Research Article

Cooperation of Nectin-1 and Nectin-3 Is Required for Maintenance of Epidermal Stratification and Proper Hair Shaft Formation in the Mouse

Toshiyuki Yoshida,1 Yoshimi Takai,2 and Irma Thesleff3

1 Institute of Advanced Biomedical Engineering and Science, Tokyo Women’s Medical University, 8-1 Kawada-cyo, Shinjuku-ku, Tokyo 162-8666, Japan
2 Division of Molecular and Cellular Biology, Department of Biochemistry and Molecular Biology, Kobe University Graduate School of Medicine, Kobe 650-0047, Japan
3 Institute of Biotechnology, University of Helsinki, P.O. Box 56, 00014 Helsinki, Finland

Correspondence should be addressed to Toshiyuki Yoshida; tshkshd1@gmail.com

Received 13 February 2014; Accepted 1 June 2014; Published 30 June 2014

Academic Editor: De-Li Shi

Copyright © 2014 Toshiyuki Yoshida et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Nectins constitute a family of four cell adhesion molecules which are localized on cell membrane. Mutations in NECTIN-1 gene cause the human ectodermal dysplasia syndrome (CLPED1) manifesting severe defects in skin and its appendages. However, nectin-1 null mutant mice have only a mild defect in epidermal stratification suggesting compensation by other nectins. We have analysed the epidermal and hair phenotypes of nectin-1; nectin-3 compound mutants. Epidermis was fragile and displayed severe defects in stratification, hair follicles were hypoplastic, and hair shaft structure was abnormal. Immunohistochemical analysis revealed severe defects in cell-cell junctions including adherens and tight junctions as well as desmosomes. It is therefore likely that the phenotypes were caused by impaired cell adhesion. The expression patterns of nectin-1 and nectin-3 together with the phenotypes in compound mutants indicated that heterophilic interactions between the two nectins are required for proper formation of epidermis and hair in mice. The nectin-1; nectin-3 compound mutant mice partially reproduced the phenotype of human CLPED1 patients.

1. Introduction

Nectins are immunoglobulin- (Ig-) like, calcium-independent cell adhesion molecules involved in various cellular and physiological processes including proliferation, migration, polarization, and adhesion [1]. The nectins are encoded by PVRL genes and the family is composed of four members, nectin-1 to nectin-4. Each nectin has an N-terminal extracellular domain with three Ig loops, a transmembrane domain, and a C-terminal intracellular domain. Nectins interact with nectins or other cell surface molecules on adjacent cells through the Ig loops. The nectin-nectin interactions are either homophilic or heterophilic, that is, interactions between the same or a different nectin, respectively [2]. The nectin interaction elicits intracellular signalling via the binding of the C-terminal domain to afadin [3]. Of the cellular functions regulated by nectins, cell adhesion has been extensively studied.

Nectin signalling recruits cadherins to the junctional site forming an adherens junction, and this is followed by the recruitment of claudins forming a tight junction [4–6]. Recent studies in mouse show that nectin interactions are involved in formation and/or maintenance of desmosomes as well [7, 8]. The identification of mutations in NECTIN-1 (PVRL1) gene as the cause of severe human ectodermal dysplasia (ED) syndrome Cleft Lip/Palate-Ectodermal Dysplasia Syndrome 1 (CLPED1, MIM 225060) indicates an important role for nectin in the development of mammalian skin and its appendages [9, 10]. In addition, during the course of our study mutations in NECTIN-4 were reported to cause ED [11]. EDs are congenital disorders characterized by abnormalities in two or more ectodermal organs such as teeth, hair, epidermis,
and several exocrine glands. The ectodermal organ defects in CLPED1 patients include kinky and sparse hair and thickening of palm skin [12–14]. The mutations identified in CLPED1 lead to truncated protein that lacks the transmembrane domain and C-terminus [9, 15]. As the interaction of the C-terminus with afadin is required for signalling, the mutations are thought to cause the loss of nectin signalling and therefore, the defects of CLPED1 patients are thought to be caused by lack of nectin-1 signalling.

Mammalian skin is composed of epidermis, dermis, and hypodermis and its appendages include pelage hair. The epidermis forms the primary barrier against the external environment and prevents water loss, and pelage hair supports this function [16]. The epidermis comprises four distinct cell layers: the basal, spinous, granular, and cornified layers [17]. The basal cells are attached to the basement membrane and proliferate to supply cells for the turnover of epidermis. During epidermal stratification epithelial cells differentiate progressively and express specific proteins for each cell layer, eventually forming the denuded and keratinized cornified envelope [16]. Pelage hair is formed by hair follicles composed of a dermal papilla which is surrounded by epithelial tissue including matrix and the inner and outer root sheath (IRS and ORS, resp.) [18]. The matrix cells proliferate actively and give rise to IRS cells and hair shaft. IRS is composed of several layers and supports the hair shaft towards the surface of epidermis. Both in the epidermis and hair follicle, epithelial cells are connected via cell-cell junctions including adherens and tight junctions and desmosomes [19–21]. Previous studies demonstrated that mutant mice exhibiting modulation of cell adhesion and loss of junctional proteins show abnormal differentiation and structure of both epidermis and hair follicles [22]. These reports indicate the importance of cell-cell junctions for skin development and homeostasis.

