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TITLE:

Neuronatin is related to keratinocyte differentiation by up-regulating involucrin

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ABSTRACT

Background Neuronatin (Nnat), which is a neuronal developmental and differentiation molecule, is expressed in the endoplasmic reticulum of non-neuronal cells and is involved in insulin secretion from pancreatic β-cells by plausibly modulating their intracellular calcium concentration. However, the role of Nnat in keratinocyte differentiation remains unclear.

Objective To unveil a possible integration of Nnat in controlling the keratinocyte differentiation markers such as involucrin, cytokeratin1, filaggrin, loricrin and S100A7.

Methods Immunohistological staining was done using psoriasis, chronic eczema, lichen planus and normal skin. Immunofluorescence staining, Western blotting and semi-quantitative real-time PCR were performed for detecting Nnat, involucrin, cytokeratin1, filaggrin, loricrin and S100A7 using human keratinocytes with or without Nnat gene transfection. Small interference RNA was applied to knockdown the Nnat gene expression.

Results Nnat existed in normal human epidermis and cultured keratinocytes. In the hyperplastic epidermis of psoriasis, chronic eczema and lichen planus, over-expression of Nnat was evident along with involucrin and cytokeratin1 expression. Coordinate up-regulation of Nnat and involucrin, but not cytokeratin1, was demonstrated in cultured keratinocytes under differentiation stimuli such as extracellular calcium elevation, exposure to phorbol myristate acetate, and increased cell density. Transfection of small interference RNA for Nnat decreased the mRNA levels of Nnat and involucrin, but not of cytokeratin1. Furthermore, a gene transfection assay showed increased involucrin expression in the Nnat-transfected keratinocytes than in mock-transfected counterparts, without any appreciable influence on cytokeratin1, filaggrin, loricrin and S100A7 expression.

Conclusions These data indicate that Nnat is related to keratinocyte differentiation by up-regulating involucrin expression.

[247 words]

Key words:

Neuronatin, involucrin, cytokeratin1, filaggrin, keratinocyte

1. Introduction

Neuronatin (Nnat) was first identified as a gene that is predominantly expressed in the neonatal rat brain. The expression of Nnat becomes prominent during late gestation in the central and peripheral nervous systems, primarily in post-mitotic and differentiating neuroepithelial cells [1]. In the brains of adult rats, Nnat expression is markedly down-regulated [2,3], which suggests its involvement in neuronal cell development and differentiation.

In addition to the neuronal expression of Nnat, it is also expressed in non-neuronal tissues such as the pituitary glands, lung, adrenal glands, uterus, skeletal muscles, ovaries, and pancreas [4,5]. Nnat is expressed in pancreatic β-cells and is possibly involved in ion-channel transport or channel modulation [6,7]. Nnat and insulin are co-localized in the cytoplasm of pancreatic β-cells, and knockdown of Nnat dramatically decreases insulin secretion after glucose challenge [6,7]. Additional data from pancreatic cells and 3T3-L1 cells showed that Nnat resides in the endoplasmic reticulum and modulates intracellular Ca^{++} stores [5-7].

Interestingly, our previous immunohistological studies have showed that human keratinocytes as well as cutaneous nerves are target tissues for Nnat expression [8,9]. Keratinocytes are the major cell type of the multilayered stratified squamous epidermis that covers the body surface. To establish the epidermal structure and skin barrier, keratinocytes undergo fine-tuned differentiation from basal to spinous and granular layers, thereby resulting in the formation of a cornified layer. The accumulation of involucrin (Inv) and cytokeratin1 (CK1) is a well-characterized hallmark of epidermal differentiation [10,11]. Although the expression of Nnat is evident in keratinocytes that are located in the basal to spinous layers of the epidermis,

sweat glands, and sebaceous glands [10], the role of Nnat in epidermal differentiation has not been clarified. This study, to our knowledge, is the first to elucidate that 1) cultured keratinocytes indeed express Nnat, 2) Nnat is over-expressed in the acanthotic epidermis of psoriasis,chronic eczema and lichen planus, and 3) Nnat is related to keratinocyte differentiation by up-regulating Inv expression.

