

Osmotic Stress Regulates Mineralocorticoid Receptor Expression in a Novel Aldosterone-Sensitive Cortical Collecting Duct Cell Line

Say Viengchareun^{1,2}, Peter Kamenicky^{1,2,3}, Marie Teixeira^{4,5}, Daniel Butlen^{4,5}, Geri Meduri^{1,2,6}, Nicolas Blanchard-Gutton¹, Christine Kurschat¹, Aurélie Lanel¹, Laetitia Martinerie^{1,2}, Shoshana Sztal-Mazer¹, Marcel Blot-Chabaud⁷, Evelyne Ferrary^{4,5}, Nadia Cherradi⁸, Marc Lombès^{1,2,3*}.

¹Inserm, U693, 63 rue Gabriel Péri, 94276, Le Kremlin-Bicêtre, France;

²Univ Paris-Sud, Faculté de Médecine Paris-Sud, UMR-S693, Le Kremlin Bicêtre, 94276, France;

³Assistance Publique-Hôpitaux de Paris, Hôpital de Bicêtre, Service d'Endocrinologie et Maladies de la Reproduction, Le Kremlin Bicêtre, F-94275, France;

⁴Inserm, Unit-M 867, 16 rue Henri Huchard, 75018 Paris, France;

⁵Université Paris 7 Denis Diderot, 75018, Paris, France;

⁶Assistance Publique-Hôpitaux de Paris, Hôpital de Bicêtre, Service de Pharmacogénétique, Biochimie Moléculaire et Hormonologie, Le Kremlin Bicêtre, 94275, France;

⁷Inserm, U608, UFR de Pharmacie, 13385 Marseille cedex 5, France;

⁸Inserm, U878, Grenoble F-38054, France.

Corresponding Author: Dr Marc Lombes, MD, PhD;

Tel : 33 1 49 59 67 09; Fax: 33 1 49 59 67 32; E-mail: marc.lombes@u-psud.fr

Abbreviated Title: Osmotic Stress and MR Expression

Precis: Osmotic Stress Regulates Mineralocorticoid Receptor Expression in Cortical Collecting Duct Cells

Disclosure Statement: the authors have nothing to disclose

Keywords: Mineralocorticoid Receptor, TonEBP, Renal Cell Line, Targeted Oncogenesis, Tis11b

Grant Support: This work was supported by grants from Inserm, the University Paris-Sud 11 and from the Bonus Qualite Recherche 2003 of the University Paris-Diderot. PK was a recipient of fellowship from the Ministère de l'Enseignement Supérieur et de la Recherche, France.

1 **Abstract**

2 Aldosterone effects are mediated by the mineralocorticoid receptor (MR), a transcription factor highly
3 expressed in the distal nephron. Given that MR expression level constitutes a key element controlling
4 hormone responsiveness, there is much interest in elucidating the molecular mechanisms governing MR
5 expression. To investigate whether hyper- or hypotonicity could affect MR abundance, we established
6 by targeted oncogenesis a novel immortalized cortical collecting duct (CCD) cell line and examined the
7 impact of osmotic stress on MR expression. KC3AC1 cells form domes, exhibit a high transepithelial
8 resistance, express 11 β -hydroxysteroid dehydrogenase 2 and functional endogenous MR, which
9 mediates aldosterone-stimulated Na^+ reabsorption through the epithelial sodium channel activation. MR
10 expression is tightly regulated by osmotic stress. Hypertonic conditions induce expression of TonEBP,
11 an osmoregulatory transcription factor capable of binding TonE response elements located in MR
12 regulatory sequences. Surprisingly, hypertonicity leads to a severe reduction in MR transcript and
13 protein levels. This is accompanied by a concomitant tonicity-induced expression of Tis11b, a mRNA-
14 destabilizing protein which, by binding to the AU-rich sequences of the 3'-UTR of MR mRNA, may
15 favor hypertonicity-dependent degradation of labile MR transcripts. In sharp contrast, hypotonicity
16 causes a strong increase in MR transcript and protein levels. Collectively, we demonstrate for the first
17 time that optimal adaptation of CCD cells to changes in extracellular fluid composition is accompanied
18 by drastic modification in MR abundance *via* transcriptional and post-transcriptional mechanisms.
19 Osmotic stress-regulated MR expression may represent an important molecular determinant for cell-
20 specific MR action, most notably in renal failure, hypertension, or mineralocorticoid resistance.

21 **Introduction**

22 The regulation of sodium balance and water homeostasis is ensured by the steroid hormone
23 aldosterone, which is the main mineralocorticoid in humans (1). In polarized epithelial tissues such as
24 the kidney, aldosterone actions are mediated by the mineralocorticoid receptor (MR), a ligand-
25 dependent transcription factor belonging to the nuclear receptor superfamily. Indeed, aldosterone by
26 binding to the MR, directly stimulates expression of ionic transporters, such as the Epithelial Na⁺
27 Channel (ENaC) and the Na⁺,K⁺-ATPase. MR also induces early expression of other target genes
28 encoding important mediators of the mineralocorticoid response that stimulate ENaC-mediated Na⁺
29 reabsorption in the kidney: Serum and Glucocorticoid-regulated Kinase 1 (SGK1), Ubiquitin-specific
30 protease Usp2-45, KS-WNK1 kinase (With No lysine K) and Glucocorticoid-Induced Leucine Zipper
31 (GILZ) transcription factor (2).

32 Several lines of evidence suggest that a key step in the mineralocorticoid response is the regulation
33 of the MR expression level. Indeed, MR knockout mice die shortly after birth from renal sodium loss
34 (3) whereas AQP2-Cre-MR mice, with targeted MR inactivation in the cortical collecting duct (CCD),
35 present with sodium wasting when subjected to low salt diet (4). Moreover, RNAi inhibition of MR
36 prevents the development of cold-induced hypertension and renal damage in mice (5) and induces in a
37 transgenic rat model a syndrome resembling autosomal dominant pseudohypoaldosteronism type 1
38 (PHA1) (6). In humans, PHA1 patients carrying a heterozygous MR mutation which leads to MR
39 haploinsufficiency (7), present with typical features of aldosterone resistance. On the other hand,
40 increased MR expression also causes deleterious effects in mice and humans. Indeed, MR
41 overexpressing mice develop kidney dysfunction and mild cardiomyopathy associated with
42 arrhythmias (8) whereas conditional cardiac MR overexpression leads to life-threatening arrhythmias
43 (9). Interestingly, Quinkler et al., demonstrated a 5-fold increase in renal MR expression in patients
44 with renal failure and heavy proteinuria, linking MR activation to kidney disease (10).

45 In the kidney, MR is expressed mostly in the distal convoluted tubules (DCT) and in the CCD of
46 the distal nephron, with higher expression levels in the renal cortex compared to that observed in the
47 medulla (11, 12). However, little information is available on the molecular mechanisms regulating MR
48 expression in the nephron segments exposed to extreme fluctuations of extracellular fluid composition,

49 given the ability of the kidney to dilute urine below plasma osmolality or to concentrate the urine
50 above plasma osmolality. Indeed, water excretion relies on the corticopapillary gradient which is
51 tightly regulated most notably by hormones (13).

52 In the renal medulla, where MR expression is low, the driving force for water reabsorption is
53 provided by elevated urea and NaCl concentrations, which generate high osmolarity levels
54 (1200 mosmol/kg). Thus, inner medullar renal cells must accumulate intracellular osmolytes in order
55 to reduce intracellular ionic strength. This adaptation, which represents a protective mechanism from
56 hypertonic stress in mammalian cells, is mediated by the Tonicity-responsive Enhancer Binding
57 protein (TonEBP or NFAT5/OREBP), a transcription factor belonging to the Rel family (14, 15).
58 TonEBP activity is upregulated by hypertonicity through combined enhanced TonEBP transcription
59 and increased TonEBP nuclear localization. This leads to increased transcription of sodium-chloride-
60 betaine cotransporter (BGT1), sodium-myoinositol cotransporter (SMIT), aldose reductase (AldoR),
61 and taurine transporter, which mediate intracellular accumulation of betaine, myoinositol, sorbitol, and
62 taurine, respectively (16). In the renal cortex, where MR expression is high, the tubular lumen is rather
63 hypotonic reaching 50 mosmol/kg because the apical membrane of epithelial cells is impermeable to
64 water leading to sustained osmotic differences.

65 To the best of our knowledge, no study has ever reported whether hypertonicity or hypotonicity
66 influences MR expression. Here, we investigated MR expression under osmotic stress in a new
67 immortalized and differentiated CCD-derived cell line which we established by a targeted oncogenesis
68 strategy in mice. We demonstrate that fluctuations in extracellular fluid composition both *in vitro* and
69 *in vivo* is accompanied by drastic modification in renal MR abundance *via* transcriptional and post-
70 transcriptional mechanisms. Elucidating such underlying mechanisms is clearly of major importance
71 in the design of new pharmacological strategies for modulating cell specific MR action.

72 **Results**

73 **Expression of Functional MR in the Immortalized KC3AC1 Cell Line**

74 The KC3AC1 cell line has been established from microdissected cortical collecting ducts of a
75 transgenic mouse by means of a targeted oncogenesis strategy in which the P1 proximal promoter of
76 the human MR gene drove the expression of the SV40 large T Antigen (TAg) in all aldosterone target
77 tissues (17). When grown on collagen I-coated Petri dish, KC3AC1 cells form islets of epithelial-like
78 cells and rapidly develop numerous domes at confluency (Fig. 1A). Positive nuclear immunostaining
79 of TAg further confirmed that KC3AC1 cells are immortalized (see inset in Fig. 1A). Electron
80 microscopic analyses revealed that these cells grow as a monolayer of epithelial cells, when cultivated
81 on filters, and exhibit desmosomes and apical microvilli (see insert), two typical features of polarized
82 cells (Fig. 1B). The presence of functional MR in KC3AC1 cells was assessed by Scatchard analysis.
83 Specific aldosterone binding sites were detected in cytosolic fractions of KC3AC1 cells with a Kd
84 compatible with the affinity of aldosterone for MR ($\sim 1.3 \pm 0.3$ nM) and the estimated MR
85 concentration was at ~ 30 fmol/mg protein (Fig. 1C). Competition studies, using 100-fold excess of
86 unlabeled aldosterone (Aldo) and the mineralocorticoid antagonist, spironolactone (Spiro), enabled
87 identification of these specific [3 H]-aldosterone binding sites as MR (Fig. 1D). To further substantiate
88 MR protein identification in KC3AC1 cells, MR immunocytochemistry and western blot analyses
89 were performed with a novel polyclonal anti-MR antibody raised against the first 18 amino acids of
90 human MR (hMR) which are conserved in the mouse and rat species. Purified 39N antibody allowed
91 the immunodetection of MR protein in the cytoplasm of KC3AC1 cells cultured in serum-starved
92 medium (Fig. 1E) and in the nucleus of cells after 30 min incubation with 10 nM aldosterone
93 (Fig. 1F). Direct western blotting revealed a band at a molecular mass of ~ 130 kDa in KC3AC1 cell
94 lysates (Fig. 1G). As a positive control, we used rabbit M cells, stably overexpressing hMR (18) and
95 as expected, a band was present in M cell homogenates but absent in lysates of parental RCSV3 cells
96 (19), devoid of MR protein. Taken together, these complementary approaches clearly demonstrated
97 that KC3AC1 cells express functional MR protein.

