

## Low Renal Mineralocorticoid Receptor Expression at Birth Contributes to Partial Aldosterone Resistance in Neonates

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**Abbreviated title:** MR and renal maturation.

**Precis:** Our study provides first evidence for a low renal mineralocorticoid receptor expression level at birth, in human and mouse, which could account for the physiological partial aldosterone resistance in neonates.

**Key words:** mineralocorticoid receptor, aldosterone, renal development.

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## ABSTRACT

The human neonatal period is characterized by renal immaturity with impaired capacity to regulate water and sodium homeostasis, resembling partial aldosterone resistance. Since aldosterone effects are mediated by the mineralocorticoid receptor (MR), we postulated that this hormonal unresponsiveness could be related to low MR expression in the distal nephron. We measured aldosterone and renin levels in umbilical cord blood of healthy newborns. We used qPCR and immunohistochemistry to analyze the expression of MR and key players of the mineralocorticoid signaling pathway, during human and mouse renal development. High aldosterone and renin levels were found at birth. MR mRNA was detected in mouse kidney at day 16 postcoitum (E16), peaking at E18, but its expression was surprisingly very low at birth, rising progressively afterwards. Similar biphasic temporal expression was observed during human renal embryogenesis, with a transient expression between 15 and 24 weeks of gestation but an undetectable immunoreactive MR in late gestational and neonatal kidneys. This cyclic MR expression was tightly correlated with the evolution of the 11 $\beta$ -hydroxysteroid dehydrogenase type 2 and the epithelial sodium channel  $\alpha$ -subunit. In contrast, glucocorticoid and vasopressin receptors, and aquaporin 2 followed a progressive and sustained evolution during renal maturation. Our study provides first evidence for a low renal MR expression level at birth, despite high aldosterone levels, which could account for compromised postnatal sodium handling. Elucidation of regulatory mechanisms governing MR expression should lead to new strategies for the management of sodium waste in preterms and neonates.

## INTRODUCTION

In mammals, sodium and water homeostasis is mainly controlled by the kidney. In human neonates, the kidney exhibits a certain degree of tubular immaturity, observed during the first months of life, which is responsible for impaired sodium and water reabsorption. This may predispose to failure to thrive and dehydration under pathological circumstances (1). It has been suggested that alteration of sodium reabsorption during perinatal life could be related to a partial and transient tubular resistance to aldosterone (2). Indeed, high plasma levels of aldosterone have been described throughout the first year of life and reached normal adult values at 12 months of age (3). Numerous cases of transient pseudohypoaldosteronism have also been reported, all in neonates under 3 months of age who presented with dehydration associated with hyponatremia, hyperkalemia and high plasma aldosterone and renin levels, during an episode of pyelonephritis. Clinical symptoms and biological parameters normalized after resolution of the infection (4, 5). All these observations suggest a partial renal unresponsiveness to aldosterone in the newborn, whose pathogenesis has never been determined.

Most aldosterone effects are mediated by the mineralocorticoid receptor (MR), a transcription factor member of the nuclear receptor family (6). The human MR gene (*hMR*) is localized on chromosome 4 in the q31.1 region (7) and spans over approximately 450 kb (8). The gene is composed of ten exons. The first two exons, 1 $\alpha$  and 1 $\beta$ , are untranslated and their alternative transcription gives rise to two splice variants: MR $\alpha$  and MR $\beta$ , expressed in various human tissues including the kidney (9). Importantly, both isoforms encode the same MR protein (6).

In the cortical collecting duct (CCD) cells, aldosterone, by binding to MR, regulates various genes implicated in sodium and water transport such as the  $\alpha$  subunit of the epithelial sodium channel ( $\alpha$ ENaC) (10), Na-K-ATPase (11) and aquaporin 2 (AQP2) (12). In epithelial cells, the enzyme 11 $\beta$ -hydroxysteroid dehydrogenase type 2 (11 $\beta$ HSD2) controls, at a pre-receptor level, the selectivity of the aldosterone-MR signaling pathway by

metabolizing cortisol into cortisone which is unable to bind to MR (13, 14). Two other proteins expressed in CCD cells are important for sodium and water homeostasis: the glucocorticoid receptor (GR), which shares ligands, hormone responsive elements and molecular partners with MR (6), and the arginine-vasopressin receptor (V2R), which regulates AQP2 intracellular shuttling (15).