During mouse development, nectins are expressed in ectodermal tissues including epidermis and hair follicle [8, 23, 24]. Nectin-1, nectin-2, and nectin-3 have been knocked out in mice, but none of the single mutant mouse lines exhibits severe abnormalities in ectodermal organs. Neither epidermis nor hair abnormalities have been reported in nectin-2 null mutants [25, 26]. Nectin-3 null mutants exhibit mild defects in dental enamel, eye, and neurogenesis [8, 27–29]. Intriguingly, although CLPED1 patients exhibiting mutations in NECTIN-1 gene manifest severe aberrations in hair and tooth development, nectin-1 deficient mice have only mild defects in skin including fragile epidermis with reduced expression of the granular layer marker loricrin, and minor defects in enamel [7, 8, 30]. In addition, nectin-1 null mutants show mild defects in the eye and neurogenesis [27, 29]. Hence, nectin-1 null mutant mice do not recapitulate the phenotypes of human CLPED1 and they cannot be used as an appropriate animal model to examine the pathogenesis of CLPED1 or the role of nectins in the morphogenesis of ectodermal organs. As previous studies have reported that overlapping expression of nectins in skin and nectins may act via heterophilic nectin-nectin interactions, we hypothesized that the function of nectin-1 may be compensated by other nectins in mice [8, 24]. As the interaction between nectin-1 and nectin-3 is the strongest among nectin interactions [2] and nectin-1 and nectin-3 mutant mice have overlapping abnormalities, nectin-3 appeared as a good candidate for the compensating nectin.

We generated compound mutant mice of nectin-1 and nectin-3 [8] and observed more severe skin defects than in single null mutants. The epidermis of compound mutant mice exhibited a cornification defect and abnormalities in the basal epithelial layer, and the structure of the pelage hair was aberrant. The expressions and localization of cell adhesion molecules associated with adherens and tight junctions and desmosomes were severely impaired in the compound mutant epidermis and hair follicles. These abnormalities were not observed during embryonic development. Our results indicated that cooperation of nectin-1 and nectin-3 is important for postnatal development and homeostasis of mouse epidermis and pelage hair.

2. Materials and Methods

2.1. Animals. Wild-type NMRI mice were used for gene expression analysis. The nectin-1 and nectin-3 single mutants were in the C57BL/6 background and double heterozygotes were generated by crossing them [29]. Compound mutants were generated by crossing the male and female double heterozygotes as previously described [8]. The appearance of a vaginal plug was taken as embryonic day 0.

2.2. Preparation of Tissues and Histology. Skin from mouse pups and embryos was dissected, fixed in 4% paraformaldehyde, and processed for paraffin sections. The sections were cut at 5 μm and used for haematoxylin and eosin (HE) staining, in situ hybridization, and immunohistochemistry.

2.3. In Situ Hybridization. In situ hybridization using 35S-UTP labeled riboprobes was carried out as previously described [8, 31]. Plasmids for generating riboprobes against nectin-1 and nectin-3 were previously described [29].

2.4. Immunohistology. Immunostaining was performed as described earlier [8, 23]. The primary antibodies used were against E-cadherin (13900, Zymed, 1:200), laminin (L9393, Sigma, 1:25), keratin 14 (RB-9020-P0, Thermo Scientific, 1:250), ZO-1 (61-7300, Zymed, 1:100), keratin 10 (PRB-159P, Covance, 1:400), loricrin (PRB-145P, Covance, 1:500), filaggrin (PRB-417P, Covance, 1:200), Ki67 (RM-9106-St, Neo Markers, 1:200), p63 (MS-1081-Pt, Neo Markers, 1:500), keratin-17 (a kind gift from Jean Pierre Coulambe, 1:200), and desmoglein1/2 (61002, Progen, 1:10).

3. Results

3.1. Expression Patterns of Nectin-1 and Nectin-3 in Epidermis and Hair Follicle. We first investigated the expression of nectins in the embryonic and postnatal skin. Since the expression of nectin-3 could not be detected by immunohistochemistry, we used radioactive in situ hybridization (ISH)
in this study. Previous studies have reported that nectin-1 is expressed in the suprabasal layer of epidermis and in all cells of the whisker follicle except for outer root sheath (ORS) [23, 24, 30], while no obvious expression of nectin-3 was observed in these tissues [24]. Consistent with these previous studies we observed nectin-1 expression in the spinous and granular layers of epidermis and hair follicle at embryonic day 17 (E17), while no specific signal for nectin-3 was detected (see Supplementary Figure S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/432043). At postnatal day 10 (P10), both nectin-1 and nectin-3 were expressed in the suprabasal layers (spinous and granular layers) of epidermis (Figures I(a) and I(b)). In hair follicle, nectin-1 expression was observed in inner root sheath (IRS), whereas nectin-3 mRNA expression was detected in ORS (Figures I(d) and I(e)).

3.2. The Macroscopic Phenotype of Nectin-1 and Nectin-3 Compound Mutant Mice. Nectin-1 and nectin-3 compound mutants were generated by crossing double heterozygote mice as previously described [8]. Until postnatal day 5, no obvious phenotype was observed in the overall phenotype of the pups. Thereafter, the growth of compound mutants was retarded and sparse hair was observed in all compound mutants (nectin-1−/−; nectin-3+/− (N = 5), nectin-1+/−; nectin-3−/− (N = 4), and nectin-1−/−; nectin-3−/− (N = 1), all these genotypes were called “compound mutant”), but not in wild type, single, or double heterozygote mice (all these genotypes were used as controls). Due to poor health, the compound mutant mice had to be sacrificed at P10. There was significant phenotypic variation among the compound mutant mice with regard to appearance of the hair, but interestingly, the severity of the skin phenotype did not correlate with specific compound genotypes. Only one double KO (nectin-1−/−; nectin-3−/−) mouse was available for analysis at P10 and it exhibited a similar, rather mild skin abnormality in histological and immunohistochemical analysis as compared with some of the nectin-1−/−; nectin-3−/− mice (see Supplementary Figure S3). The phenotypes of nectin-1+/−; nectin-3−/− mice were comparable to those of nectin-1−/−; nectin-3+/− (see Figure 3). We mainly describe the phenotype of nectin-1−/−; nectin-3+/− mice in this report. At P10, the compound mutants had variable deviations in body size (Figures 1(g)-1(i)) and hair thickness. The long guard hairs visible in macroscopic examination of controls (arrowhead in Figure 1(g)) were not observed in the mutants. However, they were actually present as shown by microscopy (see below). Obvious hair abnormalities were observed also in ventral skin in several compound mutant mice (Supplementary Figure S2).

