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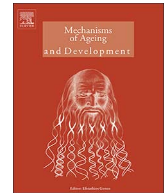

Laminin-332 regulates differentiation of human interfollicular epidermal stem cells

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A B S T R A C T

Interfollicular epidermal stem cells (IFE-SCs) have self-renewal and differentiation potentials, and maintain epidermal homeostasis. Stem cells *in vivo* are regulated by the surrounding environment called niche to function properly, however, IFE-SC niche components are not fully understood. In order to elucidate the mechanisms of keeping epidermal homeostasis and of skin aging, and also to develop new therapeutic technologies for skin diseases, we searched for niche factors that regulate IFE-SCs. We found that laminin-332, a basement membrane component, was highly expressed at the tips of the dermal papillae, where IFE-SCs are localized, and that the stem cells by themselves expressed laminin-332. Knockdown of laminin-332 during the culture of IFE-SC-model cells to construct 3-dimensional epidermis in vitro resulted in failure to form proper structure, although no significant change was observed in either cell growth or apoptosis. Pre-coating of the culture insert with laminin-332 restored the normal formation of 3-dimensional epidermis. From these results, it was shown that laminin-332 is an essential niche component for the proper differentiation of IFE-SCs.

1. Introduction

Studies on epidermal cells for the last three decades showed that interfollicular epidermal stem cells (IFE-SCs) have self-renewal and differentiation potentials, and maintain epidermal homeostasis (Ghadially, 2012). In 1987, clonal culture of human epidermal cells revealed that they formed three types of colonies; cells that formed a large colony were termed as holoclones, small colonies were formed by differentiating cells termed as paraclones, and meroclones formed a clone intermediate in size. It was considered that holoclones are epidermal stem cells and originate hierarchy of cells (Barrandon and Green, 1987). Subsequently, human epidermal cells that highly express integrin β1 on the cell surface were shown to have a high proliferation potential, and isolation of IFE-SCs has been carried out utilizing high expression of integrin β1 (Jones and Watt, 1993). Since then, various IFE-SC markers have been identified. It has been reported that IFE-SCs reside at the tips of the dermal papillae in the human epidermis, and genes such as CD271, MCSP, Delta1, and Lrig1 are specifically expressed in IFE-SCs (Akamatsu et al., 2016; Ghali et al., 2004; Jensen and Watt, 2006). Our previous studies on CD271-expressing IFE-SCs demonstrated that they played important roles in skin wound healing and that they decreased in number with chronological age (Akamatsu et al., 2016; Iwata et al., 2013).

Stem cells *in vivo* are regulated by the surrounding environment called niche to function properly (Pajonk and Vlashi, 2013). Stem cell niche is comprised of surrounding cells, extracellular matrix (ECM), and soluble factors (growth factors, cytokines, and chemokines) (Brizzi et al., 2012; Choi et al., 2015; Gattazzo et al., 2014), which regulate behavior of stem cells such as self-renewal, migration and differentiation. For example, mesenchymal cells in the bone marrow express stem

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cell factor (SCF) and CXCL12, both of which play a role in maintaining hematopoietic stem cells (Sugiyama et al., 2006; Omatsu et al., 2010). Hair follicle stem cells are maintained themselves by expressing a hemidesmosome protein, COL17A1 (Matsumura et al., 2016), and also played a critical role to keep an undifferentiated state of melanocyte stem cells located next to them by expressing TGF- β1 (Tanimura et al., 2011).

To elucidate the mechanisms of keeping epidermal homeostasis and of skin aging, as well as to develop new therapeutic technologies for skin diseases, further understanding of interactions between stem cells and stem cell niche is required. However, IFE-SC niche components are not fully understood. Here, we compared gene expression between IFE-SCs at the tips of the dermal papillae and non-stem keratinocytes at the rete ridges to identify components of the niche for IFE-SCs. Furthermore, the role of niche component for IFE-SCs was examined *in vitro* using HDK1, a human epidermal stem cell line (Inoue et al., 2014).

2. Materials and methods

2.1. Human skin biopsies

Skin samples were collected from patients at Fujita Health University Hospital. This study was approved by the ethics committees of Fujita Health University and Nippon Menard Cosmetic Research Laboratories, and written informed consent was obtained from each subject or subject's legal guardian prior to surgery. Non-affected tissues were fixed with 4% paraformaldehyde to prepare paraffin-embedded sections (n = 10 each for young and old group: young male, n = 5, 20.4 ± 6.7 years old; young female, n = 5, 19.8 ± 9.2 years old; old male, n = 5, 76.0 ± 6.4 years old; old female, n = 5, 80.2 ± 4.9 years old). For laser microdissection, fresh frozen sections of skin samples were cut without fixation.