Very little is known about the sequential organization and regulation of these different markers of epithelial CCD cells during embryogenesis. Renal ontogenesis in mammals comprises the succession of three different embryonic structures, the pronephros, mesonephros and metanephros (16). Only the latter will develop and form the adult kidney. In human and mouse, renal ontogenesis takes place during different periods of development. In the mouse, renal formation starts at 8 days postcoitum and is completed 7 days after birth (mouse gestation is 20-21 days long), whereas in human, kidney formation occurs during gestation between 3 and 36 weeks (16).

We hypothesized that the partial and transient aldosterone resistance observed in neonates might be due to a late onset or variable MR expression during renal development. No quantitative investigation of MR expression during the perinatal period has ever been published. Here, we report direct evidence for the activation of the renin-angiotensin-aldosterone system in newborn infants. We also performed a comprehensive analysis of mouse and human MR mRNA and protein expression during renal ontogenesis, using quantitative real time PCR (qPCR) analysis and immunohistochemistry. In parallel, we have studied the expression of the different MR mRNA isoforms (MR $\alpha$  and MR $\beta$ ), as well as other molecular determinants of the mineralocorticoid pathway ( $\alpha$ ENaC and 11 $\beta$ HSD2) and other important factors implicated in sodium and water transport (GR, V2R and AQP2).

We provide first evidence that MR is expressed at extremely low levels in human and mouse kidneys during the perinatal period, notwithstanding high aldosterone levels. Such a finding is an important step towards a better understanding of the physiology of neonatal sodium and water handling and of the

pathogenesis of aldosterone resistance in newborns.

## MATERIALS AND METHODS

### *Aldosterone and renin measurements*

Aldosterone and renin were measured in surplus plasma of umbilical cords taken for diagnostic purposes of 48 full term eutrophic newborns (24 females and 24 males) immediately after delivery. Informed and written consent was obtained from their mothers. Hormonal levels were assessed using radioimmunoassay kits, the Aldo-Riact, (Cisbio international, Gif sur Yvette, France), and the Renin III generation (Cisbio, Gif sur Yvette, France).

### *Mouse and human renal samples*

Wild-type mouse kidneys were collected at different developmental stages from 15 days of gestation to 15 days postnatal. For each animal, one kidney was snap-frozen in liquid nitrogen for qPCR analyses or Western Blot and the other one was fixed in buffered formol and embedded in paraffin for immunohistochemistry. Animal housing and experiments were conducted according to the French legislation.

Thirty-seven archival formol-fixed paraffin-embedded fetal, neonatal and infantile kidneys were selected from the collections of several departments of pathology according to the French legislation (Table 1). Quality and integrity of human samples were verified by immunostaining with vimentin (as a control of appropriate formol fixation) and a low molecular weight cytokeratin (as a control of epithelial tubular cells integrity). The human adult kidney sample, used as a positive control, was obtained from the healthy part of a nephrectomy for cancer, with the patient's written consent, in adherence to the Declaration of Helsinki.

Snap-frozen kidney samples from nineteen fetuses aged from 14 to 40 weeks (wk) of gestation, obtained from the Foetopathology Department of Robert Debre Hospital were used for qPCR (Table 1). All fetal samples were collected after parental informed and written consent and after declaration to the French Biomedical Agency (Decree n°003812, 09/22/2006).