3.3. The Compound Mutant Epidermis Exhibits Stratification Defects, Abnormal Basal Cell Layer, and Junctional Defects. During the dissection of the skin and its processing for histology, their skin sections detached readily from the glass slides during sectioning and staining procedures. In histological analysis, the compound mutant skin detached between dermis and subcutis (Figures 2(a), 2(b), 2(f), and 2(g)). Higher magnifications revealed morphological abnormality of basal cell nuclei and epidermal layers in the compound mutant epidermis (Figures 2(b), 2(c), 2(g), and 2(h)). Hair follicles of the compound mutants were hypoplastic and exhibited abnormal structure compared with that of the controls (Figures 2(d), 2(e), 2(i), and 2(j)). Notably, the nuclei of the compound mutant hair follicles could be observed only after prolonged haematoxylin staining or nuclear staining using DAPI (inset of Figure 2(j) and see Figure 5(l')).

Next we addressed the differentiation of the compound mutant epidermis using immunohistochemical analysis of markers for each epidermal cell layer. The following antibodies against proteins specific for basal, spinous, granular, and cornified layers were used for immunostaining: keratin 14 (K14), K10, loricrin, and filaggrin, respectively [17]. Loricrin expression was previously shown to be reduced in nectin-1 single mutants, while expressions of the markers for other cell layers were indistinguishable from controls [30]. In the compound mutants, the expressions of K14, loricrin, and filaggrin as well as basement membrane protein laminin were severely affected, while the expression of spinous layer marker K10 was observed in upper layers (Figures 3(a)–3(i), and 3(l)). In the basal layer, the expressions of cell proliferation marker Ki67 and basal layer marker p63 were almost lost in some compound mutants (Figures 3(j), 3(k), 3(m), and 3(n)), while in some mutants residual expression was apparent (Figure 3(o) and 3(p)). K14 is a known target of p63 [32] and in line with this, the triple immunostaining indicated that their levels of expression correlated in individual cells (Figures 3(o) and 3(p), Supplementary Figure S3). In conclusion, our observations indicated that the stratification of the epidermis and the proliferation and morphology of cells in the basal layer were affected by reduced nectin expression.

Previous studies have reported that nectin-nectin interactions recruit adherens and tight junctions [2] and desmosomes [7] and that disturbed cell adhesion results in abnormal differentiation of the epidermis [33–35]. We therefore investigated the expression of different junctional proteins to clarify the pathogenesis of the observed defects in epidermis. Adherens junctions and desmosomes form throughout epidermis, while tight junctions mainly form between granular layer cells [36, 37]. We used antibodies against E-cadherin and ZO-1 to detect adherens and tight junctions, respectively. Desmosomes were detected by an antibody recognizing both desmoglein 1 and 2 (desmoglein 1/2). As desmoglein 1 is present in the suprabasal layers and desmoglein 2 in the basal layer [38] the antibody detects desmosomes throughout epidermis. The epidermis of the compound mutants exhibited impaired expression and localization of these marker proteins of the adherens and tight junctions and desmosomes (Figures 4(a)–4(f)). It is likely that the observed defects in the epidermal structure and cell adhesion contributed to the weakness of the skin and the poor general condition of the mice.

3.4. The Hair Follicles and Hair Shafts of the Compound Mutants Exhibit Disturbed Structure and Impaired Cell Adhesion. The pelage hair of the compound mutants exhibited
Figure 1: ((a)–(f)) Expression of nectin-1 and nectin-3 in postnatal mouse epidermis and hair follicles. Sagittal sections of mouse skin at postnatal day 10 (P10), during first anagen, analysed by in situ hybridization. Both nectin-1 and nectin-3 are expressed in the spinous and granular layer of epidermis ((a) and (b)). In hair follicle, nectin-1 expression is observed in IRS, not in ORS (d). Nectin-3 expression is observed in ORS, not in IRS (e). Asterisk indicates nonspecific staining due to pigment of hair shaft. Dashed lines in (a), (b), and (c) indicate basement membrane (black) and border between granular and cornified layers (white) and in (d)–(f) they indicate outline of hair shaft and melanocytes (white) and border between ORS and IRS (black). Scale bars are 50 µm in (a), (b), and (c) and 20 µm in (d), (e), and (f). ((g)–(i)) Hair abnormality of the nectin-1−/−; nectin-3+/− compound mutants. Macroscopic appearance of one control and two nectin-1−/−; nectin-3+/− mice at P10. (g'), (h'), and (i') show magnifications of (g), (h), and (i). The nectin-1−/−; nectin-3+/− mice are smaller in size compared with the control ((g')–(i')). The control mouse has thick hair and the long and straight guard hairs are visible in lateral inspection (arrowhead in (g')). The density and length of hair varied among the nectin-1−/−; nectin-3+/− mutants, but the long guard hairs were not visible ((h') and (i')). Scale bar is 1 cm.
severe abnormalities. We first analysed the structure and types of hair plucked from mice of controls and all compound mutant genotypes at P10. Analysis of the plucked hair under stereomicroscope indicated that comparable hair types to the control (guard, awl, and zigzag) were present in all compound mutants (data not shown). This indicates that the long guard hair had developed in the mutants although they were not observed in the macroscopic examination of the mice (Figures 1(h) and 1(f)). Higher magnification revealed, however, that the air cells in the guard hair shafts of the compound mutants exhibited disorganized patterns (Figures 5(a) and 5(d)). A similar air cell abnormality was also observed in the other hair types (data not shown). The expression pattern of K17 in hair shafts [39] and nuclear staining using DAPI also showed abnormal localization of the hair matrix protein and arrangement of nuclei, respectively (Figures 5(b) and 5(e)). Abnormal distribution of K17 immunoreactivity and lack of laminin expression in ORS [40, 41] also revealed an abnormal structure of ORS (Figures 5(b), 5(c), 5(e), and 5(f)).

