Abstract. The amiloride-sensitive epithelial sodium channel is the limiting step in salt absorption. In mice, this channel is composed of three subunits (α, β, and γ), which are encoded by different genes (Scnn1a, Scnn1b, and Scnn1c, respectively). The functions of these genes were recently investigated in transgenic (knockout) experiments, and the absence of any subunit led to perinatal lethality. More defined phenotypes have been obtained by introducing specific mutations or using transgenic rescue experiments. In this report, these approaches are summarized and a current gene-targeting strategy to obtain conditional inactivation of the channel is illustrated. This latter approach will be indispensable for the investigation of channel function in a wide variety of organ systems.

The highly amiloride-sensitive epithelial sodium channel (ENaC) is a membrane constituent of many salt-reabsorbing epithelia that facilitates Na⁺ movement across the tight epithelia that line the distal renal tubule, the distal colon, the ducts of salivary and sweat glands, and the lung (1). ENaC is a heterotetramer composed of three homologous subunits, i.e., α, β, and γ; each subunit contains intracellular amino and carboxyl termini, two membrane-spanning domains, and a large extracellular loop (2–4). cDNA encoding the three homologous subunits of this channel have been characterized in several species, including humans and mice (5–7). Here we summarize transgenic experiments addressing channel function in mice and present the three different approaches that we have used, i.e., classic knockout experiments, transgenic rescue experiments, and the introduction of a specific mutation in the β-subunit. Finally, we discuss an additional tool for analyzing the channel in vivo, namely conditional gene inactivation.

Role of the Channel Subunit Genes

Gene targeting is defined as the introduction of site-specific modifications into the genome. It allows the in vivo analysis of diverse aspects of gene function. The classic gene-targeting (knockout) approach leads to inactivation of a gene in all tissues of the body, from the onset of development through the entire lifespan. Phenotypes of targeted mouse mutants cannot always be predicted from the presumed function of the given gene product and/or the pattern of expression of the gene. For example, constitutive inactivation of the α subunit of ENaC revealed an important role for the channel in lung liquid clearance after birth (8). αENaC knockout mice died within 40 h after birth, as a result of respiratory failure. Measurements of the amiloride-sensitive trans-epithelial potential differences in αENaC−/− (Scnn1a<sup>α<sup>−/−</sub></sup>/Scnn1a<sup>α<sup>−/−</sup></sup>) mice revealed that ENaC activity was completely abolished (Table 1). This suggests that channels composed of βγ subunits alone do not confer sufficient activity to compensate for loss of the α subunit in the lung. In human subjects, mutations in all three Scnn1 genes do result in ENaC hypofunction and are expected to induce a salt-wasting syndrome similar to pseudohypoaldosteronism type 1 (PHA-1) (9). In contrast to the αENaC deficiency in mice, PHA-1 variants in human subjects might retain sufficient residual ENaC activity to rescue or attenuate the lung phenotype (10,11). In an in vitro expression system, none of the mutations causing PHA-1 has been shown to be a null mutation and those tested still confer ENaC-mediated sodium transport activity (albeit diminished) (11). However, pulmonary ENaC dysfunction and excess airway liquid have been demonstrated in patients with PHA-1 (12). Species-specific differences, such as anatomic immaturity of newborn mouse lungs (13) or the presence of additional ENaC subunits in human subjects (e.g., δENaC) (14), might also explain the phenotypic differences.

Introduction of Specific Mutations into Scnn1 Genes

Direct evidence that ENaC dysfunction is involved in pathologic processes has come from the molecular analysis of two
human genetic diseases, i.e., Liddle’s syndrome (aldosteronism) and PHA-1 (9,17). In patients with Liddle’s syndrome, mutations in the Scnn1b and Scnn1c genes are responsible for a monogenic form of salt-sensitive hypertension (for review, see reference (10)). These mutations are clustered in the last exons of the Scnn1b and Scnn1c genes, and, in the resulting protein, PPxY motifs are affected or deleted. It has been shown that these PPxY motifs act as binding sites for a protein called Ned4 (18). Normally, ENaC is ubiquitinated and degraded, and the number of channels at the cell surface is tightly controlled. In patients with Liddle’s syndrome, lack of this protein–protein interaction has been proposed as a cause for the regulatory defect. Interference of the mutant ENaC proteins with the clathrin-mediated endocytosis pathway has also been discussed (19). As a consequence of the mutations, increased sodium channel activity is obtained through increased channel numbers and greater open probability. This renders the channel constitutively active (20). In patients with PHA-1 (a saltwasting syndrome), mutations have been identified in all three Scnn1 genes. In the variant proteins, mutations are well spread throughout the functional domains, including the gating domain, which renders the channel hypoactive (for review, see references (10) and (21)).