### *Primary Antibodies*

Four different antibodies were used: monoclonal anti-MR clone 6G1, generously provided by Dr Gomez-Sanchez (University of Mississippi, USA) (17), polyclonal anti-mouse GR (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), monoclonal anti-human GR (AbCys SA, Paris, France) and monoclonal anti-AQP2 (Santa Cruz) (18). Dilutions used were 1:120, 1:400, 1:10 and 1:1000, respectively. Monoclonal anti-vimentin clone V9 (Biogenex Laboratories, San Ramon, CA) and anti-keratin 19 antibodies (Progen, Queensland, Australia) were used at 1:120 dilution. For MR western blot analysis, rabbit polyclonal anti-MR antibody (39N) was generated using the human MR 1-18 peptide and purified by affinity chromatography (Double X/XP boosting antibody production program, Eurogentec, Seraing, Belgium).

### *Immunohistochemistry*

Five  $\mu\text{m}$ -thick tissue sections were deparaffinized and rehydrated in successive baths of toluene and graded alcohols and subjected to 15 min microwave antigen retrieval in pH 6 citrate buffer (Tris-EDTA buffer pH 9 for the anti-human GR). After cooling and 15 min of pre-incubation with a blocking serum (Vector, Burlingame, CA), slides were incubated with primary antibodies overnight at 4°C in a humid chamber. Bound immunoglobulins were revealed with the appropriate ImmPRESS anti-mouse or anti-rabbit immunoglobulins kit (Vector) according to the manufacturer's instructions.

### *Histological studies*

The histological quality of the tissues and the stage of renal development were assessed by hematoxylin-eosin staining.

### *Quantitative real time RT-PCR*

Two  $\mu\text{g}$  of total RNA, isolated from frozen samples, were subjected to DNase I treatment (Invitrogen) and reverse-transcribed with 200 units of reverse transcriptase (Superscript II, Invitrogen). PCR were performed with 100 ng cDNA in the presence of qPCR<sup>TM</sup> Mastermix Plus for Sybr<sup>TM</sup> Green I (Eurogentec, Seraing, Belgium) containing 300 nM of specific primers (Supplemental Table 1 and 2). qPCR was carried on an ABI 7300 Sequence Detector (Applied Biosystems, Foster City, CA). For standards preparation, amplicons

were subcloned into pGEMT-easy plasmid (Promega) and sequenced to confirm the identity of each sequence. Standards for different MR isoforms were determined by use of specific primers designed in exons 2 and 3 for the MR, exons 1 $\alpha$  and 2 for MR $\alpha$  and exons 1 $\beta$  and 2 for MR $\beta$ . Standard curves were generated using serial dilutions of linearized standard plasmids. Samples were amplified in duplicate or triplicate. Ribosomal 18S was used as an internal control for data normalization. Relative expression of a given gene is expressed as the ratio attomoles of specific gene/femtomoles of 18S.

### **Western Blot Analyses**

Total protein extracts were prepared from pool of frozen murine kidneys at various developmental stages (E18, D0, D8, adult). Briefly, frozen kidneys were ground in a mortar under liquid nitrogen and lysis buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 30 mM Na pyrophosphate, 50 mM Na fluoride, 0.1% TritonX100, 1X protease inhibitor cocktail (Sigma) was added to the resulting powder. Homogenates were obtained with a Teflon glass potter at 4°C and centrifuged at 13,000 rpm at 4°C for 20 min. Aliquots of supernatant were frozen in liquid nitrogen until further use. Immunoblots were incubated overnight in 5% milk-TBST followed by incubation for 1 h at room temperature with affinity purified anti-MR 39N (1:2000) and with peroxidase-conjugated goat anti-rabbit antibody, dilution 1:15000 (Vector) and visualized by the ECL<sup>+</sup> detection kit. For loading normalization, membranes were incubated with anti  $\alpha$ -tubulin antibody (Sigma). Quantitative analysis of specific signals was performed using Quantity One software (Biorad).

### **Statistical analyses**

Results represent mean  $\pm$  SEM for the mouse samples with at least 6 samples for each developmental stage. For human samples, results are expressed as mean of at least three independent analyses of three different reverse-transcribed samples. Statistical analyses were performed using a non parametric Mann Whitney test (Prism4, Graphpad Software, Inc., San Diego, CA). Correlations between two parameters were

obtained by Spearman regression analysis, with significant threshold at 0.05.