98 We next examined whether the 11 beta hydroxysteroid dehydrogenase type 2 enzyme (11 β HSD2),
99 one of the mechanisms conferring MR selectivity, was expressed in KC3AC1 cells. Initially, the

100 presence of 11 β HSD2 mRNA was demonstrated by RT-PCR analysis (data not shown). Figure 1H
101 (upper panel) presents a typical HPLC chromatogram illustrating the metabolic conversion of [3 H]-
102 corticosterone, compound [B], to 11 dehydro [3 H]-corticosterone, compound [A], after incubation of
103 KC3AC1 lysates with 1 mM NAD (a specific 11 β HSD2 cofactor), for 15 min at 37°C. The robust
104 metabolic conversion of compound [B] to [A] is highly suggestive of the presence of the 11 β HSD2
105 enzyme in these cells. We then investigated the cofactor specificity of this enzymatic activity and
106 showed that the metabolic conversion of [B] was clearly dependent on the presence of NAD cofactor,
107 but not on NADP (a specific cofactor for 11 β HSD1), thus confirming the expression of 11 β HSD2 in
108 these CCD cells (Fig. 1H, lower panel).

109

110 **Aldosterone Stimulates Na⁺ Reabsorption in KC3AC1 Cells Through ENaC**

111 Given that in the CCD, Na⁺ reabsorption is mainly mediated by the ENaC, we searched for its
112 expression in KC3AC1 cells and showed by RT-PCR (Fig. 2A) and western blot analyses (Fig. 2B)
113 that α , β and γ subunits of ENaC are expressed in these cells. When grown on filters with complete
114 medium, KC3AC1 cells developed a high transepithelial resistance ($R_T \sim 3000-5000 \Omega \text{cm}^2$) associated
115 with high short-circuit current values ($I_{sc} \sim 25 \mu\text{A} \text{cm}^2$) and a transepithelial voltage V_T of $\sim +12.5$
116 mV. We further investigated the functional integrity of ENaC. Apical administration of 10^{-5} M
117 amiloride, a specific inhibitor of this channel, resulted in a strong (>90%) and rapid decrease of I_{sc}
118 (Fig. 2C), consistent with ENaC-dependent Na⁺ transport in KC3AC1 cells.

119 We next investigated whether KC3AC1 cells remained sensitive to aldosterone action. We showed
120 that nanomolar aldosterone concentrations for 18 h significantly increased I_{sc} , indicating an enhanced
121 ionic transport in KC3AC1 cultured on filters (Fig. 2D). Aldosterone exposure also increased Na⁺
122 concentration in the basolateral compartment (Fig. 2E, left panel) and K⁺ concentration in the apical
123 compartment (Fig. 2E, right panel). Aldosterone-stimulated Na⁺ reabsorption and K⁺ secretion are
124 mediated by MR since simultaneous administration of spironolactone prevented these ionic transports.
125 Finally, we examined the ability of endogenous MR to act as a ligand-dependent transcription factor.
126 Quantitative RT-PCR (qRT-PCR) analysis showed that aldosterone exposure rapidly induced a 2- to

127 3-fold increase in the expression of two prototypical aldosterone target genes, the kinase Sgk1 (Fig.
128 2F, left panel) and the α subunit of ENaC (Fig. 2F, right panel).

129 Altogether, these results clearly demonstrate that KC3AC1 are highly differentiated CCD-derived cells
130 possessing all the necessary molecular components for aldosterone action, most notably endogenous
131 expression of functional MR. Thus, they constitute a suitable cell based model to further investigate
132 the regulatory mechanisms controlling MR expression.

133

134 **Analysis of MR Transcript and Protein Stability**

135 We first demonstrated that MR expression was dependent upon cell differentiation status. Indeed, as
136 determined by qRT-PCR, steady-state levels of MR mRNA were higher in dome-forming cells at day
137 7 than in KC3AC1 cells just before the cells became confluent (day 2) (Supplemental Fig. S1). We,
138 therefore, decided to analyze MR expression only when KC3AC1 cells were forming domes at day 7.

139 We studied the stability of MR transcripts by qRT-PCR in KC3AC1 cells. As illustrated in Fig. 3A,
140 we showed that MR mRNA rapidly declined after actinomycin D exposure with a calculated half-life
141 time ($t_{1/2}$) of approximately 3 h. We further examined MR translation product by performing western
142 blot analysis of whole KC3AC1 cell protein extracts. Fig. 3B reveals that, as expected, a 50% decrease
143 in MR protein expression was observed after 24 h exposure to actinomycin D. We also examined the
144 half-life of MR protein by submitting KC3AC1 to cycloheximide, an inhibitor of protein synthesis. As
145 demonstrated in Fig. 3C, MR protein levels also declined rapidly with an estimated half-life of 12 h.

146

147 **Hypertonic Stress Inhibits MR Expression**

148 Given the major role played by MR expression level in determining mineralocorticoid responses, we
149 were interested in examining whether variations in extracellular osmolarity, known to occur in the
150 tubular segments of the distal nephron, could affect renal MR expression. We first examined whether
151 hypertonicity could influence endogenous MR expression. Hypertonic stress exerted a dramatic effect
152 on cell morphology, the domes rapidly disappearing after exposition to raffinose for 6 h or 24 h
153 (Fig. 4A and B). TonEBP (*TonE Binding Protein*) plays a pivotal role in protecting renal cells from
154 hypertonic stress. This osmoregulatory transcription factor binds Tonicity Response Elements (TonE),

155 located in the promoter sequences of target genes such as *Aldose Reductase* gene (*AldoR*). We
156 therefore examined whether TonEBP was expressed in KC3AC1 cells. Using qRT-PCR, we showed a
157 5-fold increase of basal TonEBP transcript levels when cells were subjected to a hypertonic stress
158 mimicked by raffinose exposure (Fig. 4C). Western blot analyses performed with an anti-TonEBP
159 polyclonal antibody confirmed that TonEBP expression is clearly induced under hypertonic conditions
160 (Fig. 4D). As expected, TonEBP activated transcription of *AldoR* whose expression increased by 17-
161 fold after raffinose exposure (Fig. 4E).

162 Characterization of the 5'-flanking region of the human *MR* gene allowed us to identify six TonE in
163 the promoter region of hMR, referred to as T1 to T6 (Supplemental Fig. S2A). They all share sequence
164 motifs similar to the consensus sequence TGGAAANNYYN, suggesting that TonEBP might be
165 involved in the regulation of MR expression in renal cells. Electrophoretic mobility shift assays
166 (EMSA) showed that raffinose-induced TonEBP specifically binds the T1 sequence (Supplemental
167 Fig. S2B) with high affinity ($K_d \sim 0.45$ nM, Supplemental Fig. S3). Transient transfection assays using
168 wild type and mutant T1-driven luciferase reporter constructs, together with dominant-negative
169 TonEBP, further demonstrated that TonEBP functions as a hypertonicity-induced transcription factor
170 via binding to functional hMR TonE elements in KC3AC1 cells (Supplemental Fig. S2C and S2D).

171 Unexpectedly, raffinose exposure led to a time-dependent decrease of MR mRNA steady state levels
172 as measured by qRT-PCR, suggesting that hypertonic stress may inhibit MR gene transcription and/or
173 accelerate MR transcript degradation (Fig. 4F). This effect seems specific to MR since hypertonicity
174 did not affect β -actin nor glucocorticoid receptor expression (data not shown). To further elucidate the
175 molecular mechanisms involved in this raffinose-induced repression of MR expression, KC3AC1 cells
176 were further incubated in the absence or presence of cycloheximide and subjected to raffinose
177 exposure. Cycloheximide treatment alone induced a significant 1.5 fold-increase in MR mRNA levels
178 suggesting a critical role of putative destabilizing proteins in regulating MR expression. Of interest,
179 hypertonic stress, even in the presence of cycloheximide, was still able to significantly reduce MR
180 transcripts, consistent with repression of *MR* gene transcription independently of the neosynthesized
181 proteins (Fig. 4G). This was also true at the protein level, since a 70% decrease in MR expression was
182 observed after 18 h-raffinose exposure in the absence or in the presence of cycloheximide (Fig. 4H).

183 Taken together, our results are consistent with the hypothesis that extracellular hypertonicity inhibits
184 MR expression both by altering MR gene transcription and by controlling MR mRNA decay.

