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Review

Knockout animals and natural mutations as experimental and diagnostic tool for studying tight junction functions *in vivo*

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ABSTRACT

Two sides of functions of tight junctions; the barrier and the channel in the paracellular pathway are believed to be essential for the development and physiological functions of organs. Recent identification of molecular components of tight junctions has enabled us to analyze their functions by generating knockout mice of the corresponding genes. In addition, positional cloning has identified mutations in the genes of several components of tight junctions in hereditary diseases. These studies have highlighted *in vivo* functions of tight junctions.

Contents

1. Introduction

Tight junctions (TJs) contribute to epithelial and endothelial barrier functions by restricting the diffusion of solutes through the paracellular pathway in vertebrate species [1]. This function of TJs enables isolation from the external environment and maintenance of distinct fluid compartments within the body, which are fundamental aspects for the functions of most organ systems [1]. TJs are not complete barriers, but rather contain pores through which ions and small molecules pass passively with charge and size selectivities. Importantly, the barrier properties of TJs vary among different types of epithelia depending on their physiological functions [2–6]. Transepithelial electric resistance (TER), which is the easiest and most

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sensitive measure of barrier strength toward ions, varies from 5 to 31, 000 $\Omega \times cm^2$ [52]. The selectivities between cations and anions in the paracellular pathway also vary among different types of epithelia *in vivo* and *in vitro* [2–6]. Not only for leaky epithelia but also for tight epithelia, TJs are important routes of epithelial transport, which is the sum of transcellular and paracellular transport.

Although these functions of TJs are believed to be essential for the development and physiological functions of organs, there have been no experimental demonstrations of these aspects due to the lack of appropriate methods for specifically modulating the functions of TJs *in vivo*. However, the recent identification of molecular components of TJs has enabled us to analyze their functions by generating knockout mice of the corresponding genes (Table 1). In addition, positional cloning has identified mutations in the genes of several TJ components in hereditary human and cattle diseases, further demonstrating critical roles for TJs in various organs (Table 2). In this review, the pathologies of knockout or knockdown mice and natural mutations of the genes of TJ-associated structural proteins are summarized, and the roles of TJs *in vivo* are discussed.

2. Knockouts and natural mutations of claudin family genes

Accumulating evidence has revealed that claudins are the major barrier-forming proteins of TJs [2–6]. When claudins are overexpressed in cultured fibroblasts, exogenous claudins are concentrated from both sides of adjacent cells into cell–cell contact planes, where well-developed TJ strands, the core structures of TJ barriers, are formed *de novo* [7]. Claudins comprise a multigene family containing 24 members in the mouse and human genomes [3,4]. In most cell types, multiple claudin types are coexpressed in individual cells and the combinations and proportions of different claudins vary among cell types [9]. This manner of claudin expression is thought to provide functional diversity for the barrier properties of TJs, such as conductance and charge selectivity of ions, depending on the environment of the extracellular domains of the claudins [10]. Indeed, a number of studies have revealed that overexpression of a certain claudin in cultured epithelial cells changes the barrier or channel properties of TJs, as evaluated by measuring the TER and diffusion potential, which indicate the charge selectivity of TJs [10–18]. The effects of overexpression of each claudin depend not only on the type of claudin overexpressed but also on the cell lines used, since each cell line has its own barrier properties of TJs based on its unique background expression pattern of claudins [19]. The aspects that can be measured in such experiments are the barrier properties of TJs generated from particular combinations of claudins, namely the endogenous claudins and the overexpressed claudin. It is possible, however, that the effects reflect the barrier-forming or channel-forming properties of the added claudin types, with positive and negative charge selectivities. Thus, the overall barrier properties of TJs in each epithelial type are determined by the combination of claudin types expressed in each cell type.