The introduction of a Liddle mutation into mice should permit elucidation of the causal relationship between dysfunction of the Scnn1 genes, dietary salt intake, and hypertension. We recently introduced the Liddle mutation R566STOP into the mouse Scnn1b gene locus and thus established two mouse models, i.e., a model for salt-dependent PHA and a model for salt-induced Liddle’s syndrome (22,23). First, to reproduce Liddle’s syndrome in mice, we introduced the stop codon in the last exon of the mouse Scnn1b gene. This mutation corresponds to the R566STOP mutation in human subjects (17) (Figure 1). In the targeting vector, the neomycin selection marker (neo), flanked by two loxP sites, followed this mutation. Phenotypically, mice homozygous mutant for this new allele (Scnn1bneo) were indistinguishable from wild-type mice in appearance, growth rate, and fertility (22). However, the introduction of this mutation resulted in large decreases in Scnn1b mRNA and protein levels in all organs tested and reduced in vivo ENaC activity in the colon. This was unexpected, because this mutation should not affect RNA levels and instead should lead to increased ENaC density and activity (24). The only difference between our mutated mouse Scnn1b locus and that in human subjects is the presence of the selection marker neo controlled by the phosphoglycerate kinase (PGK) promoter, which are needed to select for stable transfection in embryonic stem cell culture (Figure 1). It is possible that the insertion of an additional selection marker (neo) into 3′ untranslated sequences resulted in destabilization and/or degradation of the Scnn1b mRNA transcripts, thus interfering with

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**Table 1. Transgenic models targeting ENaC function**

<table>
<thead>
<tr>
<th>Allele and Genotype</th>
<th>ENaC Activity (%)</th>
<th>Organ</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scnn1a&lt;sup&gt;tm1&lt;/sup&gt;/Scnn1a&lt;sup&gt;tm1&lt;/sup&gt;</td>
<td>0</td>
<td>Lung (kidney)</td>
<td>Perinatal lethal</td>
<td>8</td>
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<tr>
<td>βENaC&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>ND</td>
<td>Kidney</td>
<td>Perinatal lethal</td>
<td>15</td>
</tr>
<tr>
<td>Scnn1c&lt;sup&gt;tm1&lt;/sup&gt;/Scnn1c&lt;sup&gt;tm1&lt;/sup&gt;</td>
<td>&lt;15</td>
<td>Kidney</td>
<td>Perinatal lethal</td>
<td>16</td>
</tr>
<tr>
<td>Mutant alleles</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scnn1b&lt;sup&gt;neo&lt;/sup&gt;/Scnn1b&lt;sup&gt;neo&lt;/sup&gt;</td>
<td>~20</td>
<td>Kidney</td>
<td>PHA-1</td>
<td>22</td>
</tr>
<tr>
<td>Scnn1b&lt;sup&gt;Lid&lt;/sup&gt;/Scnn1b&lt;sup&gt;Lid&lt;/sup&gt;</td>
<td>&gt;100</td>
<td>Kidney</td>
<td>Liddle’s syndrome</td>
<td>23</td>
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<td>Transgenic rescue</td>
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<td></td>
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<td>Scnn1a&lt;sup&gt;tm1&lt;/sup&gt;/Scnn1a&lt;sup&gt;tm1&lt;/sup&gt; TgrαENaC</td>
<td>~15</td>
<td>Kidney</td>
<td>PHA-1</td>
<td>29</td>
</tr>
</tbody>
</table>

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*a* According to the International Committee on Standardized Genetic Nomenclature, the actual gene symbol for the epithelial sodium channel (ENaC) is *Scnn1* (sodium channel, non-voltage-gated 1). Genes encoding the three subunits are *Scnn1a*, -b, and -c (41). The βENaC null allele was not indicated in the naming of the different alleles, because it has been produced elsewhere. PHA-1, pseudohypoaldosteronism type 1; ND, not determined.