185

186 **Identification of *Tis11b* as a Direct-Tonicity Induced Target Gene**

187 Among the proteins that control mRNA stability, we hypothesized that Tis11b (tetradecanoyl phorbol
188 acetate-inducible-sequence 11b) might play a role in the control of MR mRNA decay. This protein
189 belongs to a family of RNA-binding proteins (comprising also TIS11/TTP/tristetraprolin and Tis11d)
190 that share characteristic tandem CCCH-type zinc-finger domain. All members of the family are
191 involved in the regulation of several short-lived cytokine mRNA and appear to share similar AU-rich
192 elements (AURE)-binding and mRNA-destabilizing activities (20, 21). Of particular importance, *in*
193 *silico* analysis of the human *Tis11b* promoter revealed the presence of 4 TonE elements, referred to as
194 TA to TD, suggesting that TonEBP might also be involved in the regulation of Tis11b expression in
195 renal cells. Interestingly, we noticed that the TonE elements TB to TD were highly conserved among
196 human, mouse and rat species (Fig. 5A). As expected, when KC3AC1 cells were exposed for 6 h
197 under a hypertonic stress, a significant time-dependent increase in TonEBP expression was observed
198 (Kruskal-Wallis test, $p < 0.0001$; post-test: Dunn's multiple comparison test, $** P < 0.01$ and $*** P$
199 < 0.001). This was accompanied by a concomitant 4-fold increase (Kruskal-Wallis test, $p < 0.0001$;
200 post-test: Dunn's multiple comparison test, $** P < 0.01$ and $*** P < 0.001$) in Tis11b mRNA levels
201 (Fig. 5B). Moreover, actinomycin D almost completely abolished the increase of Tis11b mRNA
202 induced by raffinose, suggesting a direct transcriptional control of tonicity-induced Tis11b expression
203 (Fig. 5C). Western blot analysis, using a polyclonal antibody that recognizes both Tis11d and Tis 11b
204 isoforms, confirmed at the protein level that Tis11b expression was increased after a 18 h-raffinose
205 exposure, while Tis11d expression was clearly reduced (Fig. 5D). Tis11b was shown to interact with
206 AU-rich elements (AURE) thus promoting rapid degradation of AURE-harboring mRNAs. These
207 regulatory motifs consist of pentamers of AUUUA, of nonamers of UUAUUUA(U/A)(U/A) or U-rich
208 elements, which are generally located in the 3'-UTR of labile transcripts such as cytokines, growth
209 factors and proto-oncogenes (22). Interestingly enough, *in silico* analysis revealed that MR mRNA
210 harbors several AURE (A1 to A6) in its extended 3'-UTR, most of which are highly conserved
211 between species (Fig. 5E).

212 **Hypotonic Stress Stimulates MR Expression**

213 Contrary to the results obtained under hypertonicity, hypotonic conditions induced an increase both in
214 dome formation and size (Fig. 6A and B) and dramatically enhanced MR expression by 5-fold at
215 messenger RNA level (Fig. 6C). To further clarify the potential underlying molecular mechanisms,
216 KC3AC1 cells were subjected to hypotonic conditions in the absence or presence of actinomycin D.
217 qRT-PCR (Fig. 6D) and western blot analyses (Fig. 6E) revealed that hypotonic stress increases MR
218 expression both at the mRNA and protein levels, irrespective of actinomycin D. Collectively, these
219 findings strongly suggest that hypotonicity-regulated MR expression is not consequent to stimulation
220 of MR transcription but rather to increased mRNA and protein stability.

221

222 **Renal MR expression is Altered by Water Deprivation and Water Intoxication in Mice**

223 To further address the physiological relevance of our *in vitro* findings, we subjected male mice to
224 archetypal water imbalance situations (Fig. 7). Water deprivation for 18 h, a condition known to
225 increase urine osmolality and renal medullary sodium concentration (23), induced a significant 50%
226 decrease in MR protein levels in the whole kidney of mice subjected to such hypertonic stress. In
227 contrast, acute water intoxication following oral administration of hypotonic fluid (3% vol /BW) and
228 concomitant intraperitoneal injection of vasopressin, a method for inducing positive water balance
229 (24), led to a 2-fold increase in renal MR expression. Taken together, these *in vivo* results support our
230 findings previously obtained with KC3AC1 cells.

231 **Discussion**

232 MR expression level in a target cell dictates aldosterone responsiveness. This assertion is
233 particularly relevant in several animal models and in human diseases, where alteration of renal MR
234 expression leads to severe pathophysiological consequences of impaired or excessive aldosterone
235 responses. This underlines the importance of maintaining appropriate levels of MR expression and the
236 necessity to elucidate the regulatory mechanisms of MR expression. However, such investigations
237 have been hampered by the lack of appropriate cellular models expressing endogenous MR and
238 responsive to aldosterone action. Although several useful human and mouse cell lines have been
239 established from the CCD (19, 25-30), these models have some limitations (31, 32). For instance,
240 some of them do not maintain endogenous MR expression and/or only responded to pharmacological
241 doses of aldosterone (micromolar concentrations). Others do not form domes or display suboptimal
242 electrophysiological properties (minimal transepithelial resistance, low short-circuit current).

243 In the present study, we aimed at analyzing MR expression level under physiological conditions in
244 renal cells prone to be exposed to large variations of extracellular fluid composition. This prompted us
245 to establish a novel CCD cell line by means of a targeted oncogenesis strategy in mice, using the SV40
246 T Antigen under the control of the P1 promoter of the *hMR* gene. This strategy has already proven to
247 be an efficient experimental means of deriving several differentiated mineralocorticoid-sensitive cell
248 lines (17, 33, 34).

249 KC3AC1 cells possess all features of polarized epithelial cells and form domes at confluency
250 suggesting the occurrence of vectorial ionic transport. We showed that these cells express functional
251 ENaC and 11 β HSD2 enzyme. The NAD-dependent 11 β HSD2 catalytic activity was calculated at ~ 50
252 fmol/ μ g prot/1 h, a very high activity, which was of the same order of magnitude as that estimated in
253 isolated rat CCD (35) and in renal cells stably transfected with 11 β HSD2 cDNA (36). Binding assays
254 and immunodetection enabled us to unambiguously demonstrate the presence of functional MR in
255 KC3AC1 cells. More importantly, we established that these cells remain capable of responding to
256 mineralocorticoids since physiological aldosterone concentrations stimulated Na⁺ reabsorption
257 associated with induction of *SCNNIA* (α ENaC) and *Sgk1* gene expression, in accordance with
258 previous studies (28, 37-39). Altogether, functional properties of KC3AC1 cells indicate that this cell

259 line represents a valuable model to analyze MR expression and to decipher the mineralocorticoid
260 signaling pathway in the CCD.

261 MR steady-state level results from equilibrium between its synthesis and degradation. Thus, MR
262 abundance may increase following a rise in the transcription/translation rate or alternatively may
263 decrease because of modifications of mRNA or protein stability. To the best of our knowledge, few
264 data exist on the mechanisms controlling MR expression levels. We previously demonstrated that hMR
265 gene activation is under the control of two alternative promoters (P1 and P2), which give rise to two
266 mRNA isoforms (hMR α and hMR β) (40), yet questioning the molecular events involved in the control
267 of MR gene transcription (41). Multiple protein variants are generated from one single gene by
268 mechanisms of alternative RNA splicing and alternative translational initiation, offering the possibility
269 of combinatorial patterns of receptor expression (42).

270 Herein, we provide new information regarding MR expression, both at messenger and protein
271 levels. We demonstrate that MR transcript has a relatively short $t_{1/2}$ (3 h), suggesting that MR mRNA
272 is an unstable molecule, very likely subjected to strict regulatory mechanisms of mRNA turnover,
273 while the MR protein also has a short half-life estimated at 12 h. More importantly, our study brings
274 new insights into the mechanisms modulating MR levels in the CCD under hyper- and hypotonic
275 conditions. *In vivo*, hypertonicity represents a genuine threat to renal cells exposed to extraordinarily
276 high levels of NaCl and urea since such osmotic stress causes numerous cellular perturbations
277 (cytoskeletal rearrangement, apoptosis, alteration of DNA replication, transcription and translation)
278 (43). Renal cells can accommodate hypertonic stress by developing an adapted cellular response that
279 relies on an enhanced TonEBP activity *via* increased nuclear translocation, transactivation and
280 abundance (44). This osmoregulatory transcription factor binds TonE elements present in promoter
281 sequences of several osmotically regulated genes, resulting in an enhanced production of intracellular
282 osmolytes, which maintain appropriate intracellular ionic strength. TonEBP also stimulates
283 transcription of the heat shock protein 70 (hsp70) (45) and contributes to urinary concentration by
284 direct stimulation of aquaporin-2 (AQP-2) (46) and UT-A urea transporter (47) expression. Several
285 recent studies reported a role of hypertonicity in the expression of genes either involved in tight
286 junction properties, including MUPP1 (multi-PDZ protein-1), ZO1 (Zonula occludens 1), Af6 and

287 claudin-4 (*Cldn4*) (48, 49) and/or belonging to the mineralocorticoid signaling pathway such as
288 increased expression and activity of *Sgk1* (23, 50, 51). This is particularly interesting given the pivotal
289 role played by *Sgk1* in regulating Na^+ reabsorption in the CCD and controlling cell volume (52).

290 Using KC3AC1 cells, we showed that *TonEBP* transcript and protein levels increased dramatically
291 under exposure to 200 mM raffinose or 150 mM NaCl, an effect associated with a 17-fold increase in
292 *AldoR* gene expression. Identification of several functional *TonE* elements in the P1 promoter and
293 intron A of the *hMR* gene suggests to us that activation of *TonEBP* signaling pathway can directly
294 stimulate *MR* expression. However, counterintuitively, hypertonicity decreased by more than 50% *MR*
295 transcript and protein levels after short (3 h) and long-term (18 h) exposure, excluding a direct
296 involvement of *TonEBP* in the down regulation of *MR* expression under hypertonic stress.
297 Alternatively, given that a decrease in *MR* abundance may be the consequence of a decline in *MR*
298 gene transcription and/or a modification in *MR* stability, we hypothesize that a putative destabilizing
299 protein, whose activity is regulated by hypertonicity, may participate to the control of *MR* expression.
300 Indeed, we identified *Tis11b* as a direct tonicity-induced target gene and demonstrated that it fulfills
301 all the criteria of *MR* mRNA destabilizing protein. We showed that *Tis11b* promoter contains several
302 highly conserved *TonE* responsive elements and its expression, at both mRNA and protein level,
303 increases under raffinose exposure. As previously demonstrated for several short-lived mRNA
304 including *VEGF* (53) and *Star* (54), the 3'-UTR of *MR* mRNA contains multiple highly conserved
305 *AURE* to which *Tis11b* may bind and thus facilitate its degradation. Direct binding of *Tis11b* to the
306 3'-UTR of *MR* and its functional consequences in terms of *MR* stability remains to be elucidated.
307 Taken together, we propose that hypertonic stress activates the *TonEBP* signaling pathway in
308 KC3AC1 cells, leading to a parallel increase of the mRNA-destabilizing protein *Tis11b* expression,
309 which may favor hypertonicity-dependent degradation of labile *MR* transcripts. We therefore
310 demonstrate for the first time a post-transcriptional regulation of *MR* mRNA as a pivotal process in
311 the control of *MR* expression under hypertonic conditions.