We next localized cell adhesion molecules to investigate the formation of cellular junctions in the abnormal hair shafts and hair follicles of the compound mutants. Previous studies show that E-cadherin localizes to the adherens junctions between IRS and ORS [42] and that the desmosomal and tight junction proteins desmoglein 1/2 and ZO-1 are present in IRS, ORS, and matrix in hair follicles [37]. Although we observed the expression of the markers of all three types of junctions in the hair follicles of compound mutants, the localizations of these junctional proteins were diffuse, and no obvious membrane localization was detected (Figures 5(g)–5(l)). We did not observe severe loss of Ki67 and p63 expression in the compound mutant hair follicles (Supplementary Figure S4). This suggested that the hair follicle and hair shaft abnormalities were caused by the defects in cell adhesion due to the deficiency of nectin-1 and nectin-3.

3.5. No Significant Skin Abnormalities Were Observed in the Embryos of Compound Mutants. The embryonic epidermal and hair follicle phenotypes of the compound mutants were investigated at E14 and E17. At E14, the external appearance of the skin of the mutant embryos was indistinguishable from the controls (Supplementary Figure S5) and histological examination of the epidermis and hair follicles did not reveal abnormalities either in cell and tissue morphology or in protein localization (Supplementary Figure S5). Notably, the placodes of the first wave of hair follicles giving rise to guard hair were apparent in the E14 compound mutants with similar patterns to those in the control embryos. The localization of E-cadherin in E14 hair placodes and whisker follicles was normal in the compound mutants (Supplementary Figure S5). At E17, the mutant embryos appeared to be similar to the controls when observed under stereomicroscope (Supplementary Figure S6). In histological sections, morphology of E17 compound mutant epidermis was similar to controls, and the follicles of guard hair as well as awl hair were present both in the compound mutants and controls with indistinguishable histological features (Figures 6(a) and 6(f)). Immunohistochemical analysis revealed that the basal and cornified cell layer markers were slightly reduced in intensity in the compound mutant epidermis (Figures 6(b), 6(c), 6(g), and 6(h)). However, no obvious abnormalities were observed either in the expression or in the localization of adherens junction and desmosomal proteins in the compound mutant epidermis and hair follicle in the embryos (Figures 6(d),
Figure 3: Defects in cell differentiation and cell-cell junctions in compound mutant epidermis. Immunohistochemical analysis of dorsal epidermis of control ((a)–(d), (i)–(k), and (o)), nectin-1−/--; nectin-3+/− ((e)–(h), (l)–(n), and (p)), and nectin-1+/−; nectin-3−/− (p) at P10. The continuous expression of laminin in the basement membrane of the control skin (arrowhead in (a)) is mostly lost in the nectin-1−/--; nectin-3+/−, with only little remaining expression (arrowheads in (e)). K14 expression in the basal layer is weaker and the structure is disorganized in the nectin-1−/--; nectin-3+/− ((b) and (f)). Arrowheads in (b) and (f) indicate the region magnified in inset. K10 expression is observed in the spinous layer both in the control (c) and the nectin-1−/--; nectin-3+/− (g). Loricrin and filaggrin are severely reduced in the nectin-1−/--; nectin-3+/− compared with the control ((d), (i), (h), and (l)). Ki67 and p63 are completely lost from the basal layer cells in the nectin-1−/--; nectin-3+/− ((j), (k), (m), and (n)). ((o) and (p)) Triple staining with antibodies against p63 and K14 and with DAPI for the nuclei. Arrowheads indicate the same cell in different staining. In nectin-1+/−; nectin-3−/− mutant epidermis, the cells that are negative for p63 exhibit disrupted or no expression of K14 (arrowheads in (p)). Dashed lines: basement membrane. Scale bars are 30 μm in (a)–(i), (l), (o), and (p) and 10 μm in (j), (k), (m), and (n).
Developmental Biology Journal 7

Control

(a)  

(b)  

(c)  

Nectin-1−/−
Nectin-3+/−

(d)  

(e)  

(f)  

Desmoglein 1/2
ZO-1
E-cadherin

Figure 4: Junctional defects of the compound mutant epidermis. Immunohistochemical analysis of dorsal epidermis of control ((a)–(c)) and nectin-1−/−; nectin-3+/− ((d)–(f)) at P10. E-cadherin is significantly reduced throughout the epidermis and ZO-1 is reduced in granular layer in the nectin-1−/−; nectin-3+/− epidermis ((a), (b), (d), and (e)). The membrane localization of desmoglein 1/2 is lost in the nectin-1−/−; nectin-3+/− ((c) and (f)). Dashed lines: basement membrane. Scale bar: 10 µm in (a)–(f).

4. Discussion

4.1. Nectin-1 and Nectin-3 Are Required for Cell Adhesion and Differentiation in the Epidermis and Hair Follicle. The compound mutant epidermis was fragile, and the severely reduced or absent expression of filaggrin and loricrin indicated defects in the granular and cornified cell layers. The adherens and tight junctions as well as desmosomes were all severely impaired as shown by the dispersed or completely absent expression of their specific markers and abnormal ultrastructure. Mice lacking the tight junction protein claudin-1 exhibit barrier defect in skin and die because of excess water loss [43], while mutant mice that lack components of other junctions in epidermis survive longer [33, 35, 44, 45]. Therefore, it is possible that defective tight junctions in the nectin compound mutants are the primary cause of the compromised fitness and fragility of their skin. Since nectin interaction recruits tight junctions through adherens junction formation [1], the cornification defects in the nectin mutants may be secondary to the lack of adherens junctions, and in line with this, a mouse strain lacking E-cadherin in skin also lacks tight junctions [34]. Interestingly, similar to the nectin mutants reported here, the mice lacking E-cadherin in epidermis exhibit reduction of filaggrin and loricrin but retain the expression of K10 in the suprabasal cells [33, 35].

