

## Transgenic mouse models to study human mineralocorticoid receptor function in vivo

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**Transgenic mouse models to study human mineralocorticoid receptor function in vivo.** The mineralocorticoid receptor (MR) is a transcription factor that mediates aldosterone action. MR is expressed in a wide variety of tissues, most notably in sodium-transporting epithelia, but also in nonepithelial cells of the cardiovascular and central nervous systems. However, molecular mechanisms underlying mineralocorticoid signaling and the primary mineralocorticoid-regulated genes are not fully identified. We recently showed that the human MR (*hMR*) gene possesses two first 5'-untranslated exons 1 $\alpha$  and 1 $\beta$ , and demonstrated that the 5'-flanking regions of these exons, named P1 and P2, respectively, are functional promoters that differ by their basal and corticosteroid-regulated transcriptional activities. To gain insight into the tissue-specific expression and function of MR, we have established transgenic mouse models using both targeted oncogenesis and receptor overexpression strategies. P1 and P2 promoters were used to direct expression of the large T antigen (TAg) of SV40 in constitutively MR-expressing cells. P1.TAg mice developed lethal hibernomas, while P2.TAg animals died from cerebral neuroectodermal tumors and leiomyosarcomas. Quantification of TAg messenger RNA levels revealed that P1 and P2 were differentially utilized. P1 promoter was transcriptionally active in all MR-expressing tissues and importantly directed an appropriate transgene expression in the distal nephron. Conversely, P2 activity was weak and spatially restricted. Several immortalized cell lines were established, thus constituting valuable models to investigate on aldosterone-regulated proteins. We also used P1 and P2 to target overexpression of hMR cDNA in mice. Phenotypic characterization of these mice is currently under investigation. Some transgenic lines should represent useful systems to further explore multiple functions of MR in vivo.

Aldosterone is a major regulator of salt balance and blood pressure, exerting its effects via the mineralocorticoid receptor (MR), a transcription factor belonging to the steroid receptor superfamily [1]. MR is closely related to the glucocorticoid receptor (GR), and it can indifferently bind mineralocorticoid and glucocorticoid hormones. MR is widely expressed, but at a relatively low level, in a large variety of tissues. MR participates in

the regulation of hydroelectrolytic homeostasis in sodium-transporting tight epithelia such as distal nephron [2, 3], colon [4, 5], lung [6], and salivary and sweat glands [2, 7]. In such epithelial target cells, the mineralocorticoid specificity of aldosterone action is given by the enzyme 11 $\beta$ -hydroxysteroid dehydrogenase 2 (11HSD2) [8], which converts active glucocorticoids into inactive metabolites [9–11]. However, other mineralocorticoid selectivity-conferring mechanisms are necessary to fully account for receptor specificity [12]. For instance, at the receptor level, MR is able to discriminate between aldosterone and glucocorticoids in ligand-dependent conformational changes and stabilization of the ligand-binding domain [13, 14], as well as in transcriptional activation [15, 16]. MR is also expressed in nonepithelial tissues such as the heart [17, 18], some areas of the brain [19–21], large blood vessels [22, 23], and mononuclear leukocytes [24], in which specific aldosterone actions have been described, but their mineralocorticoid specificity is not fully understood.

The ligand-activated MR is translocated in the nucleus and acts as a transcription factor after its interaction with the consensus glucocorticoid response element (GRE) sequences [25]. It is assumed that MR can directly interact with coactivators or corepressors, which will modulate the activity of the general transcription machinery [26]. Thus, MR could activate or inhibit transcription of target genes whose identification is under intense investigation.