312 At variance with hypertonicity, hypotonic conditions not only preserved the presence of domes but
313 seemed to increase their size. It has been proposed that hypotonic stress initially leads to cell swelling
314 followed by normalization of cell volume through a regulatory volume decrease, involving a reduction
315 of intracellular Cl^- . This biphasic mechanism was reported to modify gene expression and the activity

316 of some proteins, including ion channels (55). Thus, intracellular Cl^- was thought to act as a signal
317 increasing Na^+ reabsorption via p38 MAPK kinase and c-Jun N-terminal Kinase in A6 epithelial renal
318 cells (56, 57). Recently, hypotonicity was also shown to modify cell architecture associated with actin
319 cytoskeletal remodeling (58).

320 Here, we demonstrated in KC3AC1 cells that hypotonicity increased MR transcript and protein
321 levels and this effect was not prevented by actinomycin D, suggesting that MR expression level
322 increased without affecting *MR* gene transcription but rather, through an increase of its mRNA and
323 protein stability. Importantly, this increase of MR levels under hypotonic stress was consistent with
324 recent reports on hypotonic-induced activation of the mineralocorticoid signaling pathway. Indeed,
325 hypotonicity has been shown to increase Na^+, K^+ -ATPase cell surface expression in the renal
326 mpkCCD_{c14} cell line (59) and Na^+ reabsorption in A6 cells *via* up-regulation in β and γ ENaC subunits
327 (60). *Sgk1* expression has also been enhanced by hypotonic stress in renal A6 cells (61). This increase
328 in both *ENaC* and *Sgk1* gene expression could be mediated by intracellular Ca^{2+} , as a second
329 messenger in hypotonic stress-induced Na^+ transport (62).

330 Collectively, our results provide clear evidence that osmotic stress (hyper- and hypotonicity)
331 greatly affects the expression of a nuclear receptor both at messenger and protein levels, in accordance
332 with our *in vivo* findings indicating that renal MR expression is subjected to tight osmoregulatory
333 control and varies as a function of hydroelectrolytic homeostasis. From a phylogenetic point of view,
334 our results are also reminiscent of variations of MR expression observed in salmon during their
335 acclimation from fresh to salt water. MR expression increases in their gills during de-smoltification
336 (from salt to fresh water) and it is down-regulated after transfer to salt water during smoltification
337 (63).

338 Finally, it would be important to determine whether MR expression variations under osmotic
339 stress occur in other Na^+ transporting epithelia (inner ear, sweat and salivary glands) and to evaluate
340 whether other types of cellular stress such as hypoxia, redox status, pH, and ATP depletion may also
341 contribute to the regulation of MR expression.

342 **Materials and Methods**

343 **Establishment of the KC3AC1 Cortical Collecting Duct Cell Line**

344 The KC3AC1 cell line was isolated from a 4-week-old P1-TAg transgenic mouse (founder #3) (34).
345 Microdissection of cortical collecting ducts (CCD) was performed as reported earlier. Briefly, kidneys
346 were perfused under sterile conditions via the abdominal aorta at RT with 4 ml of solution A
347 containing DMEM/HAM's F12 medium (1:1), 20 mM HEPES, pH 7.4, 100 U/ml penicillin, 100
348 µg/ml streptomycin, 2 mM glutamine, and 20% fetal calf serum (FCS) and 0.25% collagenase (CLS
349 type I, 146 U/mg from *Clostridium histolyticum*, Worthington Biochemical; Freehold, NJ, USA).
350 Kidneys were removed, cut into slices which were further incubated for 20 min at 30°C in an aerated
351 0.07% collagenase solution A. After carefully rinsing, tissue was microdissected by hand with the help
352 of thin needles under stereomicroscopic observation in ice-cold medium A devoid of collagenase.
353 CCD were recognized according to morphological criteria reported earlier (64) and fragments of about
354 0.5 mm were transferred to collagen-coated 12-well plates, and cultured in fresh medium. The latter
355 was removed every 2-day for 2 weeks, until the formation of a monolayer of cells, which were
356 selected for their epithelioid morphology and the nuclear immunodetection of SV40 TAg. The
357 KC3AC1 cell line was first cultured until passage 10 then was subcloned by limit dilution to obtain a
358 homogeneous cell population which was routinely cultured at 37°C, in a humidified incubator gassed
359 with 5% CO₂, in an epithelial medium composed of DMEM/HAM's F12 (1:1); 2 mM glutamine;
360 50 nM dexamethasone (Sigma, St Louis, MO, USA); 50 nM sodium selenite (Sigma); 5 µg/ml
361 transferrin; 5 µg/ml insulin (Sigma); 10 ng/ml EGF (Tebu, Le Perray en Yvelines, France); 2 nM T3
362 (Sigma); 100 U/ml penicillin/streptomycin; 20 mM HEPES, pH 7.4 and 5% dextran charcoal-treated
363 serum (DCC). All reagents were purchased from Invitrogen (Cergy Pontoise, France), except when
364 stated otherwise.

365

366 **Cell Culture**

367 KC3AC1 cells (passages 6-20) were seeded either on collagen I-coated (Institut Jacques Boy, Reims,
368 France) Transwell/Snapwell filters (Corning Costar, Brumath, France) or on collagen I-coated Petri
369 dishes with the epithelial medium. To study corticosteroid actions, the epithelial medium was replaced

370 by a minimal medium (MM) which has the same composition as the epithelial medium but lacks
371 dexamethasone and DCC.

372

373 **Hormones and Drugs**

374 Aldosterone was purchased from Acros Organics (Noisy Le Grand, France) whereas raffinose,
375 cycloheximide, actinomycin D, spironolactone, and amiloride were purchased from Sigma.

376

377 **Hypertonic and Hypotonic Stresses**

378 Hypertonic medium (500 mosmol/kg) was composed of isoosmotic medium (300 mosmol/kg)
379 supplemented with 200 mM raffinose whereas hypotonic medium (150 mOsm/kg) represented a 1:1
380 dilution of isoosmotic medium with sterile culture water. Control experiments performed with 50%
381 medium diluted with sterile 150 mM NaCl resulted in no appreciable difference from isoosmotic
382 conditions.

383

384 ***In vivo* Water deprivation and Water Intoxication Studies**

385 Two-month-old male B6D2 mice, weighing approximately 25 g, were used in this study in accordance
386 to the guidelines for the care and use of laboratory animals. Animals had free access to water and
387 standard chow except the water-restricted group, which was subjected to water deprivation for 18 h.
388 For water intoxication, animals were once orally fed with 3% vol/kg of body weight of a 10 mM
389 glucose solution prepared with tap water (approx 800 µl/mouse), using gavage feeding needles
390 (Phymep, Paris). Mice received simultaneously an intraperitoneal injection of dDAVP (Minirin®,
391 Ferring GmbH, Kiel, Germany) at a concentration of 400 ng/kg BW. After 6 h, animals were
392 sacrificed and kidneys were collected and snap-frozen in liquid nitrogen for subsequent western blot
393 analysis as previously described (65).

394

395 **Electron Microscopic Analyses**

396 KC3AC1 cells were grown on Transwell filters with complete epithelial medium. Electron microscopic
397 analyses were performed as described previously (34).

398

399 **Hormone Binding Assays**

400 Whole cell specific binding of [³H]-aldosterone and [³H]-dexamethasone was determined in KC3AC1
401 cells grown on a collagen I-coated 12-well plate, as described previously (34). Values of binding
402 parameters were determined at equilibrium by Scatchard analysis of [³H]-aldosterone and [³H]-
403 dexamethasone binding to KC3AC1 cytosolic fractions as described previously (66).

404

405 **Western Blot Analyses**

406 Total protein extracts were prepared from KC3AC1 cells cultured on collagen I-coated Petri dishes
407 under isotonic, hyper- or hypotonic medium or from mice kidneys. Briefly, cells were lysed in lysis
408 buffer, as described previously (67). For ENaC western blotting, immunoblots were incubated
409 overnight in 5% milk-Tris buffer saline/ 0.1% Tween (TBST) before incubation with rabbit anti-
410 α , β , γ ENaC antibodies (1:5000) for 1 h at room temperature. After extensive washes, membranes
411 were incubated with a goat anti-rabbit peroxidase-conjugated second antibody (1:8000) for 1 h at room
412 temperature. For MR western blotting, immunoblots were incubated overnight in 5% milk- TBST
413 followed by incubation for 1 h at room temperature with anti-MR 39N (1:2000) and with peroxidase-
414 conjugated goat anti-rabbit antibody, dilution 1:15000 (Vector). Rabbit polyclonal anti-MR antibody
415 (39N) was generated using the human MR 1-18 peptide and purified by affinity chromatography
416 (Double X/XP boosting antibody production program, Eurogentec, Seraing, Belgium). For TonEBP
417 western blotting, blots were incubated at room temperature for 1 h with polyclonal anti-TonEBP
418 (1:2000) followed by incubation with peroxidase-conjugated goat anti-rabbit antibody, dilution
419 1:15000. For Tis11b and Tis 11d western blot blotting, immunoblots were incubated in 5% milk-
420 TBST for 1 h at room temperature. The blot was probed for 2 h in PBS containing 0.1% Tween with
421 antibodies to a C-terminal peptide fragment (amino acids 300-320) of TIS11b (1:1000 dilution) which
422 recognize both TIS11b and its family member TIS11d. The membrane was thoroughly washed with
423 the same buffer (3 x 10 min), and then incubated for 1 h with horseradish peroxidase (HRP)-labelled
424 goat anti-rabbit IgG. In all cases, membranes were washed and the antigen-antibody complex was
425 visualized by the ECL⁺ detection kit (GE Helthcare). For loading normalization, membranes were
426 incubated with anti α -Tubulin or anti β -actin antibody (Sigma).