We observed abnormal cell shapes and intracellular structures in light and electron microscopy of epidermal cells in the nectin compound mutants. Similar observations were reported in mice lacking intermediate filament proteins K14 and K17 [46, 47] and it is known that a desmosome is connected to intermediate filaments through desmoplakin [38]. We observed diffuse localization of desmoglein 1/2 throughout the epidermis and abnormal desmosomes between cells of the basal and suprabasal layers in the compound mutants. We hypothesized that nectin deficiency in the suprabasal cells caused the defects in desmosomes, which was followed by abnormal organization of intermediate filaments resulting in the abnormal ultrastructure of epidermal cells.

Interestingly, the compound mutant epidermis exhibited severe defects also in the basal layer although expression of neither nectin-1 nor nectin-3 was detected in these cells. The shapes of basal cell nuclei were abnormal and the expression of laminin, K14, and p63, all required for the maintenance and proliferation of basal layer cells [47–49], was reduced. In addition, expression of E-cadherin and desmoglein 1/2 was impaired. As desmosomes affect cell shape and elicit intracellular signalling involved in cellular viability [50, 51], it is conceivable that the defects in the basal cells were secondary to the nectin deficiency in the spinous layer and may have resulted from compromised function of desmosomes.

6(h), 6(i), and 6(l)). The expression of Ki67 and p63 in the basal layer cells of the E17 compound mutant epidermis was also comparable to the control (Figures 6(j), 6(k), 6(m), and 6(n)). These results suggested that the epidermal and hair phenotypes of the compound mutants become apparent mainly postnatally.
The immunohistochemical observation of hair follicles indicated similar defects in cellular junctions and intracellular structures as in epidermis, and the aberrant hematoxylin and eosin (HE) staining of hair matrix cells conceivably resulted from these structural defects. The hair follicles of the compound mutants were hypoplastic and the hair shafts were structurally abnormal as indicated by disorganized air cells and disturbed expression of the hair shaft protein K17. This may have contributed to reduced stiffness of the hair and to our failure to observe long guard hair in macroscopic examination of mutant fur. Since the deficiency of adherens junctions and desmosomes results in abnormal structure of the hair follicle and hair shaft [33, 35, 44], it is likely that the similar phenotypes seen in the nectin mutants were caused by impaired cell adhesion.

4.2. Heterophilic Interaction between Nectin-1 and Nectin-3 Is Required for Recruitment of Cell-Cell Junctions in Postnatal Epidermis and Hair Follicles. The phenotypes of the compound mutants were significantly more severe than those of nectin-1 or nectin-3 single mutants, indicating cooperative roles of these two nectins. The defects in epidermis and hair exhibited marked variation between the mice of the same genotype, and interestingly, we did not detect obvious differences between nectin-1−/−; nectin-3+− and nectin-1+−; nectin-3−/− genotypes. The interaction between nectin-1 and

![Figure 5: Structural abnormalities in hair bulb and hair shaft of the compound mutant. Microscopic appearance of plucked hair and immunohistochemical analysis of the hair shaft and bulb of control ((a)–(c) and (g)–(i)) and nectin-1−/−; nectin-3+/− ((d)–(f) and (j)–(l)) at P10. The air cells of nectin-1−/−; nectin-3+/− hair shaft exhibit irregular shape and disorganized arrangement as compared to control ((a) and (d)). The localization of K17 at ORS (arrowhead in (e)) and hair shaft (arrow in (e)) is diffuse in the nectin-1−/−; nectin-3+/− hair follicle as compared to control ((b) and (e)). The arrangement of nuclei is also disturbed in the nectin-1−/−; nectin-3+/− ((b'); (e'), DAPI staining of sections in (b) and (e)). Laminin expression in ORS is lost in the nectin-1−/−; nectin-3+/− hair follicle ((c) and (f)). The membrane localization of E-cadherin and desmoglein 1/2 in ORS and IRS is lost in the nectin-1−/−; nectin-3+/− hair follicles ((g), (h), (j), and (k)). The membrane localization of ZO-1 in matrix cells is severely disturbed in the nectin-1−/−; nectin-3+/− ((i), (i'), (l), and (l')). Arrowheads in (g)–(l) indicate the magnified regions of insets. Scale bars: 50 μm in (a) and (d), 30 μm in (b) and (e), 100 μm in (c) and (f), and 50 μm in (g)–(l).]
Figure 6: Embryonic development of the skin is not affected in the nectin mutants. Hematoxylin and eosin staining and immunohistochemical analysis of dorsal skin of control ((a)–(d) and (i)–(k)) and nectin-1−/−; nectin-3+/- mice ((e)–(h) and (l)–(n)) at E17. The nectin-1−/−; nectin-3+/- epidermis and developing hair follicle appear histologically comparable to the controls ((a) and (e)). In nectin-1−/−; nectin-3+/- epidermis, K14 expression is restricted to one row of basal epithelial cells facing the basement membrane, while in the control K14 is expressed intensely in 2-3 cell layers ((b) and (f)). The expression of filaggrin is slightly reduced in the nectin-1−/−; nectin-3+/- epidermis compared with control ((c) and (g)). The expressions of E-cadherin and DSG in nectin-1−/−; nectin-3+/- epidermis and hair follicle are comparable to the control ((d), (h), (i), and (l)). Ki67 and p63 expressions in basal layer cells of the nectin-1−/−; nectin-3+/- epidermis are similar to the control ((j), (k), (m), and (n)). Dashed lines: basement membrane. Scale bars: 100 μm in (a) and (e) and 20 μm in (b)–(d) and (f)–(n).