## **RESULTS**

### ***Activation of the renin-angiotensin-aldosterone system at birth***

High levels of aldosterone and renin levels were observed in umbilical cord blood of 48 healthy newborns (mean and median of 817.1 and 662.5 pg/ml [range 105-2211] and 78.8 and 50.0 pg/ml [range 12-288], respectively), statistically different ( $P < 0.001$ ) from normal plasma aldosterone and renin concentration values in healthy adults (n=50) were  $99 \pm 43$  and  $8.1 \pm 3.7$  pg/ml, respectively.

### ***Expression of MR and its signaling partners during mouse renal development***

#### *Quantitative mRNA expression*

Murine MR (mMR) mRNA expression was quantified by qPCR at various developmental stages between 15 days of gestation (E15) and 15 days postnatal (D15) (Fig. 1A) and compared to the day of birth (D0) chosen as the statistical reference. At E15, mMR transcript is hardly detectable. Its expression begins to increase significantly at E16 and reaches a maximum (4-fold increase) at E18 (relative expression 0.016 amol/fmol 18S). During the period surrounding birth (E19 and D0), mMR mRNA expression is surprisingly low, with levels comparable to E16 (0.004 amol/fmol). Thereafter, it increases again progressively until D15.

We next examined the relative expression of mMR $\alpha$  and mMR $\beta$  isoforms, using specific primers. We found a similar expression profile for mMR $\alpha$  isoform (Fig.1B), with an onset of expression at E16, a 2.5-fold increase at E18 and a significant decrease at E19 and D0. A high positive correlation between mMR and mMR $\alpha$  isoform expression profiles is found (Fig. 1C). Similar results are obtained with the mMR $\beta$  isoform (data not shown). □

To investigate whether other genes of the mineralocorticoid signaling pathway had the same evolution profile, we quantified  $\alpha$ ENaC and 11 $\beta$ HSD2 mRNA throughout development. Like mMR, their renal expression increases from E16 to E18 and is down-regulated at E19 and D0 before

increasing again after birth (Fig. 1D and 1E). There is a highly significant correlation between mMR and the expression profiles of 11 $\beta$ HSD2 (Fig. 1F) and  $\alpha$ ENaC (data not shown), suggesting a comparable maturation process for different markers of the late distal nephron.

We also analyzed GR, V2R and AQP2 gene expression during renal development (Supplemental Fig. 1). At variance with mMR, renal GR expression has a very different profile. GR mRNA is already present at E15, increases slightly at E16 but does not vary significantly at other developmental stages. Like MR, V2R and AQP2 transcripts are first detected at E16 as previously described (19, 20). In contrast, unlike MR, V2R and AQP2 expression increases progressively until D15, with no nadir at birth.

#### *MR, GR and AQP2 protein expression*

Immunohistochemical studies, using a monoclonal antibody directed against MR first 18 amino-acids (17), revealed that the MR protein seems to be mostly detected in the nuclei of the CCD cells at all developmental stages from E16 to D15 and in adult kidney (Fig. 2B to F, and Supplemental Fig 2). No staining is observed at E15 (Fig. 2A). At E16, MR immunodetection is faint and increases throughout the developmental stages studied, with an extension to additional CCD cells. Importantly, MR is never detected in the glomeruli, in the proximal convoluted tubule, in the arteries, or in the interstitial cells. On the contrary, GR protein, which is already present at E15, is detected not only in the nuclei of the CCD cells (Fig. 2G), but also, in the glomeruli, in different segments of the nephron (proximal and distal convoluted tubules, Henle's loop), and in endothelial and interstitial cells (Fig. 2G to 2L). The AQP2 protein is first expressed at E16. In contrast to the weak MR expression, strong cytoplasmic immunostaining, particularly at the apical membrane of the CCD cells is observed till D15 (Fig. 2M to 2R). These findings corroborate our qPCR results, as both AQP2 transcript and protein appear initially on E16.