2.2. Laser microdissection and cDNA microarray analysis

Skin samples were embedded in OCT compound, frozen, and sectioned on a cryostat (Carl Zeiss, Thornwood, NY, USA), and samples were attached on slide glasses (Leica Microsystems, Wetzler, Germany). The sections were fixed in 75% ethanol and stained with 0.05% toluidine blue. After the sections were dried, basal cells at the tips of the dermal papillae where IFE-SCs reside and the rete ridges in the epidermis were separately isolated with a laser microdissection system Leica LMD6000 (Leica Microsystems). Then, total RNA was extracted using RNeasy Micro Kit (Qiagen, Hilden, Germany). The Ovation PicoSL WTA System V2 (NuGEN Technologies, San Carlos, CA) was used to amplify cDNA. The cDNA was labeled with SureTag DNA Labeling Kit (Agilent technologies, Santa Clara, CA) for gene expression analysis using SurePrint G3 human GE Microarray 8 × 60 K Ver. 2.0 (Agilent Technologies). Gene expression and statistical analyses were performed using the Subio Platform v1.18 (Subio Inc., Nagoya, Japan). The values of raw signals were logarithmically transformed and normalized. To identify genes which were highly expressed in IFE-SCs, the genes whose IFE-SCs: rete ridge expression level ratios were > 2 were selected, and Mann-Whitney's *U* test was used to see if the differences were significant ($P < 0.05$). Identified 3149 probes for 2957 genes were listed in Table 2 and S1. Extracellular matrix or basement membrane components encoded by the identified genes (Table 2) were selected as candidate constituents of IFE-SCs niche.

2.3. Immunohistochemistry

Paraffin-embedded skin samples were sectioned, deparaffinized, and boiled in Target Retrieval Solution (Dako, Glostrup, Denmark). After washed with phosphate-buffered saline (PBS), these sections were blocked with 2% BSA for 1 h and then incubated with primary antibodies against CD271 (Acris Antibodies, Herford, Germany), LAMB3 (Sigma-Aldrich, St. Louis, MO, USA) overnight at 4 °C. Thereafter, the samples were incubated with Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies (Life Technologies, Carlsbad, CA, USA). For nuclear staining, 4′,6-Diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA) was used. Sections of reconstructed human epidermis (RHE) were processed similarly to the skin samples for immunostaining against filaggrin (FLG; abcam, Cambridge, MA, USA), keratin 10 (KRT10), CD271, LAMB3, Yes-associated protein 1 (YAP1; Novus Biologicals, CO, USA), and Notch intracellular domain (NICD; Bios Antibodies, MA, USA).

Table 1
Microdissected samples for microarray analysis.

No.	Age	Sex	Collection site
1	12	F	Groin
2	14	F	Neck
3	34	F	Neck
4	36	M	Abdomen
5	48	M	Abdomen
6	59	M	Upper arm

(F, female; M, male).

Table 2
List of genes encoding extracellular matrix component up-regulated in IFE-SCs.

No.	Fold change	Gene symbol	Gene name
1	9.19	LAMA4	laminin, alpha 4
2	8.47	SPON1	spondin 1, extracellular matrix protein
3	6.95	ECM1	extracellular matrix protein 1
4	5.33	LGALS3BP	lectin, galactoside-binding, soluble, 3 binding protein
5	5.01	MFGE8	milk fat globule-EGF factor 8 protein
6	4.72	LUM	lumican
7	4.22	COL6A2	collagen, type VI, alpha 2
8	3.97	COL1A1	collagen, type I, alpha 1
9	3.93	PRSS2	protease, serine, 2 (trypsin 2)
10	3.72	COL21A1	collagen, type XXI, alpha 1
11	3.63	MGP	matrix Gla protein
12	3.56	BGN	biglycan
13	3.43	VCAN	versican
14	3.35	SERPINE2	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2
15	3.31	COL5A1	collagen, type V, alpha 1
16	3.24	VEGFA	vascular endothelial growth factor A
17	3.08	ECM2	extracellular matrix protein 2, female organ and adipocyte specific
18	3.01	MMP14	matrix metallopeptidase 14 (membrane-inserted)
19	2.77	COL6A3	collagen, type VI, alpha 3
20	2.72	SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
21	2.62	THBS2	thrombospondin 2
22	2.57	ASPN	asporin
23	2.54	FREM2	FRAS1 related extracellular matrix protein 2
24	2.42	COL14A1	collagen, type XIV, alpha 1
25	2.29	LEPREL1	leprecan-like 1
26	2.23	CRIP2	cysteine-rich protein 2
27	2.22	LAMA1	laminin, alpha 1
28	2.20	CFP	complement factor properdin
29	2.19	LAMA3	laminin, alpha 3
30	2.17	ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1
31	2.16	LAMC3	laminin, gamma 3
32	2.12	LTBP1	latent transforming growth factor beta binding protein 1
33	2.07	MFAP4	microfibrillar-associated protein 4
34	2.07	LAMC2	laminin, gamma 2
35	2.00	LAMB3	laminin, beta 3

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