The genomic structure of the human MR (*hMR*) gene has recently been elucidated. This gene contains 10 exons, two different 5' untranslated exons, namely exon 1 $\alpha$  and 1 $\beta$ , spliced alternatively onto the exon 2, which contains the MR translation start site, giving rise to two distinct mRNA isoforms: hMR $\alpha$  and hMR $\beta$  [27]. In situ hybridization studies indicated that these two transcripts were coexpressed in human tissues at approximately the same level in aldosterone target tissues examined [28]. Furthermore, a third 5' untranslated exon (exon 1 $\gamma$ ) has been identified in the rat [29], sharing 75% homology with a human sequence located upstream of exon 1 $\beta$ ;

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however, we failed to detect such a splice variant in human kidney [30]. Collectively, this raises the question of how receptor isoforms are generated and whether regulation of hMR expression might result from differential utilization of alternative promoters. As a result, we have analyzed the 5'-flanking regions of exons 1 $\alpha$  and 1 $\beta$ , named P1 and P2, respectively. Their sequences are GC rich and possess numerous Sp1 sites, but they lack a TATA box. Transient transfection assays in different cell types demonstrated that P1 and P2 are functional promoters [28].

Study of hMR promoters in an in vivo context is an essential step in understanding the regulation of hMR expression fully. The generation of transgenic mouse models using P1 or P2 fragments to direct expression of a reporter gene constitutes an appropriate experimental approach. Our strategy is illustrated in Figure 1. On one hand, targeted oncogenesis, which consists in driving expression of the oncogene large T antigen (TA $\alpha$ ) of the SV40 virus by either P1 or P2 promoter, was initially used. This allows us at the same time to analyze the tissue-specific utilization of alternative promoters and to establish novel cell lines given the high transforming properties of TA $\alpha$ . On the other hand, in order to better elucidate MR function in different types of tissues, a targeted overexpression of recombinant hMR driven by P1 or P2 was initiated in transgenic mice to create potential physiopathologic models. This review briefly describes some of our recent data and views on the role played by MR in physiological and pathophysiological processes.

#### CHARACTERIZATION OF HUMAN MINERALOCORTICOID RECEPTOR PROMOTERS IN VITRO

To identify the regulatory elements of the hMR gene, a phage library had been constructed from a YAC DNA containing the entire *hMR* gene. One phage clone was isolated and mapped by restriction enzyme digestion and Southern blot analysis. It contained approximately 15 kb of hMR 5'-flanking region. A 1.2-kb *HindIII-AvaII* fragment containing 216 bp of exon 1 $\alpha$  and 965 bp of its 5'-flanking region (P1) was subcloned, as well as a 1.8 kb *SspI-SspI* fragment containing 1673 bp upstream sequences (P2) and 123 bp of exon 1 $\beta$ . Both CG-rich sequences present numerous Sp1-binding sites characteristic of TATA-less promoters. A series of deletion mutants of these regions was inserted into a luciferase reporter plasmid and used in transient transfection assays to determine functional activities of the proximal P1 or distal P2 fragments [28]. We showed that the BH construct (-341, +128) containing 535 bp of P1 exhibited the highest basal activity, suggesting the existence of silencer regulatory elements upstream -341 in the HA fragment. The P2 sequences possessed weak promoter activities,

approximately one tenth that of P1. No difference in normalized luciferase activities was observed between the longest *SspI-SspI* fragment of P2 and the shorter 431 bp length *SacII-SspI* construct. Interestingly, both P1 and P2 promoter activities were up-regulated by glucocorticoids, but only the distal P2 promoter was stimulated by aldosterone treatment. We also demonstrated that MR and GR were able to synergistically activate the distal promoter, suggesting MR/GR heterodimerization as previously described [31]. Even though two GRE-like sequences have been identified in P1 and P2 regions, no binding of hGR, hMR, or both was detected by gel shift or DNase protection assays, suggesting an indirect effect of corticosteroid receptors in these promoters.

The influence of corticosteroid hormone status on MR mRNA or proteins expression is still a matter of controversy. Indeed, several animal studies had reported opposite results on the effect of adrenalectomy on MR levels in rat kidney and hippocampus [29, 32-34], whereas no variation was observed in rat colon [35, 36]. These different results could be attributed to the experimental conditions used and tissues and/or species analyzed. More importantly, the results might also largely depend on the MR level examined either at the mRNA or protein level. Transcriptional or post-transcriptional events are probably involved in the modulation of synthesis rate, stability of transcripts, or the receptor protein. Thus, regulatory mechanisms controlling intracellular MR levels are extremely complex and may involve differential utilization of alternative promoters with variable basal transcriptional strength and sensitivity to hormonal modulation [28].