Table 1
Tight junction gene knockout (KO) and knockdown (KD) mice

Gene	Phenotype	Ref.
Cldn-1 KO	Skin barrier defect to the water loss	[23]
Cldn-5 KO	Blood-brain barrier defect	[30]
Cldn-11 KO	CNS myelin defect, Blood-testis barrier defect	[34]
	Deafness	[36,37]
Cldn-14 KO	Phenocopy of human non-syndromic deafness	[41]
Cldn-15 KO	Megaintestine	[44]
Cldn-16 KD	Phenocopy of familial hypomagnesemia with hypercalciuria and nephrocalcinosis	[49]
Cldn-19 KO	Schwann cell barrier defect	[57]
Occludin	Viable with complex phenotype	[63]
ZO-1	Embryonic lethal at E10.5	[77]
ZO-2	Embryonic lethal	[85]
ZO-3	No phenotype	[72,85]

Table 2
Tight junction-associated hereditary diseases

Gene	Disease	Ref.
Cldn-1	Neonatal ichthyosis and sclerosing cholangitis	[24]
Cldn-14	Non-syndromic deafness (*DFNB29*)	[8]
Cldn-16	Familial hypomagnesemia with hypercalciuria and nephrocalcinosis (human)	[48]
	Chronic interstitial nephritis and renal tubular dysplasia (bovine)	[53,54]
Cldn-19	hypomagnesemia with hypercalciuria and nephrocalcinosis with visual impairment	[59]
Tricellulin	Non-syndromic deafness (*DFNB49*)	[67]
ZO-2	Familial hypercholamenia	[78]

To date, many claudin gene knockout mice and mutations in claudin genes related to hereditary human and cattle diseases have been reported. Various pathologies have therefore come to be interpreted from the viewpoint of TJ barrier/channel deficits. In addition, mutation and morpholino suppressions of claudin genes in zebrafish have been reported.

2.1. Claudin-1-deficient mice and claudin-1 gene mutations

The mammalian epidermis consists of a stratified epithelium with four types of layers, namely the stratum basale, stratum spinosum, stratum granulosum and stratum corneum. It is well known that cornified cell envelopes and the lipid lamella between them create a strong barrier in the skin against physical stress, infection and water dispersion [20]. On the other hand, the roles of TJs in the epidermis have been ignored for a long time due to the difficulties associated with identifying TJs in the skin by electron microscopy [21]. However, the recent identification of TJ-associated proteins has enabled us to re-examine the existence of TJs in the mammalian epidermis. Immunofluorescence microscopy analyses have identified continuous TJs surrounding keratinocytes in the stratum granulosum that include, at least, occludin, claudin-1 and claudin-4 [22,23].

Claudin-1-deficient ($Cldn1^{-/-}$) mice are born alive, but die within 1 day of birth due to excessive dehydration from the skin [23]. The skin of $Cldn1^{-/-}$ mice looks macroscopically normal at birth, but gradually begins to show a wrinkled appearance. The epidermis in $Cldn1^{-/-}$ mice does not exhibit any overt abnormalities in the organization of the keratinocyte layers, except that the stratum corneum appears to be more compact than those of wild-type and heterozygous mice under conventional fixation/embedding conditions during sample preparation for microscopy [23]. Morphological analyses by immunofluorescence and electron microscopy suggested the presence of continuous TJs containing occludin and claudin-4 in the stratum granulosum in $Cldn1^{-/-}$ mice [23]. However, these TJs are permeable to a water-soluble tracer of ~600 Da injected subcutaneously, whereas this tracer cannot pass through TJs in wild-type mice. The lipid lamella formed between corneocytes appears normal in $Cldn1^{-/-}$ mice [23]. Although claudin-1 was demonstrated to be essential for the TJ barrier in granular cells[23], the barrier function of the stratum corneum to the water loss in these mice may also be affected, which should be examined in the future study. The reason why claudin-1-deficient TJs, which still include at least claudin-4, become leaky is also an open question.

Mutations in the claudin-1 gene have been identified in neonatal ichthyosis and sclerosing cholangitis (NISCH) syndrome [24]. Originally, this syndrome was reported as autosomal recessive ichthyosis with scalp hypotrichosis, scaling alopecia, sclerosing cholangitis, oligodontia, enamel dysplasia and leukocyte vacuolization in four patients in two inbred kindreds of Moroccan origin [25]. The disease gene was mapped to chromosome 3q27–28, where a deletion of two nucleotides led to a frameshift mutation that resulted in a premature stop codon at amino acid 67 of claudin-1 [24]. Subsequently, a different frameshift mutation leading to a premature stop codon at