427 **Immunocytochemical Analyses**

428 Immunodetection of the SV40 T Antigen was performed as previously described (68). For MR
429 immunodetection with 39N antibody (1:500), cells were cultured in Lab-Tek chambers (Nunc, Dutscher,
430 Brumath, France) and were processed as previously described (69).

431

432 **11 β Hydroxysteroid Dehydrogenase Type 2 Catalytic Activity**

433 KC3AC1 cells were seeded at 6×10^5 cells per well on collagen I-coated 12-well plates and were
434 cultured for 48 h in epithelial medium before incubation overnight in MM. 11 β HSD2 activity was
435 determined as previously described (34).

436

437 **Electrophysiological Studies**

438 The measurement of short-circuit current ($I_{sc}:\mu A \times cm^2$), transepithelial voltage ($V_T:mV$) and
439 transepithelial resistance ($R_T:\Omega \times cm^2$) were performed on KC3AC1 cells grown on collagen I-coated
440 Snapwell filters as previously described (27).

441

442 **Ions Measurements**

443 Cells were seeded on collagen I-coated Transwell filters and were cultured for 5 days in
444 epithelial medium. Thereafter, medium was replaced for 24 h by MM before hormonal
445 stimulation performed in this MM. The next day, K^+ , Na^+ and Cl^- ions were measured as
446 previously described (34).

447

448 **RT-PCR**

449 Total RNA was extracted from cells with TRIZOL reagent (Invitrogen) according to the
450 manufacturer's recommendations and RNA was thereafter processed for RT-PCR, as previously
451 described (34).

452 **Quantitative RT- PCR**

453 Briefly, 1 µg of total RNA was treated using the DNase I Amplification Grade procedure
454 (Invitrogen), then RNA was reverse-transcribed using the High Capacity cDNA RT kit from Applied
455 Biosystems. Samples were diluted 10-fold, then 1/20 of the reverse transcription reaction was used for
456 qRT-PCR using the Power SYBR[®] Green PCR Master Mix (Applied Biosystems). Final primer
457 concentrations were 300 nM for each primer. Reaction parameters were carried out on an ABI 7300
458 Sequence Detector (Applied Biosystems) and were as follows: 95°C for 10 min followed by 40 cycles
459 at 95°C for 15 sec and 60°C for 1 min. For preparation of standards, amplicons were purified from
460 agarose gel and subcloned into pGEMT-easy plasmid (Promega, Charbonnières-les-Bains, France),
461 then sequenced to verify the identity of each fragment. Standard curves were generated using serial
462 dilutions of linearized standard plasmids, spanning 6 orders of magnitude and yielding correlation
463 coefficients > 0.98 and efficiencies of at least 0.95, in all experiments. Standard and sample values
464 were determined in duplicate from 3 independent experiments. Relative expression within a given
465 sample was calculated as a ratio (amol of specific gene/fmol of 18S). Results are mean ± SEM and
466 present the relative expression compared to that obtained with control cells, which were arbitrary set
467 at 1. Table 1 indicates primer sequences of genes analyzed by RT-PCR and by qRT-PCR.

468

469 **Electrophoretic Mobility Shift Assays**

470 Differentiated KC3AC1 cells were starved for 3 h in MM and exposed to hypertonic stimulation.
471 Nuclear protein extraction and gel mobility shift assays using [³²P]-dCTP labeled double stranded
472 oligonucleotides (specific activity of approximately 10⁸ cpm/µg of DNA) were performed as described
473 elsewhere (70). Oligonucleotide sequences were as follows:

474 T1 TonE sense: 5'AGCTTGAAGTAGTGGAAAAATCCAATA-3' and T1 TonE antisense

475 5'AGCTTATTGGATTTTTCCACTAGTTCA-3';

476 T1M TonE mutant sense 5'AGCTTGAAGTTTGAAAAGCCAATA-3' and T1M TonE mutant

477 antisense 5'AGCTTATTGGCTTTTTCAACTAGTTCA;

478 T2 TonE sense 5'-TAACCGTGCTTGGAAAAACCCTTTA-3' and T2 TonE antisense 5'-

479 TGTTTAAGGGTTTTCCAGCACG-3'.

480

481 **Transfection Assays**

482 For transient transfections, cells were seeded in a 6-well plate at 5×10^5 cells per well in complete
483 medium. The next day, cells were transfected in Optimem Medium (Invitrogen) with
484 Lipofectamine 2000 (Invitrogen), according to the manufacturer's recommendations. After a 6 h
485 incubation period, medium was replaced by isotonic, hypertonic or hypotonic medium. The following
486 day, cells were harvested and β -galactosidase and luciferase assays were performed as described
487 elsewhere (71). Luciferase activities were normalized to β -galactosidase activities and expressed as a
488 percent of relative transcriptional activities compared with controls.

489

490 **Statistical Analyses**

491 Data are expressed as the mean \pm SEM. Mann Whitney U-test was used to determine significant
492 differences between two groups. For multiple comparisons, Kruskal-Wallis test followed by Dunn's
493 post-test was performed using the computer software Prism 4 (GraphPad Software, San Diego, CA).
494 Statistical significance is indicated at *P* values < 0.05 , 0.01 and 0.001 .

495 **Acknowledgments**

496 This work was supported by grants from Inserm, the University Paris-Sud 11 and from the Bonus
497 Qualite Recherche 2003 of the University Paris-Diderot. The authors are indebted to Jacqueline
498 Bauchet and the “Centre d’Exploration Fonctionnelle Intégrée” for ionic measurements (IFR Claude
499 Bernard, Paris), Annick Ganieux (IFR de Bicêtre, Le Kremlin Bicêtre) for plasmid preparation, and
500 Françoise Cluzeaud (CRB3, Inserm U773, Medical School Bichat, Paris) for her assistance in
501 electron-microscopy. The generous supplies of the Dominant negative-TonEBP plasmid together with
502 the TonEBP antibody from Pr H. Moo Kwon (University of Maryland, Baltimore, USA) are gratefully
503 acknowledged. Tis11b antibodies were kindly provided by Dr Martin Schmidlin from the University
504 of Basel, Switzerland.

References

1. **Bonvalet JP** 1998 Regulation of sodium transport by steroid hormones. *Kidney Int Suppl* 65:S49-56
2. **Viengchareun S, Le Menuet D, Martinerie L, Munier M, Pascual-Le Tallec L, Lombes M** 2007 The mineralocorticoid receptor: insights into its molecular and (patho)physiological biology. *Nucl Recept Signal* 5:e012
3. **Berger S, Bleich M, Schmid W, Cole TJ, Peters J, Watanabe H, Kriz W, Warth R, Greger R, Schutz G** 1998 Mineralocorticoid receptor knockout mice: pathophysiology of Na⁺ metabolism. *Proc Natl Acad Sci U S A* 95:9424-9429
4. **Ronzaud C, Loffing J, Bleich M, Gretz N, Grone HJ, Schutz G, Berger S** 2007 Impairment of sodium balance in mice deficient in renal principal cell mineralocorticoid receptor. *J Am Soc Nephrol* 18:1679-1687
5. **Sun Z, Bello-Roufai M, Wang X** 2008 RNAi inhibition of mineralocorticoid receptors prevents the development of cold-induced hypertension. *Am J Physiol Heart Circ Physiol* 294:H1880-1887
6. **Lim HY, van den Brandt J, Fassnacht M, Allolio B, Herold MJ, Reichardt HM** 2008 Silencing of the mineralocorticoid receptor by ribonucleic acid interference in transgenic rats disrupts endocrine homeostasis. *Mol Endocrinol* 22:1304-1311
7. **Geller DS, Zhang J, Zennaro MC, Vallo-Boado A, Rodriguez-Soriano J, Furu L, Haws R, Metzger D, Botelho B, Karaviti L, Haqq AM, Corey H, Janssens S, Corvol P, Lifton RP** 2006 Autosomal dominant pseudohypoaldosteronism type 1: mechanisms, evidence for neonatal lethality, and phenotypic expression in adults. *J Am Soc Nephrol* 17:1429-1436
8. **Le Menuet D, Isnard R, Bichara M, Viengchareun S, Muffat-Joly M, Walker F, Zennaro MC, Lombes M** 2001 Alteration of cardiac and renal functions in transgenic mice overexpressing human mineralocorticoid receptor. *J Biol Chem* 276:38911-38920
9. **Ouvrard-Pascaud A, Sainte-Marie Y, Benitah JP, Perrier R, Soukaseum C, Cat AN, Royer A, Le Quang K, Charpentier F, Demolombe S, Mechta-Grigoriou F, Beggah AT, Maison-Blanche P, Oblin ME, Delcayre C, Fishman GI, Farman N, Escoubet B, Jaisser F** 2005 Conditional mineralocorticoid receptor expression in the heart leads to life-threatening arrhythmias. *Circulation* 111:3025-3033

10. **Quinkler M, Zehnder D, Eardley KS, Lepenies J, Howie AJ, Hughes SV, Cockwell P, Hewison M, Stewart PM** 2005 Increased expression of mineralocorticoid effector mechanisms in kidney biopsies of patients with heavy proteinuria. *Circulation* 112:1435-1443
11. **Doucet A, Katz AI** 1981 Mineralocorticoid receptors along the nephron: [³H]aldosterone binding in rabbit tubules. *Am J Physiol* 241:F605-611
12. **Lombes M, Farman N, Oblin ME, Baulieu EE, Bonvalet JP, Erlanger BF, Gasc JM** 1990 Immunohistochemical localization of renal mineralocorticoid receptor by using an anti-idiotypic antibody that is an internal image of aldosterone. *Proc Natl Acad Sci U S A* 87:1086-1088
13. **Fenton RA, Knepper MA** 2007 Mouse models and the urinary concentrating mechanism in the new millennium. *Physiol Rev* 87:1083-1112
14. **Miyakawa H, Woo SK, Dahl SC, Handler JS, Kwon HM** 1999 Tonicity-responsive enhancer binding protein, a rel-like protein that stimulates transcription in response to hypertonicity. *Proc Natl Acad Sci U S A* 96:2538-2542
15. **Woo SK, Dahl SC, Handler JS, Kwon HM** 2000 Bidirectional regulation of tonicity-responsive enhancer binding protein in response to changes in tonicity. *Am J Physiol Renal Physiol* 278:F1006-1012
16. **Burg MB, Ferraris JD, Dmitrieva NI** 2007 Cellular response to hyperosmotic stresses. *Physiol Rev* 87:1441-1474
17. **Le Menuet D, Viengchareun S, Muffat-Joly M, Zennaro MC, Lombes M** 2004 Expression and function of the human mineralocorticoid receptor: lessons from transgenic mouse models. *Mol Cell Endocrinol* 217:127-136
18. **Massaad C, Houard N, Lombes M, Barouki R** 1999 Modulation of human mineralocorticoid receptor function by protein kinase A. *Mol Endocrinol* 13:57-65
19. **Vandewalle A, Lelongt B, Geniteau-Legendre M, Baudouin B, Antoine M, Estrade S, Chatelet F, Verroust P, Cassingena R, Ronco P** 1989 Maintenance of proximal and distal cell functions in SV40-transformed tubular cell lines derived from rabbit kidney cortex. *J Cell Physiol* 141:203-221