nectin-3 is the strongest among homophilic and heterophilic nectin interactions, but nectin-1 and nectin-3 also interact with nectin-4 and nectin-2 which are expressed in postnatal and embryonic skin [1]. Taken together, our results indicated that, in single nectin-1 and nectin-3 null mutant mice, homophilic interaction of the remaining nectin (i.e., nectin-3 in nectin-1−/− and vice versa) partially compensated for the function of the lacking nectin. Additional loss of one copy of the remaining nectin reduced the compensatory interaction and was sufficient to cause the severe epidermal and hair abnormalities. We therefore suggested that the intensity of nectin signalling, reflecting the amount and strength of nectin interactions, was critical for recruitment of cell-cell junctions in epidermis and hair follicle. Of specific interest was the localization of nectin-1 and nectin-3 in adjacent cell layers and the IRS and ORS in the hair follicle. This presents an example of heterophilic nectin interactions between adjacent cell layers required for proper development in various organs [8, 29, 52, 53].

The compound mutant epidermis exhibited more severe abnormalities than in hair follicle. The compound mutant epidermis exhibited severely reduced expression of Ki67 and p63, while only slight reductions of p63 and Ki67 expression were observed in the compound mutant hair follicle. This might be explained by the different expression pattern of nectin-1 and -3 in epidermis and hair follicle. In the epidermis, both nectin-1 and nectin-3 were expressed in suprabasal layer. In hair follicle, nectin-1 was expressed in IRS
and nectin-3 was expressed in ORS. Thus, the loss of nectin-1 and -3 was thought to cause more severe loss of signal from suprabasal layer to basal layer than that from ORS to IRS. This might lead to the more severe abnormalities in epidermis than that in hair follicle.

Although the overall health of the compound mutants was compromised at P10 and they had severe skin defects, the pups appeared macroscopically normal at P5 and no significant abnormalities were observed in the epidermis and hair follicles during embryogenesis. This was consistent with the results that nectin-1 mutants have no apparent embryonic phenotype and that nectin-3 appeared not to be expressed in the embryonic skin. However, as nectin-2 and nectin-4 are expressed in the embryonic skin [24], it is likely that they further compensate for nectin function during embryonic development.

In conclusion, our results suggest that heterophilic interaction between nectin-1 and nectin-3 is required for postnatal epidermal development and proper pelage hair formation in mice. In particular, nectin deficiency affected adherens, desmosomal, and tight junctions and reduced indirectly basal cell proliferation, thereby leading to severe skin phenotype. These findings increase the understanding of the role of nectins in epidermis and hair development.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was supported by grants from the Academy of Finland (IT) and the Sigrid Juselius Foundation (IT). The authors thank Merja Mäkinen, Riikka Santalahti, and Raija Savolainen for excellent technical assistance and Marja Mikkola for comments on the paper.

References

[1] T. Sakisaka, W. Ikeda, H. Ogita, N. Fujita, and Y. Takai, “The roles of nectins in cell adhesions: cooperation with other cell adhesion molecules and growth factor receptors,” *Current Opinion in Cell Biology*, vol. 19, no. 5, pp. 593–602, 2007.

[2] Y. Takai, W. Ikeda, H. Ogita, and Y. Rikitake, “The immunoglobulin-like cell adhesion molecule nectin and its associated protein afadin,” *Annual Review of Cell and Developmental Biology*, vol. 24, pp. 309–342, 2008.

[3] K. Takahashi, H. Nakanishi, M. Miyahara et al., “Nectin/PRR: an immunoglobulin-like cell adhesion molecule recruited to cadherin-based adherens junctions through interaction with afadin, a PDZ domain-containing protein,” *The Journal of Cell Biology*, vol. 145, no. 3, pp. 539–549, 1999.

[4] T. Hoshino, T. Sakisaka, T. Baba, T. Yamada, T. Kimura, and Y. Takai, “Regulation of E-cadherin endocytosis by nectin through afadin, Rap1, and p120ctn,” *The Journal of Biological Chemistry*, vol. 280, no. 25, pp. 24095–24103, 2005.

[5] T. Sato, N. Fujita, A. Yamada et al., “Regulation of the assembly and adhesion activity of E-cadherin by nectin and afadin for the formation of adherens junctions in Madin-Darby canine kidney cells,” *The Journal of Biological Chemistry*, vol. 281, no. 8, pp. 5288–5299, 2006.

[6] A. Yamada, N. Fujita, T. Sato et al., “Requirement of nectin, but not cadherin, for formation of claudin-based tight junctions in annexin II-knockdown MDCK cells,” *Oncogene*, vol. 25, no. 37, pp. 5085–5102, 2006.

[7] M. J. Barron, S. J. Brookes, C. E. Draper et al., “The cell adhesion molecule nectin-1 is critical for normal enamel formation in mice,” *Human Molecular Genetics*, vol. 17, no. 22, pp. 3509–3520, 2008.

[8] T. Yoshida, J. Miyoshi, Y. Takai, and I. Thesleff, “Cooperation of Nectin-1 and Nectin-3 is required for normal ameloblast function and crown shape development in mouse teeth,” *Developmental Dynamics*, vol. 239, no. 10, pp. 2558–2569, 2010.

[9] M. A. Sözen, K. Suzuki, M. M. Tolarova, T. Bustos, J. E. Fernández Iglesias, and R. A. Spritz, “Mutation of PVRL1 is associated with sporadic, non-syndromic cleft lip/palate in northern Venezuela,” *Nature Genetics*, vol. 29, no. 2, pp. 141–142, 2001.

[10] K. Suzuki, T. Bustos, and R. A. Spritz, “Linkage disequilibrium mapping of the gene for Margarita Island ectodermal dysplasia (ED4) to 11q23,” *American Journal of Human Genetics*, vol. 63, no. 4, pp. 1102–1107, 1998.