*b* The values for resting ENaC activity were estimated from *in vitro* experiments.
the expression of the corresponding gene locus (25,26). Alternatively, the PGK-neo cassette might have directly affected the transcriptional activity of the nearby Scnn1b promoter region (27). With a low-salt diet, these mice (Scnn1b<sup>neo</sup>) developed clinical symptoms of acute PHA-1, including weight loss, hyperkalemia, and decreased BP (22).

Nevertheless, we could derive a Liddle mouse model from this Scnn1b<sup>neo</sup> allele. Because the PGK-neo cassette was flanked by loxP sites, it could be deleted by mating Scnn1b<sup>neo</sup> mice with transgenic mice ubiquitously expressing Cre recombinase (EIIa-Cre) (used as a so-called deleter strain) (28). This breeding converted the Scnn1b<sup>neo</sup> allele into the Scnn1b<sup>Lid</sup> allele (Figure 1) and the salt-wasting phenotype observed in Scnn1b<sup>neo</sup> mice into a salt-retaining phenotype (23) (Figure 2).

Interestingly, the mice remained normotensive with a normal-salt diet, despite evidence of hypervolemia and increased sodium reabsorption in the large intestine. However, a Liddle phenotype, characterized by higher BP, metabolic alkalosis, and hypokalemia accompanied by cardiac and renal hypertrophy, was induced with a high-salt diet. Mice carrying the Scnn1b<sup>Lid</sup> allele thus largely reproduce the clinical symptoms observed for human patients with Liddle’s syndrome (23).

**Figure 2. Development of a mouse model for Liddle’s syndrome.** (A) Schematic diagram of the wild-type exon 13 and the mutant exon 13, carrying the R566STOP mutation within the Scnn1b gene locus. (B) Wild-type and mutant ENaC, composed of two α subunits, one β subunit, and one γ subunit. Note the lack of the carboxyl-terminal segment in the mutant β subunit. (C) ENaC activity. Hormones such as aldosterone normally tightly control ENaC activity. In patients with Liddle’s syndrome, ENaC activity (the number of channels at the cell surface as well as the open probability of the channel) is increased despite low plasma renin and aldosterone levels, leading to a salt-retention phenotype.

**Transgenic Rescue of αENaC Deficiencies**

By introducing a transgenic Scnn1a gene into the Scnn1a genetic knockout background, we were able to rescue the perinatal lethal pulmonary phenotype at birth and partially restored sodium transport in renal, colonic, and pulmonary epithelia (29). This provided further insights into the role of the Scnn1a subunit in defective Na<sup>+</sup> absorption in the kidney. Young mice (5 to 9 d of age) exhibited clinical features of severe PHA-1, with metabolic acidosis, urinary salt-wasting, growth retardation, and 50% mortality rates. Adult transgenic rescue mice exhibited compensated PHA-1, with normal acid/base and electrolyte values but sixfold elevations of plasma aldosterone levels, compared with wild-type littermate control animals. We propose that, as in patients with PHA-1, ENaC activity derived from transgene expression provides similar levels of Na<sup>+</sup>-absorbing function in the critical organs (lung, kidney, and colon). The clinical course observed for these rescued animals was similar to that observed for children with PHA-1. Such affected children have no apparent problem with clearance of fetal lung liquid in the perinatal period and generally present with clinical symptoms related to metabolic dysfunction only after the first 48 h of life (30).