#### TISSUE-SPECIFIC EXPRESSION OF HUMAN MINERALOCORTICOID RECEPTOR IN VIVO

Different isoforms of the glucocorticoid and MRs generated by alternative utilization of 5'-untranslated exons have been previously identified [27, 29, 37]. This type of genomic organization allows differential control of gene expression by alternative promoters in a tissue-specific and/or developmental manner. Analysis of the relative amount of hMR $\alpha$  and hMR $\beta$  messenger isoforms, which only differ by their first exon, 1 $\alpha$  or 1 $\beta$ , could facilitate our understanding of hMR tissue-specific expression. Because of the ethical problem of obtaining access to aldosterone target tissues in human, such a study was a difficult task. We investigated the expression of hMR transcripts in the human renal, cardiac, skin, and colonic tissue samples by in situ hybridization [38]. Discrimination between the two messenger isoforms was performed using exon 1 $\alpha$ - and 1 $\beta$ -specific riboprobes. The total amount of hMR mRNA was measured with a common exon 2 riboprobe. Specific signals were quantitated by computer-assisted densitometry. The relative level of hMR transcripts detected with the exon 2 probe was

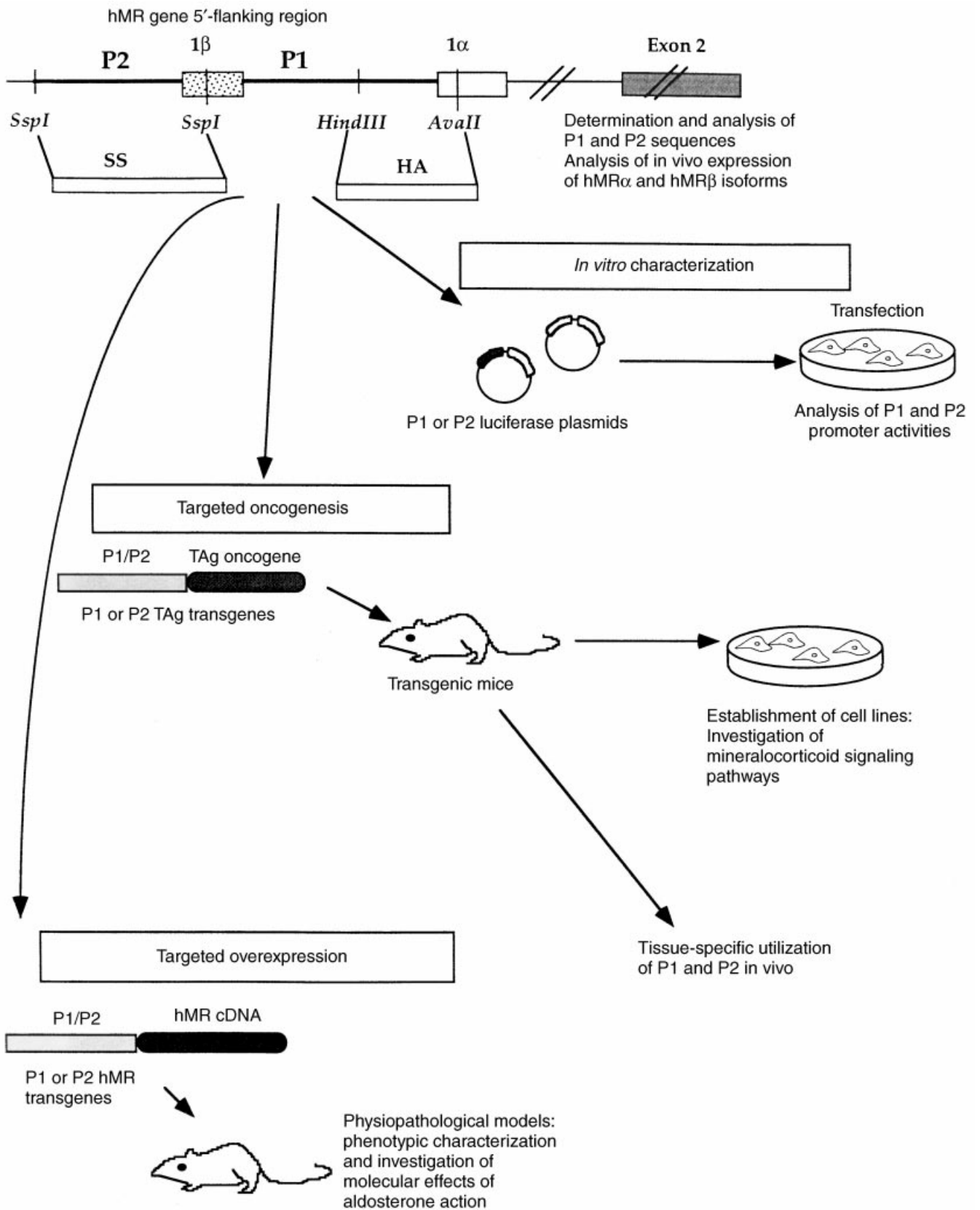


Fig. 1. Strategies used to study human mineralocorticoid receptor expression and function.

as follows: distal nephron = sweat glands = colon > epidermis > heart. More importantly, the two hMR mRNA isoforms were present in all typical aldosterone cells such as in cortical collecting ducts, colonic enterocytes, sweat gland ducts, cardiomyocytes, and keratinocytes, and were coexpressed at approximately the same level. This hMR expression pattern is at variance with that reported in the rat by RNase protection assay in which MR $\alpha$  was prevailing over MR $\beta$  isoform in kidney [29], but was equally expressed in hippocampus. However, a relative abundance of hMR $\alpha$  and  $\beta$  transcripts compared with that of exon 2 containing mRNA