[11] F. Brancati, P. Fortugno, I. Bottillo et al., “Mutations in PVRL4, encoding cell adhesion molecule nectin-4, cause ectodermal dysplasia-syndactyly syndrome,” *The American Journal of Human Genetics*, vol. 87, no. 2, pp. 265–273, 2010.

[12] T. Bustos, V. Simosa, J. Pinto-Cisternas et al., “Autosomal recessive ectodermal dysplasia: I. An undescribed dysplasia/malformation syndrome,” *American Journal of Medical Genetics*, vol. 41, no. 4, pp. 398–404, 1991.

[13] J. Zlotogora, “Syndactyly, ectodermal dysplasia, and cleft lip/palate,” *Journal of Medical Genetics*, vol. 31, no. 12, pp. 957–959, 1994.

[14] J. Zlotogora, Y. Zilberman, A. Tenenbaum, and M. R. Wexler, “Cleft lip and palate, pili torti, malformed ears, partial syndactyly of fingers and toes, and mental retardation: a new syndrome?” *Journal of Medical Genetics*, vol. 24, no. 5, pp. 291–293, 1987.

[15] K. Suzuki, D. Hu, T. Bustos et al., “Mutations of PVRL1, encoding a cell-cell adhesion molecule/herpesvirus receptor, in cleft lip/palate-ectodermal dysplasia,” *Nature Genetics*, vol. 25, no. 4, pp. 427–430, 2000.

[16] F. M. Watt, “Stem cell fate and patterning in mammalian epidermis,” *Current Opinion in Genetics & Development*, vol. 11, no. 4, pp. 410–417, 2001.

[17] X. Dai and J. A. Segre, “Transcriptional control of epidermal specification and differentiation,” *Current Opinion in Genetics and Development*, vol. 14, no. 5, pp. 485–491, 2004.

[18] S. Müller-Röver, B. Handjiski, C. van der Veen et al., “A comprehensive guide for the accurate classification of murine hair follicles in distinct hair cycle stages,” *Journal of Investigative Dermatology*, vol. 117, no. 1, pp. 3–15, 2001.

[19] J. R. McMillan and H. Shimizu, “Desmosomes: structure and function in normal and diseased epidermis,” *Journal of Dermatology*, vol. 28, no. 6, pp. 291–298, 2001.

[20] C. Jamora, R. DasGupta, P. Kocieniewski, and E. Fuchs, “Links between signal transduction, transcription and adhesion in formation of adherens junctions in Madin-Darby canine kidney cells,” *The Journal of Biological Chemistry*, vol. 281, no. 8, pp. 5288–5299, 2006.
Developmental Biology Journal 11

epithelial bud development," Nature, vol. 422, no. 6929, pp. 317–322, 2003.

[21] J. A. McGrath, J. R. McMillan, C. S. Shemanko et al., "Mutations in the plakophilin 1 gene result in ectodermal dysplasia/skin fragility syndrome," Nature Genetics, vol. 17, no. 2, pp. 240–244, 1997.

[22] J. E. Lai-Cheong, K. Arita, and J. A. McGrath, "Genetic diseases of junctions," Journal of Investigative Dermatology, vol. 127, no. 12, pp. 2713–2725, 2007.

[23] J. Laurikkala, M. L. Mikkola, M. James, M. Tummers, A. A. Mills, and I. Thesleff, "p63 regulates multiple signalling pathways required for ectodermal organogenesis and differentiation," Development, vol. 133, no. 8, pp. 1553–1563, 2006.

[24] N. Okabe, K. Ozaki-Kuroda, H. Nakanishi, K. Shimizu, and Y. Takai, "Expression patterns of nectins and afadin during epithelial remodeling in the mouse embryo," Developmental Dynamics, vol. 230, no. 1, pp. 174–186, 2004.

[25] M. J. Bouchard, Y. Dong, B. M. Medermott Jr. et al., "Defects in nuclear and cytoskeletal morphology and mitochondrial localization in spermatozoa of mice lacking nectin-2, a component of cell-cell adherens junctions," Molecular and Cellular Biology, vol. 20, no. 8, pp. 2865–2873, 2000.

[26] S. Mueller, T. A. Rosenquist, Y. Takai, R. A. Bronson, and E. Wimmer, "Loss of nectin-2 at sertoli-spermatid junctions leads to male infertility and correlates with severe spermatozoan head and midpiece malformation, impaired binding to the zona pellucida, and oocyte penetration," Biology of Reproduction, vol. 69, no. 4, pp. 1330–1340, 2003.

[27] T. Honda, T. Sakisaka, T. Yamada et al., "Involvement of nectins in the formation of puncta adherentia junctions and the mossy fiber trajectory in the mouse hippocampus," Molecular and Cellular Neuroscience, vol. 31, no. 2, pp. 315–325, 2006.

[28] M. Inagaki, K. Irie, H. Ishizaki, M. Tanaka-okamoto, J. Miyoshi, and Y. Takai, "Role of cell adhesion molecule nectin-3 in spermatid development," Genes to Cells, vol. II, no. 9, pp. 1125–1132, 2006.

[29] M. Inagaki, K. Irie, H. Ishizaki et al., "Roles of cell-adhesion molecules nectin 1 and nectin 3 in ciliary body development," Development, vol. 132, no. 7, pp. 1525–1537, 2005.

[30] K. Wakamatsu, H. Ogita, N. Okabe et al., "Up-regulation of loricrin expression by cell adhesion molecule nectin-1 through Rapl-ERK signaling in keratinocytes," The Journal of Biological Chemistry, vol. 282, no. 25, pp. 18173–18181, 2007.