20. **Carballo E, Lai WS, Blackshear PJ** 2000 Evidence that tristetraprolin is a physiological regulator of granulocyte-macrophage colony-stimulating factor messenger RNA deadenylation and stability. *Blood* 95:1891-1899
21. **Ogilvie RL, Abelson M, Hau HH, Vlasova I, Blackshear PJ, Bohjanen PR** 2005 Tristetraprolin down-regulates IL-2 gene expression through AU-rich element-mediated mRNA decay. *J Immunol* 174:953-961
22. **Guhaniyogi J, Brewer G** 2001 Regulation of mRNA stability in mammalian cells. *Gene* 265:11-23
23. **Chen S, Grigsby CL, Law CS, Ni X, Nekrep N, Olsen K, Humphreys MH, Gardner DG** 2009 Tonicity-dependent induction of Sgk1 expression has a potential role in dehydration-induced natriuresis in rodents. *J Clin Invest* 119:1647-1658
24. **Verbalis JG, Drutarosky MD** 1988 Adaptation to chronic hypoosmolality in rats. *Kidney Int* 34:351-360
25. **Vandewalle A, Ronco P, Cassingena R** 1991 Establishment of permanent renal tubule cell lines by infection with the wild-type and a thermosensitive mutant of the simian virus 40. *Am J Kidney Dis* 17:619-621
26. **Prie D, Friedlander G, Coureau C, Vandewalle A, Cassingena R, Ronco PM** 1995 Role of adenosine on glucagon-induced cAMP in a human cortical collecting duct cell line. *Kidney Int* 47:1310-1318
27. **Blot-Chabaud M, Laplace M, Cluzeaud F, Capurro C, Cassingena R, Vandewalle A, Farman N, Bonvalet JP** 1996 Characteristics of a rat cortical collecting duct cell line that maintains high transepithelial resistance. *Kidney Int* 50:367-376
28. **Bens M, Vallet V, Cluzeaud F, Pascual-Letallec L, Kahn A, Rafestin-Oblin ME, Rossier BC, Vandewalle A** 1999 Corticosteroid-dependent sodium transport in a novel immortalized mouse collecting duct principal cell line. *J Am Soc Nephrol* 10:923-934
29. **Djelidi S, Beggah A, Courtois-Coutry N, Fay M, Cluzeaud F, Viengchareun S, Bonvalet JP, Farman N, Blot-Chabaud M** 2001 Basolateral translocation by vasopressin of the aldosterone-induced pool of latent Na-K-ATPases is accompanied by alpha1 subunit dephosphorylation: study

- in a new aldosterone-sensitive rat cortical collecting duct cell line. *J Am Soc Nephrol* 12:1805-1818
30. **Gaeggeler HP, Gonzalez-Rodriguez E, Jaeger NF, Loffing-Cueni D, Norregaard R, Loffing J, Horisberger JD, Rossier BC** 2005 Mineralocorticoid versus glucocorticoid receptor occupancy mediating aldosterone-stimulated sodium transport in a novel renal cell line. *J Am Soc Nephrol* 16:878-891
 31. **Bens M, Chassin C, Vandewalle A** 2006 Regulation of NaCl transport in the renal collecting duct: lessons from cultured cells. *Pflugers Arch* 453:133-146
 32. **Chassin C, Bens M, Vandewalle A** 2007 Transimmortalized proximal tubule and collecting duct cell lines derived from the kidneys of transgenic mice. *Cell Biol Toxicol* 23:257-266
 33. **Zennaro MC, Le Menuet D, Viengchareun S, Walker F, Ricquier D, Lombès M** 1998 Hibernoma development in transgenic mice identifies brown adipose tissue as a novel target of aldosterone action. *J Clin Invest* 101:1254-1260.
 34. **Teixeira M, Viengchareun S, Butlen D, Ferreira C, Cluzeaud F, Blot-Chabaud M, Lombes M, Ferrary E** 2006 Functional IsK/KvLQT1 potassium channel in a new corticosteroid-sensitive cell line derived from the inner ear. *J Biol Chem* 281:10496-10507
 35. **Alfaidy N, Blot-Chabaud M, Robic D, Kenouch S, Bourbouze R, Bonvalet JP, Farman N** 1995 Characteristics and regulation of 11 beta-hydroxysteroid dehydrogenase of proximal and distal nephron. *Biochim Biophys Acta* 1243:461-468
 36. **Bocchi B, Fagart J, Cluzeaud F, Fay M, Rafestin-Oblin ME, Farman N** 2003 Glucocorticoid metabolism by 11-beta hydroxysteroid dehydrogenase type 2 modulates human mineralocorticoid receptor transactivation activity. *J Steroid Biochem Mol Biol* 84:239-244
 37. **Masilamani S, Kim GH, Mitchell C, Wade JB, Knepper MA** 1999 Aldosterone-mediated regulation of ENaC alpha, beta, and gamma subunit proteins in rat kidney. *J Clin Invest* 104:R19-23
 38. **Chen SY, Bhargava A, Mastroberardino L, Meijer OC, Wang J, Buse P, Firestone GL, Verrey F, Pearce D** 1999 Epithelial sodium channel regulated by aldosterone-induced protein sgk. *Proc Natl Acad Sci U S A* 96:2514-2519

39. **Naray-Fejes-Toth A, Canessa C, Cleaveland ES, Aldrich G, Fejes-Toth G** 1999 *sgk* is an aldosterone-induced kinase in the renal collecting duct. Effects on epithelial Na^+ channels. *J Biol Chem* 274:16973-16978
40. **Zennaro MC, Keightley MC, Kotelevtsev Y, Conway GS, Soubrier F, Fuller PJ** 1995 Human mineralocorticoid receptor genomic structure and identification of expressed isoforms. *J Biol Chem* 270:21016-21020
41. **Le Menuet D, Viengchareun S, Penfornis P, Walker F, Zennaro MC, Lombès M** 2000 Targeted oncogenesis reveals a distinct tissue-specific utilization of alternative promoters of the human mineralocorticoid receptor gene in transgenic mice. *J Biol Chem* 275:7878-7886
42. **Pascual-Le Tallec L, Lombes M** 2005 The mineralocorticoid receptor: a journey exploring its diversity and specificity of action. *Mol Endocrinol* 19:2211-2221
43. **Ferraris JD, Burg MB** 2007 Tonicity-regulated gene expression. *Methods Enzymol* 428:279-296
44. **Kim JA, Jeon US, Kwon MS, Lim SW, Kwon HM** 2007 Transcriptional activator TonE-binding protein in cellular protection and differentiation. *Methods Enzymol* 428:253-267
45. **Woo SK, Lee SD, Na KY, Park WK, Kwon HM** 2002 TonEBP/NFAT5 stimulates transcription of HSP70 in response to hypertonicity. *Mol Cell Biol* 22:5753-5760
46. **Hasler U, Jeon US, Kim JA, Mordasini D, Kwon HM, Feraille E, Martin PY** 2006 Tonicity-responsive enhancer binding protein is an essential regulator of aquaporin-2 expression in renal collecting duct principal cells. *J Am Soc Nephrol* 17:1521-1531
47. **Nakayama Y, Peng T, Sands JM, Bagnasco SM** 2000 The TonE/TonEBP pathway mediates tonicity-responsive regulation of UT-A urea transporter expression. *J Biol Chem* 275:38275-38280
48. **Lanaspa MA, Almeida NE, Andres-Hernando A, Rivard CJ, Capasso JM, Berl T** 2007 The tight junction protein, MUPP1, is up-regulated by hypertonicity and is important in the osmotic stress response in kidney cells. *Proc Natl Acad Sci U S A* 104:13672-13677
49. **Lanaspa MA, Andres-Hernando A, Rivard CJ, Dai Y, Berl T** 2008 Hypertonic stress increases claudin-4 expression and tight junction integrity in association with MUPP1 in IMCD3 cells. *Proc Natl Acad Sci U S A* 105:15797-15802