strikingly differs among tissues. In kidney, the signals corresponding to 1 $\alpha$  and 1 $\beta$  mRNAs were clearly lower than that given by the exon 2-specific probe and, therefore, fully accounted for the expression of the common exon 2 containing mRNA. In contrast, in the heart, 1 $\alpha$ - and 1 $\beta$ -containing transcripts are expressed in the cardiomyocytes at approximately fourfold higher levels than exon 2-containing messengers, strongly suggesting the existence of other hMR mRNA variants that could arise from tissue-specific alternative mRNA splicing.

In an attempt to study hormonal regulation of hMR isoforms expression, quantitative analysis of hMR transcripts was performed using skin biopsies of patients with hyperaldosteronism or hypoaldosteronism. The expression of exon 1 $\beta$ -containing transcripts was significantly reduced in sweat gland ducts of patients with Conn's and Liddle's syndromes, both characterized by an increased renal sodium reabsorption associated with extracellular volume expansion and hypertension, resulting from increased aldosterone production and from constitutive activation of the amiloride-sensitive epithelial sodium channel, respectively [39–41]. In contrast, hMR levels were in the normal range in a subject affected by type I pseudohypoaldosteronism, a form of mineralocorticoid resistance associated with high circulating aldosterone levels [42]. Altogether, our results strongly suggested the existence of novel tissue-specific hMR variants that we are currently trying to identify and characterize. Moreover, we have demonstrated that the expression of exon 1 $\alpha$ - and 1 $\beta$ -containing transcripts are regulated in a tissue-specific manner, and that these splice variant levels seem to be differentially affected by sodium overload rather than by plasma aldosterone concentrations. It might be particularly interesting to investigate whether modifications of intracellular osmolarity and/or sodium content could influence hMR gene expression.