Western blot analysis of kidneys obtained from murine fetuses, postnatal and adult animals at different developmental stages showed an extremely low level of MR expression in the kidneys of E18 as well as D0 mice. In sharp contrast, the renal MR expression was

dramatically up-regulated at D8, reaching adult values (Fig.3). These data fully confirm that in the mouse, renal MR, at both mRNA and protein, is very low at birth.

#### **Expression of hMR and its signaling partners during human renal development**

##### *Quantitative mRNA expression*

Nineteen human fetal renal samples (14-40 wk) (see Table 1), were used to quantify hMR mRNA expression by qPCR (Fig. 4A). Relative hMR expression is lowest at 14 wk, increases at 16 wk and peaks at 19 wk. Renal hMR mRNA levels decrease afterwards, around 25 wk of gestation. The expression of both hMR $\alpha$  and hMR $\beta$  isoforms is highly correlated with hMR expression (Supplemental Fig 3).  $\alpha$ ENaC and 11 $\beta$ HSD2 transcripts are expressed in human kidney and share the same evolution profile as hMR isoforms. The best correlation scores are found for hMR $\alpha$  and  $\alpha$ ENaC (Fig. 4B) and hMR $\alpha$  and 11 $\beta$ HSD2 (Fig. 4C).

We also quantified the relative expression of GR, V2R and AQP2 (Supplemental Fig 4). GR transcript level is stable during gestation. On the contrary, V2R mRNA expression is relatively low whereas AQP2, whose maximal values are reached later during renal maturation (24 wk), is highly expressed during development.

##### *MR, GR and AQP2 protein expression during human kidney development*

A collection of thirty-seven paraffin-embedded human kidney samples from 14 fetuses, 12 neonates (0 to 11 month-old) and 11 children (1 to 11 year-old) was used to analyze and quantify MR, GR and AQP2 protein expression during fetal and postnatal renal development (Table 1, Fig. 5 and 6). The integrity of all samples was assessed by a hematoxylin-eosin (HE) staining, which allowed assessing the stages of kidney development (Fig. 5A to 5D, and 6A to 6C). As expected, we observed that formation of glomeruli and nephrons and the architectural organization of the kidney take place between 5 and 36 wk of gestation. We noticed that, at higher magnification, podocytes in several glomeruli still retain an immature cubic shape around 40 wk of gestation (Fig. 5D), while their mature flattened shape is observed in every glomerulus only at approximately 1 year

of age (Fig. 6B). This confirms that some nephrons achieve functional maturity only during the postnatal period, accordingly to previous studies (21). Tissue integrity was verified using two specific antibodies recognizing well-characterized markers of CCD cells (cytokeratin 19) (22) and mesenchymal cells (vimentin) (23).

Using the same MR specific monoclonal antibody (17), MR protein was immunodetected at 15 wk of gestation, mostly in the nuclei of the CCD cells (Fig. 5F). MR is absent in 10-wk-old samples (Fig. 5E). The immunoreactivity is present in all the seven samples from 15 to 24 wk of gestation (Fig. 5F). Interestingly, no MR is observed perinatally in human kidneys. The immunostaining is negative in all seventeen samples from 30 wk of gestation to 10 months of age (Fig. 5G-H and 6D). MR expression is again observed at 11 months after birth and is present in all the 11 samples studied, from 11 months to 11 years (Fig. 6E). The intensity of the immunostaining and the number of immunopositive cells in the CCD increase throughout postnatal development.

GR expression is detected earlier than MR, starting from 10 wk of gestation. The labeling is nuclear and localized in the nuclei of the tubular epithelial cells but also in the glomerular, endothelial and interstitial cells (Fig. 5I). The GR protein is present at all stages of fetal development (Fig. 5I to 5L) and during the first two months after birth. Afterwards, between 2 and 10 months, the immunoexpression is variable, 5 samples out of eleven being immunonegative or weakly positive (Fig. 6G). However, after 11 months, the GR protein is constantly found in all samples (Fig. 6H). These results suggest an early functional role for GR during human embryogenesis and, like MR, a variability of expression during specific periods of development.