**Classic Approach for Investigating ENaC Function in Other Tissues**

Mice deficient for ENaC (αENaC<sup>−/−</sup> or Scnn1a<sup>tm1</sup>/Scnn1a<sup>tm1</sup>) can now be used as tools to study the role of ENaC in tissues such as eye, skin, or inner ear. The role of ENaC expression is less clear in those tissues; for example, a role for ENaC in differentiation or mechanoreception has been postulated. In skin, ENaC expression has been found in amphibians (31) and mammals (32,33), specifically in hair follicles, interfollicular epidermis, and sweat glands. For the latter, a role in mediating Na<sup>+</sup> absorption has been proposed (34). The function of ENaC in the mammalian epidermis, which is a nonabsorbing epithelium, is not clear. Preliminary studies on skin sections from newborn Scnn1a<sup>tm1</sup> homozygous mutant and wild-type mice demonstrated abnormal epidermal differentiation, with hyperplasia and vacuolization of the epidermis, accompanied by premature lipid secretion. These results indicate that ENaC is essential for normal epidermal differentiation and barrier formation, presumably through adjustment of ion transport required for normal epidermal development (E.H., M.G., T.M., unpublished observations).

Furthermore, a role for ENaC in the cochlea has been suggested (35–37). It has been proposed that ENaC might be implicated in mechanically gated transducer channels, which are involved in hearing. To test this hypothesis, we examined mice deficient in Scnn1a (Scnn1a<sup>tm1</sup>/Scnn1a<sup>tm1</sup>) and therefore in ENaC function. First, neonatal Scnn1a<sup>tm1</sup>/Scnn1a<sup>tm1</sup> mice exhibited vestibular reflexes not different from those of wild-type littermates, indicating normal vestibular function (38). In organotypic cultures of cochlear outer hair cells, we could show that hearing function, as monitored by measurement of transducer currents in whole-cell voltage-clamp experiments, was not impaired in these mice (38). Therefore, the mechani-
cally gated transducer channel is different from ENaC, but ENaC might play a role in regulating the Na\(^+\) concentration within the endolymph, thus being directly or indirectly involved in auditory function. Because Scnn1a knockout mice (with the Scnn1a\(^{tm1}\) allele) die soon after birth, conditional knockout of the Scnn1a gene is one of the possible methods to address this topic in the future.

**Investigating ENaC Function with Conditional Gene Targeting**

More recently, methods aimed at controlling gene targeting in a time- and tissue-dependent manner have been developed. This approach is appropriate in cases where complete gene inactivation leads to a lethal or complex phenotype or where it may be difficult to distinguish cell-autonomous lesions from more complex lesions, as in the case of the Scnn1 genes. Conditional gene targeting involves the use of the site-specific recombinase Cre ("causes recombination") from phage P1, which recognizes and binds to a 34-bp, partly palindromic, target sequence called loxP ("locus of crossover in P1"). Cre recombinase has the ability to efficiently excise any sequence placed between two loxP sites ("floxed") of the same relative orientation by intramolecular recombination. As a result, one loxP site remains within the genome (39). Gene inactivation can be restricted to a particular cell type in vivo by crossing a mouse strain harboring the floxed allele with a transgenic strain expressing Cre recombinase under the control of a cell type-specific promoter, e.g., the aquaporin 2 promoter (40). Moreover, tissue-specific gene inactivation may define physiologic roles of Scnn1 gene products in a given tissue, without compromising other functions of the organism.

Therefore, we have planned a targeting vector that contains homologous sequences of the endogenous Scnn1a locus, the vital coding exon (here exon 1) flanked by loxP sites, and a neomycin selection marker (neo), followed by a third loxP site (Scnn1a\(^{neo}\) allele) (Figure 3). In embryonic stem cells, the neomycin selection marker is removed by transient transfection using Cre recombinase, leaving the floxed exon 1 (Scnn1a\(^{floxed}\) allele) untouched. In mice, mating of mice carrying Scnn1a\(^{neo}\) or Scnn1a\(^{floxed}\) allele with a germline deleter strain (e.g., El la-Cre) (28) induces excision of the flanked sequences, thereby creating the Scnn1a\(^{mut}\) mutared allele. This should inactivate the Scnn1a gene locus and abolish ENaC activity (Figure 3). Conditional gene targeting will be possible using Cre-expressing mouse strains and mice homozygous for either the Scnn1a\(^{floxed}\) or Scnn1a\(^{neo}\) allele. Control of gene targeting will allow differentiation of the effects of chronic versus acute depletion of proteins and analysis of functions at different time points in development. Gene inactivation at a specific time point, leaving gene function intact throughout development, should prevent adaptive responses; therefore, phenotypes might be different in conditional, compared with conventional, knockout mice.

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