#### TARGETED ONCOGENESIS AND TISSUE-SPECIFIC USE OF P1 AND P2 PROMOTERS

To analyze P1 or P2 hMR alternative promoters functions in vivo and to establish new cell lines derived from MR-expressing tissues, we have generated transgenic

mice carrying large TAg cDNA under the control of P1 or P2 sequences. The phenotypic features of P1 and P2.TAg mice were somehow unexpected. Whereas no tumor was apparent in classic aldosterone target tissues, all of the 10 P1.TAg founder animals developed lethal dorsal liposarcomas from brown fat origin, so-called hibernomas [43]. Death occurred within the first weeks of life (2 to 17 weeks), preventing the generation of any transgenic line. Three P2.TAg transgenic lines have been obtained. The founder 55 and the eight F1 animals line 45 died before breeding. All founders and F1 P2.TAg animals developed cerebral primitive neuroectodermal tumors (PNETs). Some of them also presented thymus hyperplasia and died between six months and one year of age. The formation of PNET did not systematically occur in F2 generation animals of line 54, however, facial leiomyosarcomas generally arose from the lips or eyelids. The distinct phenotype among P1 and P2.TAg animals already demonstrated differences in hMR promoter utilization in vivo [Le Menuet et al, *J Biol Chem* 2000 (in press)].

The mouse MR expression pattern was initially examined in wild-type animals by ribonuclease protection assays (RPA), and quantitative analysis of specific signals allowed the establishment of the relative abundance of mMR transcripts among tissues that was subsequently used as a basis to compare transgene expression driven by each of the *hMR* promoters. We showed that colon always contains the highest levels of mMR mRNA. The receptor was also expressed at a relatively high level in the lung, salivary glands, brain, and testis. The uterus, kidney, heart, skin, muscle, and spleen were also shown to express substantial amounts of mMR.

The transgene messenger pattern of expression was then examined in various tissues of transgenic animals together with that of endogenous mMR by RNase protection assays. We first showed that the presence of TAg did not modify mMR expression. In the P1.TAg mice, TAg and mMR were coexpressed in all tissues tested. As expected, hibernomas and brown adipose tissue (BAT), which kept a normal histologic appearance in younger animals, expressed very high levels of TAg. The relative tissue-specific distribution of TAg in P1.TAg mice compared with that of mMR revealed some differences: the transgene messenger amount was low in colon but far exceeded that of mMR in salivary glands and testis. Transgene content was lower than that of mMR in kidney, but immunohistochemistry with an anti-TAg antibody showed that cellular localization of TAg was similar to renal expression of MR, with a nuclear labeling exclusively observed over the distal parts of the nephron. Thus, the P1 promoter seems to be transcriptionally active in all MR-expressing tissues since P1-driven transgene expression recapitulates that of the endogenous receptor. In addition, our data strongly suggested that

P1 promoter could be used to drive expression of any transgene in the renal MR-sensitive cells. In contrast, P2.TAg mice exhibited a rather low transgene expression in all organs with a variable localization among transgenic mice. No transgene was detected in colon and skin in any animal. Unlike line 54, line 45 animals expressed TAg in kidney, whereas TAg was only found in the salivary glands of founder 55. This could indicate a strong influence of the transgene integration site on the tissue-specific expression driven by P2 promoter. Conversely, cerebral or facial tumors and hyperplastic thymus expressed high levels of TAg. It is worth noting that the line 54 F2 generation animals, which did not develop PNET, did not express transgene in the brain [Le Menuet et al, *J Biol Chem* 2000 (in press)].

Our results demonstrated a distinct tissue-specific utilization and strength of proximal and distal hMR promoters. Indeed, P1 promoter drives a similar expression pattern than endogenous mMR regulatory sequences, most notably in the kidney. Furthermore, both in vitro and in vivo studies showed that P1 activity is approximately 10 times stronger than that of P2. We could hypothesize that P1 might govern tissue specificity and the level of MR expression, while P2 could be implicated in the fine-tuning regulation depending of physiological states.