[31] T. Yoshida, L. A. Phylactou, J. B. Uney, I. Ishikawa, K. Eto, and S. Iseki, "Twist is required for establishment of the mouse coronal suture," Journal of Anatomy, vol. 206, no. 5, pp. 437–444, 2005.

[32] A. B. Truong, M. Kretz, T. W. Ridley, R. Kimmel, and P. A. Khavari, "p63 regulates proliferation and differentiation of developmentally mature keratinocytes," Genes and Development, vol. 20, no. 22, pp. 3185–3197, 2006.

[33] C. L. Tinkle, T. Lechler, H. A. Pasolli, and E. Fuchs, "Conditional targeting of E-cadherin in skin: Insights into hyperproliferative and degenerative responses," Proceedings of the National Academy of Sciences of the United States of America, vol. 101, no. 2, pp. 552–557, 2004.

[34] J. A. Tunggal, I. Helfrich, A. Schmitz et al., "E-cadherin is essential for in vivo epidermal barrier function by regulating tight junctions," The EMBO Journal, vol. 24, no. 6, pp. 1146–1156, 2005.

[35] P. Young, O. Boussadia, H. Halfter et al., "E-cadherin controls adherens junctions in the epidermis and the renewal of hair follicles," The EMBO Journal, vol. 22, no. 21, pp. 5723–5733, 2003.

[36] M. Perez-Moreno, C. Jamora, and E. Fuchs, "Sticky business: orchestrating cellular signals at adherens junctions," Cell, vol. 112, no. 4, pp. 535–548, 2003.

[37] K. Morita, M. Itoh, M. Saitou et al., "Subcellular distribution of tight junction-associated proteins (occludin, ZO-1, ZO-2) in rodent skin," Journal of Investigative Dermatology, vol. 110, no. 6, pp. 862–866, 1998.

[38] D. Garrod and M. Chidgley, "Desmosome structure, composition and function," Biochimica et Biophysica Acta—Biomembranes, vol. 1778, no. 3, pp. 572–587, 2008.

[39] A. A. Panteleyev, R. Paus, R. Wanner et al., "Keratin 17 gene expression during the murine hair cycle," Journal of Investigative Dermatology, vol. 108, no. 3, pp. 324–329, 1997.

[40] J. Li, J. Tzu, Y. Chen et al., "Laminin-10 is crucial for hair morphogenesis," The EMBO Journal, vol. 22, no. 10, pp. 2400–2410, 2003.

[41] D. Nanba, Y. Hieda, and Y. Nakanishi, "Remodeling of desmosomal and hemidesmosomal adhesion systems during early morphogenesis of mouse pelage hair follicles," Journal of Investigative Dermatology, vol. 114, no. 1, pp. 171–177, 2000.

[42] C. Jamora and E. Fuchs, "Intercellular adhesion, signalling and the cytoskeleton," Nature Cell Biology, vol. 4, no. 4, pp. E101–E108, 2002.

[43] M. Furuse, M. Hata, K. Furuse et al., "Claudin-based tight junctions are crucial for the mammalian epithelial barrier: a lesson from claudin-1-deficient mice," Journal of Cell Biology, vol. 156, no. 6, pp. 1099–1111, 2002.

[44] A. Klijic, H. Bazzi, J. P. Sundberg et al., "Desmosome 4 in hair follicle differentiation and epidermal adhesion: evidence from inherited hypotrichosis and acquired pemphigus vulgaris," Cell, vol. 113, no. 2, pp. 249–260, 2003.

[45] P. J. Koch, M. G. Mahoney, H. Ishikawa et al., "Targeted disruption of the pemphigus vulgaris antigen (desmoglein 3) gene in mice causes loss of keratinocyte cell adhesion with a phenotype similar to pemphigus vulgaris," The Journal of Cell Biology, vol. 137, no. 5, pp. 1091–1102, 1997.

[46] S. Kim, P. Wong, and P. A. Coulombe, "A keratin cytoskeletal protein regulates protein synthesis and epithelial cell growth," Nature, vol. 441, no. 7091, pp. 362–365, 2006.

[47] C. Lloyd, Q. Yu, J. Cheng et al., "The basal keratin network of stratified squamous epithelia: defining K15 function in the absence of K14," The Journal of Cell Biology, vol. 129, no. 5, pp. 1329–1344, 1995.

[48] D. K. Carroll, J. S. Carroll, C. Leong et al., "p63 regulates an adhesion programme and cell survival in epithelial cells," Nature Cell Biology, vol. 8, no. 6, pp. 551–561, 2006.

[49] M. Gonzales, K. Haan, S. E. Baker et al., "A cell signal pathway involving laminin-5, α3β1 integrin, and mitogen-activated protein kinase can regulate epithelial cell proliferation," Molecular Biology of the Cell, vol. 10, no. 2, pp. 259–270, 1999.

[50] D. R. Garrod, M. Y. Berika, W. F. Bardsley, D. Holmes, and L. Tabernerò, "Hyper-adhesion in desmosomes: its regulation in wound healing and possible relationship to cadherin crystal structure," Journal of Cell Science, vol. 118, no. 24, pp. 5743–5754, 2005.
[51] K. J. Green and C. L. Simpson, “Desmosomes: new perspectives on a classic,” *Journal of Investigative Dermatology*, vol. 127, no. 11, pp. 2499–2515, 2007.

[52] K. Ozaki-Kuroda, H. Nakanishi, H. Ohta et al., “Nectin couples cell-cell adhesion and the actin scaffold at heterotypic testicular junctions,” *Current Biology*, vol. 12, no. 13, pp. 1145–1150, 2002.

[53] H. Togashi, J. Miyoshi, T. Honda, T. Sakisaka, Y. Takai, and M. Takeichi, “Interneurite affinity is regulated by heterophilic nectin interactions in concert with the cadherin machinery,” *The Journal of Cell Biology*, vol. 174, no. 1, pp. 141–151, 2006.