50. **Waldegger S, Barth P, Raber G, Lang F** 1997 Cloning and characterization of a putative human serine/threonine protein kinase transcriptionally modified during anisotonic and isotonic alterations of cell volume. *Proc Natl Acad Sci U S A* 94:4440-4445
51. **Bell LM, Leong ML, Kim B, Wang E, Park J, Hemmings BA, Firestone GL** 2000 Hyperosmotic stress stimulates promoter activity and regulates cellular utilization of the serum- and glucocorticoid-inducible protein kinase (Sgk) by a p38 MAPK-dependent pathway. *J Biol Chem* 275:25262-25272
52. **McCormick JA, Bhalla V, Pao AC, Pearce D** 2005 SGK1: a rapid aldosterone-induced regulator of renal sodium reabsorption. *Physiology (Bethesda)* 20:134-139
53. **Cherradi N, Lejczak C, Desroches-Castan A, Feige JJ** 2006 Antagonistic functions of tetradecanoyl phorbol acetate-inducible-sequence 11b and HuR in the hormonal regulation of vascular endothelial growth factor messenger ribonucleic acid stability by adrenocorticotropin. *Mol Endocrinol* 20:916-930
54. **Duan H, Cherradi N, Feige JJ, Jefcoate C** 2009 cAMP-dependent posttranscriptional regulation of steroidogenic acute regulatory (STAR) protein by the zinc finger protein ZFP36L1/TIS11b. *Mol Endocrinol* 23:497-509
55. **Miyazaki H, Shiozaki A, Niisato N, Marunaka Y** 2007 Physiological significance of hypotonicity-induced regulatory volume decrease: reduction in intracellular Cl⁻ concentration acting as an intracellular signaling. *Am J Physiol Renal Physiol* 292:F1411-1417
56. **Niisato N, Post M, Van Driessche W, Marunaka Y** 1999 Cell swelling activates stress-activated protein kinases, p38 MAP kinase and JNK, in renal epithelial A6 cells. *Biochem Biophys Res Commun* 266:547-550
57. **Taruno A, Niisato N, Marunaka Y** 2007 Hypotonicity stimulates renal epithelial sodium transport by activating JNK via receptor tyrosine kinases. *Am J Physiol Renal Physiol* 293:F128-138
58. **Tamma G, Procino G, Svelto M, Valenti G** 2007 Hypotonicity causes actin reorganization and recruitment of the actin-binding ERM protein moesin in membrane protrusions in collecting duct principal cells. *Am J Physiol Cell Physiol* 292:C1476-1484

59. **Vinciguerra M, Arnaudeau S, Mordasini D, Rousselot M, Bens M, Vandewalle A, Martin PY, Hasler U, Feraille E** 2004 Extracellular hypotonicity increases Na,K-ATPase cell surface expression via enhanced Na⁺ influx in cultured renal collecting duct cells. *J Am Soc Nephrol* 15:2537-2547
60. **Niisato N, Taruno A, Marunaka Y** 2007 Involvement of p38 MAPK in hypotonic stress-induced stimulation of beta- and gamma-ENaC expression in renal epithelium. *Biochem Biophys Res Commun* 358:819-824
61. **Rozansky DJ, Wang J, Doan N, Purdy T, Faulk T, Bhargava A, Dawson K, Pearce D** 2002 Hypotonic induction of SGK1 and Na⁺ transport in A6 cells. *Am J Physiol Renal Physiol* 283:F105-113
62. **Taruno A, Niisato N, Marunaka Y** 2008 Intracellular calcium plays a role as the second messenger of hypotonic stress in gene regulation of SGK1 and ENaC in renal epithelial A6 cells. *Am J Physiol Renal Physiol* 294:F177-186
63. **Kiilerich P, Kristiansen K, Madsen SS** 2007 Hormone receptors in gills of smolting Atlantic salmon, *Salmo salar*: expression of growth hormone, prolactin, mineralocorticoid and glucocorticoid receptors and 11beta-hydroxysteroid dehydrogenase type 2. *Gen Comp Endocrinol* 152:295-303
64. **Morel F, Butlen D** 1990 Hormonal receptors in the isolated tubule. *Methods Enzymol* 191:303-325
65. **Martinerie L, Viengchareun S, Delezoide AL, Jaubert F, Sinico M, Prevot S, Boileau P, Meduri G, Lombes M** 2009 Low Renal Mineralocorticoid Receptor Expression at Birth Contributes to Partial Aldosterone Resistance in Neonates. *Endocrinology* 150:4414-24
66. **Lombes M, Oblin ME, Gasc JM, Baulieu EE, Farman N, Bonvalet JP** 1992 Immunohistochemical and biochemical evidence for a cardiovascular mineralocorticoid receptor. *Circ Res* 71:503-510
67. **Viengchareun S, Bouzinba-Segard H, J-P Laigneau JP, Zennaro MC, Kelly PA, Bado A, Lombes M, Binart N** 2004 Prolactin potentiates insulin-stimulated leptin expression and release from differentiated brown adipocytes. *J Mol Endocrinol* 33:679-691

68. **Viengchareun S, Servel N, Feve B, Freemark M, Lombes M, Binart N** 2008 Prolactin receptor signaling is essential for perinatal brown adipocyte function: a role for insulin-like growth factor-2. *PLoS ONE* 3:e1535
69. **Kamenicky P, Viengchareun S, Blanchard A, Meduri G, Zizzari P, Imbert-Teboul M, Doucet A, Chanson P, Lombes M** 2008 Epithelial sodium channel is a key mediator of growth hormone-induced sodium retention in acromegaly. *Endocrinology* 149:3294-3305
70. **Lombes M, Binart N, Oblin ME, Joulin V, Baulieu EE** 1993 Characterization of the interaction of the human mineralocorticosteroid receptor with hormone response elements. *Biochem J* 292:577-583.
71. **Pascual-Le Tallec L, Kirsh O, Lecomte MC, Viengchareun S, Zennaro MC, Dejean A, Lombes M** 2003 PIAS1 interacts with the N-terminal domain of mineralocorticoid receptor and represses its transcriptional activity - Implication of SUMO-1 modification. *Mol Endocrinol* 17:2529-2542

<i>Name</i>	<i>Accession number</i>	<i>Amplicon</i>	<i>Sense primer</i>	<i>Antisense primer</i>
<i>Primers for RT-PCR</i>				
α ENaC	AF112185	555 bp	CTAATGATGCTGGACCACACC	AAAGCGTCTGTTCCGTGATGC
β ENaC	AF112186	631 bp	GCCAGTGAAGAAGTACCTGC	CCTGGGTGGCACTGGTGAA
γ ENaC	AF112187	670 bp	AAGAATCTGCCGGTTCGAGGC	TACCACTCCTGGATGGCATTG
<i>Primers for quantitative RT-PCR</i>				
18S	X00686	66 bp	CCCTGCCCTTTGTACACACC	CGATCCGAGGGCCTCACTA
mMR	M36074	153 bp	ATGGAAACCACACGGTGACCT	AGCCTCATCTCCACACACCAAG
α ENaC	AF112185	150 bp	GGACTGGAAAATCGGCTTCC	TAGAGCAGGCGAGGTGTCG
Sgk1	AF205855	150 bp	TCACTTCTCATTCCAGACCGC	ATAGCCCAAGGCACTGGCTA
TonEBP	AY050663	127 bp	CCTCTCCTCACCGTCGTCATC	CTCCACCTCCTTCTCATTCTG
AldoR	BC085310	79 bp	GGCCGTGAAAAGTTGCTATTGA	GGTCCCGGGCTGTGAGAT
Tis11b	NM_007564.3	100 bp	CGACACACCAGATCCTAGTCCTT	TGCATAAAAACCTTCGCTCAAGTCA

Table 1 : Primer sequences of genes analyzed in RT-PCR and quantitative RT-PCR

The abbreviations of the genes, their GENBANK or NCBI accession number and 5'- to 3'- nucleotide sequences of the sense and antisense primers are presented.

516 **Figure Legends**

517 **Figure 1: Expression of Functional MR in the Immortalized KC3AC1 Cell Line**

518 **A:** Phase-contrast micrograph of confluent KC3AC1 cells grown on a Petri dish coated with collagen I
519 (passage 6). Cells form islets of characteristic epithelial cells and rapidly develop large and numerous
520 domes, suggesting vectorial transepithelial hydroelectrolytic transport (optical lens x 20). Strong
521 nuclear immunostaining was observed in all cells with a monoclonal anti-TAg antibody, confirming
522 that KC3AC1 cells are immortalized (insert). **B:** Electron microscopic micrographs of KC3AC1 cells
523 grown on filters forming monolayers of epithelioid cells and presenting microvilli at the apical side of
524 the membrane and a desmosome (see insert). **C:** Scatchard analysis. Cytosolic fractions of KC3AC1
525 cells (passage 9) were incubated with increasing concentrations of [³H]-aldosterone for 4 h at 4°C.
526 Bound (B) and unbound (U) hormone were separated by the charcoal-dextran technique. U was
527 calculated as the difference between total and bound hormone concentration. The curve was simulated
528 from the best model of interaction ($B=nU/(K_d+U)+\beta U$), where n is the maximum number of specific
529 binding sites, K_d is the dissociation constant, and β is the constant of nonspecific binding. Mean
530 values are given with the corresponding intraexperimental SD. Experimental points are means of
531 triplicate determinations. **D:** Whole cell binding. Cells were incubated in minimum medium (MM)
532 24 h prior to the experiment. Then, 10 nM [³H]-aldosterone with and without a 100-fold excess of
533 unlabeled aldosterone (Aldo) or spironolactone (Spiro) was added to cells. After 1 h incubation at
534 37°C, cells were rinsed twice with ice cold PBS, lysed with cold ethanol and the radioactivity was
535 counted. Data represent means \pm SEM of 3 independent determinations. Statistical significance: ***
536 $P<0.001$. **E, F:** Immunocytochemical detection of MR in KC3AC1 cells incubated in the absence (C)
537 or presence of 10 nM aldosterone for 30 min (D). Note the presence of cytoplasmic labelling when
538 cells were incubated in MM and nuclear labelling in presence of the ligand. **G:** Western blot analysis
539 of MR expression in KC3AC1 cells. Twenty to thirty μ g of proteins from KC3AC1 cell homogenates
540 were processed for immunoblotting with anti-MR 39N antibody. Note the presence of a band of ~130
541 kDa in KC3AC1 cell lysates. This band was clearly immunodetected in M cells lysates, which stably
542 overexpress MR, but were absent in the parental RCSV3 cell lysates, which do not express MR. **H:**
543 HPLC analysis. Cells were incubated for 1 h with 10 nM [³H]-corticosterone [B], and time-dependent

544 production of [³H]-dehydrocorticosterone [A] was measured by HPLC and the radioactivity of each
545 fraction was counted. Upper panel presents a typical chromatogram obtained after HPLC analysis.
546 Compound [B] was recovered between fractions 12 and 16 whereas compound [A] was recovered
547 between fractions 4 and 7. Lower panel: cofactor specificity of 11 β HSD2 dehydrogenase activity was
548 assessed in permeabilized KC3AC1 cells after incubation with 10 nM [³H]-corticosterone in the
549 absence or presence of 1 mM NADP or 1 mM NAD.