Another important aspect of our targeted oncogenesis strategy was the development of different cell lines derived from neoplastic as well as normal tissues of P1 and P2.TAg transgenic mice. Indeed, we have successfully established P1.TAg cell lines originating from hibernomas (T34, T37s, and T37i) [43], the whole brain (F6), hippocampus (BZ), lung (PP), salivary glands (SAL), glabrous skin of palms (PAL), and liver (LUCA) [Le Menuet et al, *J Biol Chem* 2000 (in press)]. Unfortunately, despite several attempts and appropriate transgene expression, no cellular model was obtained from isolated cardiomyocytes or various tubular segments of the distal nephron. In contrast, cellular models from P2.TAg mice could only be derived from leiomyosarcomas (JO, LIPT, and AXI). This could be related to the very low level of TAg expression in normal tissues of these animals. Incidentally, we failed to obtain cell lines from PNET.

Among the cell lines derived from P1.TAg mice hibernomas, one of them, the T37i cell line, was extensively characterized. Under normal culture conditions, T37i cells present a fibroblastic-like appearance. After confluence and treatment with insulin and triiodothyronine, which are known to induce adipocyte differentiation, cells became larger and numerous intracytoplasmic vacuoles appeared, consisting of small lipid droplets, as shown by red oil staining. Most of the cells presented with multiple nuclei and achieved terminal adipocyte differentiation. The uncoupling protein 1 (UCP1), which is

involved in thermogenesis, is a specific marker of brown fat. Unlike undifferentiated cells, after stimulation by isoproterenol or retinoic acid, fully differentiated T37i cells expressed UCP1 mRNA. Both differentiated, undifferentiated T37i, T34, and T37s cells expressed low but detectable amounts of mMR mRNA, as assessed by RPA. Specific aldosterone-binding sites were also detected in the cytosolic fraction of undifferentiated T37i, consistent with the presence of a functional mMR protein in these cells. mMR transcripts were also present in the BAT of wild-type mice and in P1.TAg mice hibernomas, indicating that brown adipocytes represent new aldosterone-target cells [43]. Furthermore, we have shown that aldosterone treatment of T37i cells induced adipocyte differentiation with a dose-dependent and mineralocorticoid-specific increase in triglyceride content. This is in accordance with previous report on aldosterone-mediated differentiation of 3T3-L1 cells, a cellular model of white adipocytes [44]. This process was accompanied by a stimulation of early adipogenic gene markers expression such as lipoprotein lipase, PPAR $\gamma$ , and adipocyte-specific fatty acid binding protein (aP2) (Penfornis, manuscript submitted for publication; abstract, Zennaro et al, 80th Annual Meeting Endocrine Society, New Orleans, 1998). Collectively, our results indicate that aldosterone and MR participate in the very early induction of brown adipocyte differentiation program and provide strong evidence that MR, besides its involvement in hydroelectrolytic homeostasis, also plays a role in the regulation of energy balance. This is supported by preliminary results demonstrating that aldosterone actually inhibited transcription and function of the mitochondrial uncoupling protein 1 in brown adipocytes (abstract; Lombès et al, 81st Annual Meeting Endocrine Society, San Diego, 1999).

Some other cellular models originating from various nonneoplastic tissues of P1.TAg mice are currently under characterization, most of them expressing TAg mRNA and protein as well as mMR messenger. Of particular interest are the PP cells from mouse 44 lung and PAL cells from mouse 18 palmary skin. The MUC-1 transcripts, a gene specifically expressed in glandular epithelial cells, for instance in bronchi [45, 46], were detected by reverse transcription-polymerase chain reaction (RT-PCR) in PP cells. We also demonstrated the presence of involucrin mRNA in PAL cells by the same technique, this gene being a specific marker of granulous and spinous layer of the epidermis [47]. If a functional mMR protein could be detected in these cell lines, they may constitute useful cellular models to further examine mineralocorticoid action in the lung and epidermis and to identify target genes, which are induced or repressed by aldosterone at the transcriptional level. Other cell lines derived from the central nervous system, the liver, or

the smooth muscle tumor could also represent potential interesting systems for the same type of investigations.

