-/-}$  (KO),  $AS^{+/-}$  (HET) and wild type (WT) mice at the day of birth (D0) and at eight-  
469 postnatal days (D8). Relative mRNA levels were determined by qRT-PCR in at least 4 different  
470 samples of each genotype and developmental stage. Results, expressed as the ratio of attomoles of  
471 specific gene per femtomoles of 18S, are means  $\pm$  SEM of three independent experiments and  
472 correspond to the relative expression compared with that obtained in  $AS^{-/-}$  mice kidneys at D0, which  
473 was arbitrarily set at 1. \*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$ .

474

475 **Figure 3: 11 $\beta$ HSD2 and  $\alpha$ ENaC expression:** 11 $\beta$ HSD2 and  $\alpha$ ENaC mRNA expression were  
476 measured in the kidney of  $AS^{-/-}$  (KO),  $AS^{+/-}$  (HET) and wild type (WT) mice on the day of birth (D0)  
477 and at eight-postnatal days (D8). Relative mRNA levels were determined by qRT-PCR in at least 4  
478 different samples of each genotype and developmental stage. Results, expressed as the ratio of  
479 attomoles of specific gene per femtomole of 18S, are means  $\pm$  SEM of three independent experiments  
480 and correspond to the relative expression compared with that obtained in  $AS^{-/-}$  mice kidneys at D0,  
481 which was arbitrarily set at 1. NS : Not significant; \*\*\*,  $p < 0.001$ .

482

483 **Figure 4: Organotypic cultures of kidneys from newborn (OCD0) or postnatal day eight (OCD8)**  
484 **wild type mice:** **A.** Organotypic cultures were grown in epithelial medium for 7 days. After  
485 dissection, single glomeruli and nephrons can be individualized in the medium. On the first day of  
486 culture (1) epithelial-like tubular cells start growing. After seven days of culture (7) a layer of  
487 epithelial-like tubular cells is observed. These cells demonstrate specific features of epithelial cells and  
488 have the capacity to form domes (arrow). **B.** Immunodetection of MR protein in organotypic cultures,

489 after 7 days of culture in epithelial medium for OCD0, or after 7 days of culture in epithelial medium  
490 followed by 24 h in minimal medium (MM) and 24 h in either MM (Control), MM + Aldosterone  $10^{-7}$   
491 M (A-7) or MM + Aldosterone  $10^{-7}$  M and Spironolactone  $10^{-6}$  M (A-7+Spiro-6) treatment for OCD8.  
492 Immunoreactive MR is absent in OCD0, but still detectable in epithelial-like tubular cells of OCD8  
493 after a week of culture. MR immunostaining is cytoplasmic in absence of aldosterone (Control) or in  
494 presence of the MR antagonist, spironolactone, (A-7+Spiro-6). MR expression in the epithelial cells is  
495 nuclear in presence of aldosterone (A-7). C. Relative MR mRNA expression was determined in OCD0  
496 and OCD8 after 7 days of culture in epithelial medium, followed by 24 h in minimal medium (MM)  
497 and 24 h in either MM (Control), MM + Aldosterone  $10^{-7}$  M (A-7) or MM + Dexamethasone  $10^{-7}$  M  
498 (D-7) treatment. Results, expressed as the ratio of attomoles of specific gene per femtomole of 18S, are  
499 means  $\pm$  SEM. The control condition was chosen as our statistical reference.

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