AQP2 is first detected at 15 wk of gestation, localized at the membrane of CCD epithelial cells with accentuation at the apical pole (Fig. 5N). Unlike MR, the staining intensity is constantly strong and the immunolabeling is present at all renal developmental stages from 15 wk of gestation to 11 years after birth and in the adult kidney (Fig. 5N to 5P, and 6J to 6L). Taken together, these results indicate that, as for the mouse, MR has a biphasic expression during human renal development, with a

transient expression during gestation that seems to be restricted to the mineralocorticoid signaling pathway. Thus, the low renal MR expression during the early postnatal period might account for the transient aldosterone resistance observed in newborns.

## DISCUSSION

Kidney growth and differentiation during embryonic and fetal development is a complex process in which the maturation of the distal nephron is directly correlated with the renal capacity to regulate sodium and water transport. Herein, we demonstrate that different key players of the mineralocorticoid signaling pathway (11 $\beta$ HSD2, MR isoforms and  $\alpha$ ENaC), involved in the control of renal sodium reabsorption (24), have a parallel temporal evolution during renal ontogenesis. Their expression in the kidney peaks at E18 in the mouse and between 19 and 24 wk in the human fetus. These results are in accordance with previous *in situ* hybridization studies which found the onset of MR mRNA expression in the murine kidney at 18 days postcoitum (25) and demonstrated the presence of MR transcripts between 6 and 16 wk (26) and of immunoreactive MR protein between 14 and 20 wk of human gestation (27). However, no quantitative analysis of MR expression in the perinatal period has ever been reported, considering the difficulty of collecting such samples. In the present study, we provide first evidence for a transient renal MR expression during both murine and human fetal life, which seems to coincide with skin keratinization, a very specific stage of fetal development. Indeed, before 20 wk of human gestation and 17 days postcoitum in the mouse, the absence of a physiological impermeable barrier facilitates the free exchange between fetal extracellular fluid and amniotic fluid (28, 29). In contrast, after full keratinization, the maintenance of sodium balance becomes a highly regulated and dynamic process prominently regulated by renal MR. The decline in renal MR expression after 24 wk (human) and 18 days (mouse) of conception could be explained by modifications in the sodium concentration of the amniotic fluid (30), whose resorption by fetal swallowing constitutes a major mechanism regulating sodium intake. This is reminiscent of variation in MR expression observed in salmon during their acclimation from fresh to salt water (31).

In any case, this transient expression seems dispensable for fetal kidney development since pseudohypoaldosteronism type 1 (PHA1a) patients, presenting with heterozygous MR inactivating mutations (32), and MR knockout newborn mice (33) exhibit normal renal morphology (34).

Interestingly, high aldosterone levels in human umbilical cords are associated with an extremely low or undetectable renal MR expression at birth. The molecular mechanisms underlying MR expression are very complex, involving the use of alternative tissue-specific promoters (35, 36). The profound hormonal changes associated with labor and delivery, are likely to be involved in MR down regulation. Whether changes in MR expression are a direct consequence of fetal acclimation from an aquatic (intrauterine) environment to extrauterine terrestrial life, remains to be elucidated.

The critical role played by MR on sodium balance during the postnatal period is now well established. Indeed, MR knockout mice die shortly after birth, around day 8, of dehydration and failure to thrive (33), and children with PHA1a present with salt wasting ascribed to MR haploinsufficiency (32). Both phenotypes are rescued by sodium supplementation (32, 37). In healthy newborns, the absence of dehydration or failure to thrive indicates that renal MR levels are sufficient to ensure appropriate sodium balance even though MR expression was below our immunodetection limit in the perinatal period. In sharp contrast, preterms have constant salt loss and an impaired capacity to concentrate urine, causing a major problem to pediatricians (1). Given the presence of AQP2 at both mRNA and protein level, this renal phenotype could be partly explained by very low levels of MR, known to be involved in AQP2 cellular trafficking and protein up-regulation (12, 38).