In summary, we have demonstrated that MR expression is broader than initially thought and involves complex regulatory mechanisms. The two *hMR* promoters clearly differ by their relative potency and their differential tissue-specific utilization *in vivo*. Finally, establishment of novel mineralocorticoid-sensitive cell lines by targeted oncogenesis should facilitate further studies on molecular mechanisms of aldosterone action.

### TARGETED OVEREXPRESSION OF HUMAN MINERALOCORTICOID

One approach to gain new insights into MR function is the generation of knockout mice. Animals with null mutation in the *MR* gene die 10 days after birth [48, 49], with symptoms resembling to pseudohypoaldosteronism type I in which mutations in *hMR* gene have been documented [50]. We decided to use an alternative genetic approach to elucidate the physiological functions played by MR in different tissues. Transgenic mouse models are currently being developed. We used the P1 or P2 promoter to drive expression of recombinant hMR in a tissue-specific manner in order to create potential physiopathologic models. Five independent transgenic lines were generated with P1.hMR construct and three with P2.hMR construct. Phenotypic characterization of these transgenic animals is currently ongoing. Preliminary observations reveal the existence of major renal abnormalities in P1-hMR line 42 animals. Transgenic mice (approximately 25%) presented renal kystic formations, often associated with renal atrophy and hypertrophy of the opposite kidney. Some of them exhibited biological stigmas of renal failure. In addition, systematic histologic examination identified nephropathies with dilation of Bowman's spaces, tubular lumen, and interstitium together with vacuolization of some tubular cells. Whether these major histologic alterations observed in most P1-hMR animals are due to a direct or indirect effect of renal hMR overexpression remains to be established. Moreover, echocardiographic assessment of ventricle functions in 12-week-old P1-hMR mice disclosed a significant increase in both end-systolic and end-diastolic dimensions of the left ventricle, leading to a decrease in the baseline shortening fraction. Although these results need to be confirmed, they underline the major role of MR as an important regulator of cardiomyocyte function. The P1-hMR animals might thus provide a model of dilated cardiomyopathy that should facilitate pathophysiological and pharmacological studies. Interestingly, as expected from previous studies on P1.TAg animals, the expression of hMR transgene in the testis was higher than that of endogenous mMR mRNA, suggesting that MR could be involved in the physiology of reproduction.

We are currently trying to analyze and correlate the tissue-specific expression of the recombinant MR at both mRNA and protein level and the phenotype of the animals. A precise evaluation of renal, cardiovascular, and endocrine functions should allow definition of functional consequences of targeted hMR overexpression. This will be performed in a center dedicated to investigations on small living animals (Centre d'Explorations Fonctionnelles Intégrées), which should open shortly in Bichat Medical School (Paris, France). At the molecular level, an extensive study on tissue-specific gene expression using a mouse cDNA array technique will be performed on normal and transgenic mice. This should provide new information on modulation of MR-sensitive target genes, most notably those induced or repressed by aldosterone at the transcriptional level. Identification and characterization of such specific genes will provide a basis for candidate genes involved in renal and cardiac pathophysiology.

### CONCLUSION

Our transgenic mouse models have provided important information on the molecular basis of hMR expression *in vivo*. We have shown that the regulation of its tissue-specific expression is controlled by a complex system that involves the use of two alternative promoters and the generation of at least two variant messengers and probably some other hMR isoforms that render the mineralocorticoid signaling system even more complex. The aldosterone-activated MR was originally described as a major regulator of sodium reabsorption in tight epithelia, we proposed that MR also plays an important role on other nonepithelial target cells. The physiopathological effects of aldosterone and mineralocorticoid selectivity-conferring mechanisms remain to be fully elucidated. Experimental MR-overexpressing animals represent suitable systems to explore the widespread and pleiotropic functions of MR *in vivo*. Other transgenic mouse models using both tissue-specific promoters, including appropriate fragments of hMR promoters and MR variants, should certainly be helpful to further examine mineralocorticoid signaling pathways.

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