550

551 **Figure 2: Aldosterone Stimulates Na⁺ Reabsorption in KC3AC1 Cells Through ENaC**

552 **A:** RT-PCR was performed as described in the Materials and Methods section and allowed the
553 detection of a 555 bp (αENaC), a 631 bp (βENaC) and a 670 bp (γENaC) amplicon. **B:** Western Blot
554 analysis allowed the detection of the three subunits of ENaC at 90 kDa (αENaC), 92 kDa (βENaC)
555 and 85 kDa (γENaC). **C:** Electrophysiological studies. Cells were grown on Snapwell filters in
556 minimum medium (MM) for 24 h. The short-circuit current ($I_{sc}; \mu A \times cm^2$), transepithelial voltage
557 ($V_T; mV$) and transepithelial resistance ($R_T; \Omega \times cm^2$) were measured as previously described (29).
558 When 10^{-5} M amiloride was applied at the apical side of the KC3AC1 cells, the short circuit current
559 was almost completely abolished within minutes. **D:** Cells, cultured on Snapwell filters as described
560 above, were incubated for 18 h in the absence (Control) or presence of 10^{-8} M aldosterone (+Aldo).
561 Each point represents mean \pm SEM of at least three independent determinations performed in duplicate
562 or in triplicate. Statistical significance: ** $P < 0.01$. **E:** Ionic measurements. Cells were seeded on
563 collagen I-coated Transwell filters and were cultured for 5 days in the epithelial medium. Cells were
564 rinsed with PBS then were cultivated in MM for 24 h in the presence of 10^{-8} M aldosterone (+Aldo) or
565 10^{-8} M aldosterone + 10^{-6} M spironolactone (+Aldo + Spiro). A= apical ; B= basolateral. 400 μ l of the
566 supernatant was recovered from the medium bathing the apical and basolateral surface of the
567 KC3AC1 cells. Na⁺ (left panel) and K⁺ ions (right panel) were then measured by the “Centre
568 d’Experimentation Fontionnelle Intégrée” of the IFR Claude Bernard (Paris, France) on an Olympus
569 automate. Data represent means \pm SEM of 3 independent determinations. Statistical significance:
570 * $P < 0.05$, and *** $P < 0.001$. **F:** qRT-PCR analysis of sgk1, and αENaC mRNA expression in
571 KC3AC1 cells after aldosterone treatment. Highly differentiated KC3AC1 cells were grown on filters

572 for 24 h in MM and subsequently treated for various periods of time (0, 0.5, 1, 2, 4, 6 or 24 h) with
573 10 nM aldosterone. Results were normalized by the amplification of 18S RNA and are expressed as
574 relative fold induction compared to basal conditions. Data represent means \pm SEM of at least three
575 independent determinations performed in duplicate. Statistical significance: * $P < 0.05$, ** $P < 0.01$ and
576 *** $P < 0.001$.

577

578 **Figure 3: Analysis of MR Transcript and Protein Stability**

579 **A:** Analysis of MR transcript by qRT-PCR. Differentiated KC3AC1 cells were incubated for various
580 periods of time in the absence or in the presence of 0.4 μ M actinomycin D. Total RNA was extracted
581 and processed for qRT-PCR. Results were normalized by the amplification of 18S RNA and were
582 expressed as relative to the control. **B, C:** Western blot analysis of MR expression in KC3AC1 cells.
583 B) Differentiated KC3AC1 cells were incubated for 18 h in the absence or in presence of 0.4 μ M
584 actinomycin D. C) Differentiated KC3AC1 cells were incubated for various periods of time in the
585 absence or in the presence of cycloheximide (5 μ g/ml). Twenty μ g of protein from KC3AC1 cell
586 homogenates were processed for immunoblotting with anti-MR 39N antibody (1/2000). α -Tubulin
587 was used as loading control. MR was normalized to α -Tubulin protein levels after digitalization on a
588 gel scanner using QuantityOne software (Bio-Rad, Marnes-la-Coquette, France). Results are presented
589 as ratio MR/ α -Tubulin and as % of control.

590

591 **Figure 4: Hypertonic Stress Inhibits MR Expression**

592 **A, B:** Phase-contrast micrographs of a monolayer of differentiated KC3AC1 cells incubated in the
593 absence (A) or in presence of 0.2 M raffinose (B) for 6 h. Note the absence of domes in raffinose
594 treated-cells. **C, E:** Hypertonicity stimulates TonEBP and Aldose Reductase expression. Differentiated
595 KC3AC1 cells were incubated in the absence or the presence of 0.2 M raffinose for 6 h. mRNA
596 expression was measured by qRT-PCR. Results are expressed as amol/fmol of 18S are mean \pm SEM
597 of 30 to 36 independent determinations. Statistical significance: *, $P < 0.05$; **, $P < 0.01$;
598 ***, $P < 0.001$. **D:** Western Blot analysis of TonEBP in KC3AC1 cell lysates exposed for 18 h to
599 raffinose. Fifteen μ g of protein from KC3AC1 cell homogenates were processed for immunoblotting
600 with anti-TonEBP SHN50 antibody. Note the presence of a specific band of \sim 180 kDa in KC3AC1

601 cell lysates exposed to raffinose. **F, G:** Analysis of MR transcript by qRT-PCR. F) Differentiated
602 KC3AC1 cells were incubated for various periods of time in the absence or presence of 0.2 M
603 raffinose. G) Differentiated KC3AC1 cells were incubated for 6 h in the absence (Iso) or in the
604 presence of 0.2 M raffinose (Hyper) and/or cycloheximide (Cyclo, 5 µg/ml). Total RNA was extracted
605 and processed for qRT-PCR as described in Fig. 3. Data represent means ± SEM of at least three
606 independent determinations performed in duplicate. Statistical significance: * $P < 0.05$ and
607 *** $P < 0.001$. **H:** Western blot analysis of MR expression in KC3AC1 cells. Differentiated KC3AC1
608 cells were incubated for 18 h in the absence (Iso) or in the presence of 0.2 M raffinose (Hyper) and/or
609 cycloheximide (5 µg/ml). Western blot analyses were performed as described in Fig. 3.

610

611 **Figure 5: Identification of *Tis11b* as a Direct Tonicity-induced Target Gene**

612 **A:** Localization of TonE responsive elements in the *Tis11b* gene 5'-flanking region. Grey boxes
613 indicate the two exons of the gene. TonE consensus sequence is presented. Four TonE (TA to TD)
614 were located in the promoter, at position -1596 bp, -1167 bp, -833 bp, and -660 bp, respectively,
615 upstream of the translational ATG start site of exon 1. Note that the TonE, TB to TD, are conserved
616 between human, mouse and rat species. **B:** Hypertonicity stimulates TonEBP and *Tis11b* expression.
617 Differentiated KC3AC1 cells were incubated in the absence or the presence of 0.2 M raffinose for
618 various periods of time up to 6 h. mRNA expression was measured by qRT-PCR. Results are
619 expressed as amol/fmol of 18S are mean ± SEM of three independent determinations performed in
620 duplicate. Statistical significance: **, $P < 0.01$; ***, $P < 0.001$. **C:** Transcriptional control of *Tis11b*
621 expression under hypertonicity. Differentiated KC3AC1 cells were incubated for 6 h in the absence
622 (Iso) or in the presence of 0.2 M raffinose (Hyper) and/or 0.4 µM actinomycin D (Actino). Data
623 represent means ± SEM of at least three independent determinations performed in duplicate. Statistical
624 significance: ** $P < 0.01$ and *** $P < 0.001$. **D:** Western Blot analysis of *Tis11b* in KC3AC1 cell
625 lysates exposed for 18 h to raffinose. Thirty µg of protein from KC3AC1 cell homogenates were
626 processed for immunoblotting using a polyclonal antibody that recognizes both *Tis11d* and *Tis11b*
627 isoforms. **E:** Localization of AU-rich elements (AURE) in the 3'-UTR of MR transcript. Grey boxes
628 indicate the last exons 8 and 9 of *hMR* gene. AURE consensus sequence is presented. Six AURE (A1
629 to A6) were identified in the 3'-UTR of MR transcript, at position 599 bases (b), 850 b, 933 b, 972 b,

630 2041b and 2456 b respectively, downstream of the stop codon TGA in exon 9 of human *MR* gene.

631 Note that AURE A1 to A4 are conserved among human, mouse and rat species.

632

633 **Figure 6: Hypotonic Stress Stimulates MR Expression**

634 **A, B:** Phase-contrast micrographs of monolayers of differentiated KC3AC1 cells incubated for 6 h
635 with isotonic (A) or a hypotonic medium (B). Note that the domes seemed larger in KC3AC1 cells
636 incubated under hypotonic conditions.

637 **C, D:** Analysis of MR transcripts by qRT-PCR. C) Differentiated KC3AC1 cells were incubated for 6
638 h with isotonic (Iso) or a hypotonic medium (Hypo). D) Differentiated KC3AC1 cells were incubated
639 for various periods of time under hypotonic conditions (Hypo) and/or in the presence of 0.4 μ M
640 actinomycin D (Actino). Total RNA was extracted and processed for qRT-PCR as described in Fig. 3.
641 Statistical significance: *** $P < 0.001$.

642 **E:** Western blot analysis of MR expression in KC3AC1 cells. Differentiated KC3AC1 cells were
643 incubated for 18 h with isotonic (Iso) or hypotonic medium (Hypo) and/or in the presence of 0.4 μ M
644 actinomycin D (Actino). Western blot analyses were performed as described in Fig. 3.

645

646 **Figure 7: Renal MR Expression is Altered by Water Deprivation and Water Intoxication in** 647 **Mice**

648 Fifty μ g protein from kidney homogenates obtained from the 3 groups (control, water-deprived, and
649 water-intoxicated mice, 3 animals per condition) were loaded on 7.5% SDS-PAGE followed by direct
650 immunoblotting with anti-MR 39N antibody (1/4000). α -Tubulin was used as loading control. MR
651 protein levels were normalized to those of α -Tubulin after digitalization on a gel scanner using
652 QuantityOne software (BioRad). Results are means \pm SEM of at least 3 independent determinations
653 and are expressed relative to MR expression measured in the control group, arbitrarily set at 1.
654 Statistical significance: * $P < 0.05$ and ** $P < 0.01$.