In mice and humans, renal MR levels progressively increase after birth, following renal maturation which is achieved 8 days postnatal in rodents and approximately one year after birth in humans (1). Our findings indicate that MR expression in the postnatal period parallels the full maturation of the late distal nephron. The regulatory mechanisms governing MR expression are currently unknown, but it is likely that the dynamic setting of a functional mineralocorticoid receptor signaling predicts the physiological

adaptation of the neonate to its new environment.

In conclusion, we provide first evidence both in mouse and human, of a biphasic temporal expression of MR and of other partners of the mineralocorticoid signaling pathway during renal development. There is a major but transient peak of MR expression during fetal life, concomitant with fetal skin keratinization, while the progressive increase after birth correlates with full renal maturation. Our results also indicate that the low MR expression at birth could constitute the molecular basis of the partial aldosterone resistance during the neonatal period. Further experiments are required to evaluate whether similar expression profiles are detected in other aldosterone target tissues. Elucidation of mechanisms regulating MR levels could ultimately lead to new therapeutic strategies for the management of sodium loss in preterms and neonates and, possibly, of renal (39-41) and heart diseases (39, 42) in adulthood.

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## FIGURE LEGENDS

**Figure 1: Ontogenesis of mMR, mMR $\alpha$ ,  $\alpha$ ENaC and 11 $\beta$ HSD2 mRNA expression during mouse kidney development. (A, B, D, E):** Relative mRNA expression levels were determined using qPCR at various developmental stages as follow: E15-19: 15-19 days postcoitum. D0: day of birth. D2-15: second to fifteenth postnatal day. D0 was used as reference for statistical analysis: \* P<0.05, \*\* P<0.01, \*\*\* P<0.001. Results, expressed as the ratio of attomoles of specific gene/femtomoles of 18S, are mean  $\pm$  SEM (E15 n=6, E16, n= 8, E18 n=10, E19 n=12, D0 n=15, D2 n=6, D4 n=6, D8 n=6, D15 n=6). **(C, F):** Correlations between the relative expression of mMR versus mMR $\alpha$  (C) or 11 $\beta$ HSD2 (F) were obtained by Spearman regression analysis. r represents the correlation coefficient.

**Figure 2: Immunodetection of MR, GR and AQP2 during renal development of murine fetus and neonates. (A-F):** Immunoreactive MR protein is absent at E15 (A) but begins to be detected at E16, most notably in the nuclei of cortical collecting duct cells (B, see inset, arrow; see also Supplemental Fig 2). MR expression is rather weak at E18 and D0 but increases afterwards at D8 and in the adult kidney. **(G-L):** GR protein is already present at E15 (G), in the nuclei (see inset) of endothelial, interstitial and epithelial cells, in different segments of the nephron and in the glomeruli (H-L). **(M-R):** A strong AQP2 immunostaining is observed at the apical membrane of the cortical collecting duct cells from E16 to adulthood (N-R). E15, E16, E18: 15, 16 and 18 days postcoitum. D0: day of birth. D8: eighth post natal day. Original magnification: x20. Insets: x100. gl: glomerulus; dct: distal convoluted tubule; ccd: cortical collecting duct.

**Figure 3: Western blot analysis of MR expressin during murine renal development.** Twenty  $\mu$ g of protein from kidney homogenates at different developmental stages (E18, 18 days postcoitum, D0: day of birth. D8: eighth postnatal day, A: adult) were loaded on 7.5% SDS-PAGE followed by direct immunoblotting with anti-MR 39N antibody (1/2000).  $\alpha$ -tubulin was used as loading control. MR was normalized to  $\alpha$ -tubulin protein levels after digitalization on a gel scanner using QuantityOne software

(Bio-Rad, Marnes-la-Coquette, France). Results are means  $\pm$  SD of 4 independent determinations and are expressed relative to MR expression measured at day 0, arbitrarily set at 1.

**Figure 4: Ontogenesis of hMR mRNA expression during human kidney development. (A):** Relative hMR expression was determined using qPCR in fetal kidney samples at various gestational ages. Each point represents the mean of three independent determinations of hMR expression, performed in triplicate in a given sample (see materials and methods), and normalized by hMR expression in one adult kidney sample ( $0.04 \pm 0.08$  amol/pmol of 18S, mean  $\pm$  sem, n=11). **(B, C):** Correlations between the expression of hMR $\alpha$  isoform versus  $\alpha$ ENaC (B) or 11 $\beta$ HSD2 (C) were obtained by Spearman regression analysis.  $r$  represents the correlation coefficient.

**Figure 5: Histology and MR, GR and AQP2 immunoreactivity in human fetal kidneys. (A-D):** H&E staining at various stages of fetal renal development. At 10 gestational weeks (GW), the human kidney is clearly immature (A). At 40 GW (D), glomeruli and nephron formation is achieved, but some podocytes conserve an immature cubic shape (inset, D). **(E-H):** MR immunodetection is observed at specific stages of renal development. Immunoreactive MR is transiently detected in distal collecting ducts at 19 GW, as illustrated by a positive staining, mostly in the nuclear compartment (inset, F). In contrast, no renal MR expression is found at 10 GW (E) and between 30 to 40 GW (G-H). **(I-L):** The GR protein is immunodetected at all developmental stages, in the nuclei of endothelial, interstitial and epithelial cells, in different nephronic segments and the glomeruli. **(M-P):** AQP2 is not expressed at 10 GW (M) whereas a strong immunostaining is detected at the apical membrane of the cortical collecting duct cells from 15 to 40 GW (N,O and P). Original magnification: x20. Insets: x100. gl: glomerulus; dct: distal convoluted tubule; ccd: cortical collecting duct.

**Figure 6: Histology and MR, GR and AQP2 immunoreactivity in human neonatal and adult kidneys. (A-C):** H&E staining at various stages of postnatal renal development. Podocytes have reached a mature flattened shape at 11 months postnatal (inset, B). **(D-F):** MR immunodetection is observed at specific stages postnatal renal development. No MR protein is detected from birth until 11

postnatal months (D). Afterwards, a MR immunoreactivity is readily detected at 11 months after birth (E) as in the adult kidney (F). **(G-I):** GR is weakly expressed during the 2-10 months postnatal period (G). Afterwards, GR protein is strongly expressed in the nuclei of endothelial, interstitial and epithelial cells, in different parts of the nephron and the glomeruli (H and I). **(J-L):** A strong AQP2 immunostaining is detected at the apical membrane of the cortical collecting duct cells from birth to adulthood. Original magnification: x20. gl: glomerulus; dct: distal convoluted tubule; ccd: cortical collecting duct.

Age		10-14 GW	15-25 GW	30-40 GW	0-6 months	7-10 months	11 months - 11 years
# human renal samples	Gene Expression	1	15	3	0	0	0
	Immunohistochemistry	2	7	5	5	7	11
MR immunostaining		0	+	0	0	0	++
GR immunostaining		+	++	+++	+	+	+++
AQP2 immunostaining		0	+++	+++	+++	+++	+++

**Table 1: Number of snap-frozen and paraffin-embedded human kidney samples obtained for different periods of development and relative quantification of MR, GR and AQP2 immunodetection.** Snap-frozen and paraffin-embedded samples were used for qPCR and immunohistochemistry, respectively. Fetal kidney samples (GW: gestational weeks) originate from *in utero* fetal deaths with rapid delivery and autopsy within 24 to 36 hours *post mortem*. Neonatal kidney samples (0-11 months) are from sudden infant deaths. Infantile kidney samples (11 months to 11 years) come from the healthy part of nephrectomies for nephroblastoma. Although not directly quantitative, the evaluation of the staining was scored by both the number of immunolabeled cells and the labeling intensity within specific target structures. At least 6 20x power fields of renal cortex were studied for each immunolabeled section, each marker being studied at least on 3 non-consecutive sections of each sample. An immunostaining score was attributed as follows 0 = below detection threshold ; + = less than 10 weakly immunolabeled cells per field ; ++ = 10-50 moderately immunostained cells per field ; +++ = more than 50 strongly stained cells per field.