2. Materials and Methods

2.1. Reagents and antibodies

Phorbol myristate acetate (PMA), calcium, and anti-Inv mouse IgG antibody (SY5) were purchased from Sigma-Aldrich Chemical (St Louis, MO). Anti-Nnat rabbit IgG antibody and anti-CK1 mouse IgG antibody (AE1) were purchased from Abcam (Cambridge, MA). Anti-GAPDH rabbit IgG antibody (FL-335), normal rabbit IgG, and normal mouse IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cell culture

Normal human epidermal keratinocytes (NHEKs), which were obtained from Clonetics-BioWhittaker (San Diego, CA), were grown in culture dishes at 37°C in a 5% CO_2 atmosphere. The NHEKs were cultured in serum-free keratinocyte growth medium (Lonza, Walkersville, MD) that was supplemented with bovine pituitary extract, recombinant epidermal growth factor, insulin, hydrocortisone, transferrin, and epinephrine. The culture medium was replaced every 2 d. At near confluence (70–90%), the cells were disaggregated with 0.25% trypsin/0.01% ethylenediamine tetraacetic acid and passaged. Second-to-fourth-passage NHEKs were used in all of the experiments.

2.3. Immunofluorescence and confocal laser-scanning microscopy analysis

NHEKs that were cultured on slides were washed with PBS, fixed with acetone for 10 min, and blocked by using 10% BSA in PBS for 30 min. Samples were incubated with primary rabbit anti-Nnat (1:50), anti-Inv (1:1000), and/or anti-CK1 (1:200) antibody in PBS overnight at 4°C. The slides were washed with PBS before incubation with anti-rabbit (Alexa Fluor 546) or anti-mouse (Alexa Fluor 488) (Molecular Probes, Eugene, OR) secondary antibody for 1.5 h at room temperature. The slides were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA). All of the samples were analyzed by using a D-Eclipse confocal laser-scanning microscope (Nikon, Tokyo, Japan).

2.4. Immunohistochemical analysis

All formalin-fixed and paraffin-embedded tissues (10 psoriasis, 10 chronic eczema, 10 lichen planus and 10 normal skin) were obtained from the archives of the Department of Dermatology of Kyushu University Hospital, Japan. Several patients were associated with metabolic syndromes; 2 hypertension, 1 diabetes and 1 coronary heart disease in psoriasis; 1 hypertension, 1 diabetes and 1 coronary heart disease in chronic eczema; and 1 hypertension in lichen planus.

The paraffin-embedded tissues were evaluated via hematoxylin and eosin staining. The paraffin-embedded tissue blocks were cut into 4-μm thick tissue sections. The sections were de-paraffinized by using xylene for 10 min, rehydrated through a graded ethanol series, and then blocked for endogenous peroxidase activity in 0.3% H_2O_2 in methanol for 30 min. Antibody-binding epitopes were retrieved by pressure-cooking the tissue sections in 10 mM sodium citrate buffer at pH 7.0 (Yatoron, Tokyo, Japan) for 10 min, and nonspecific binding was blocked by using 10% goat serum. The sections were then incubated with anti-Nnat, anti-Inv or anti-CK1 (Zymed Lab Inc., San Francisco, CA) antibody overnight at 4°C. Immunodetection was carried out by employing the avidin–biotin horseradish peroxidase method by using

3,3′-diaminobenzidine as the chromogen followed by light counterstaining with hematoxylin. Washes with Tris-buffered saline or phosphate-buffered saline were performed between each step according to the manufacturer's protocols. Appropriate positive and negative controls were included in each assay.

2.5. Reverse transcription-PCR and semi-quantitative real-time PCR analysis

Total RNA was extracted by using an RNeasy Mini kit (Qiagen, Valencia, CA). Reverse transcription was performed by using a PrimeScript RT-PCR kit (Takara Bio, Shiga, Japan). Amplification was started at 95°C for 10 s as the first step, followed by 35 cycles of PCR at 95°C for 5 s, and at 60°C for 20 s. The PCR products were electrophoresed through a 1.5% agarose gel and densitometric analysis was performed by using ImageJ software (NIH). ImageJ is a public domain, Java-based image processing program that was developed at the National Institutes of Health (NIH; Bethesda, MD). The primers from Takara Bio were as follows:

hNNAT-FW: 5′-CTCGGCTGAACTGCTCATCATC-3′

hNNAT-RV: 5′-TTCTCGCAATGGGCTGTGTC-3′

hGAPDH-FW: 5′-GCACCGTCAAGGCTGAGAAC-3′

hGAPDH-RV: 5′-TGGTGAAGACGCCAGTGGA-3′

hINV-FW: 5′-CTGCCTCAGCCTTACTGTGA-3′

hINV-RV: 5′- TGGGTATTGACTGGAGGAGG-3′

hCK1-FW: 5′- ACAAGAAGTCACTATCAACCAGAG-3′

hCK1-RV: 5′- GCTCCAGGAACCTCACCTTG-3′

hFILAGGRIN-FW: 5′- TTTCGGCAAATCCTGAAGAATCC-3′

hFILAGGRIN-RV: 5′- ACTGTGCTTTCTGTGCTTGTG-3′

hLORICRIN-FW: 5′- GCACCGATGGGCTTAGAG-3′

hLORICRIN-RV: 5′- AGAAACCAAAGAGGCTAAACAG-3′

hS100A7-FW: 5′- ATGTCTCCCAGCAAGGACAG-3′

hS100A7-RV: 5′- TGCTGACGATGAAGGAG-3′

2.6. Western blotting analysis

NHEKs, treated with or without PMA or different concentrations of Ca^{++} for 48 h, were incubated with lysis buffer (Complete Lysis-M, Roche Applied Science, Indianapolis, IN). The lysate protein concentration was measured by using a BCA Protein Assay kit (Pierce, Rockford, IL). Equal amounts of protein (20 μg) were dissolved in NuPage LDS Sample Buffer (Invitrogen) and 10% NuPage Sample Reducing Agent (Invitrogen). The lysates were boiled at 70°C for 10 min and then loaded and run on 4–12% NuPage Bis-Tris Gels (Invitrogen) at 200 V for 40 min. The proteins were transferred onto polyvinylidene fluoride membranes (Invitrogen) and were blocked in 2% BSA in 0.1% Tween-20 (Sigma-Aldrich) and Tris-buffered saline. Then, the membranes were probed with anti-Inv, anti-CK1, anti-Nnat, or anti-GAPDH antibodies overnight at 4°C. The secondary antibody that was used was anti-rabbit or anti-mouse horseradish peroxidase-conjugated IgG antibody. The protein bands were detected by using the Western Breeze kit (Invitrogen). Densitometric analysis of the proteins band was performed by using ImageJ software (NIH).

2.7. Transfection of Nnat-targeted specific si (small interference) RNA

SiRNA targeted against Nnat (Si-Nnat, s226688, Ambion, TX, USA) and siRNA consisting of a scrambled sequence that would not lead to specific degradation of any cellular message (Si-control, AM4613) were purchased from Ambion (Austin, TX, USA). NHEKs cultured in 24-well plates, were incubated with the mix from the HiPerFect Transfection Kit (Qiagen,

Courtaboeuf, France) containing 10 nM siRNA and 3.0 ml of HiPerFect reagent in 0.5 ml of culture medium, according to the manufacturer's instructions. After a 48 h incubation period, siRNA-transfected-NHEKs were collected and Western blotting was performed for detecting the amounts of CK1 and Inv.

2.8. Plasmid construction and cell transfection

The ORF of hNNAT was amplified from commercially available cDNA clones (DNAFORM, Yokohama, Japan) by performing PCR by using a sense primer (5′-CTAGCTAGCATGGCGGCAGTGGCGGCGGCCTCG-3′) that covered a NheI site and an antisense primer (5′-GGAATTCTCAGTTGGGGGCTCGCTGCCTGCGC-3′) at the stop codon that covered an EcoRI site. The PCR product was cloned into the NheI/EcoRI site of pCXN2.1(+) and designated pCXN2.1(+)-hNNAT. Accurate amplification of the insert was verified by carrying out DNA sequencing.

NHEK cells (4×10^5) on 6-well culture plates were transfected with 1 μg of pCXN2.1(+)-hNNAT or with the empty vector (pCXN2.1(+)) by using Lipofectamine LTX (Invitrogen) according to the manufacturer's protocol. All of the cells were harvested and analyzed by qRT-PCR at 48 h after transfection.

2.9. Statistical analysis

All data were presented as means ± SEM for three independent experiments and analyzed by using the unpaired Student's *t*-test or one-way ANOVA followed by Dunn's multiple comparisons post-test. All analyses were performed with GraphPad Prism5 software. A p value of less than 0.05 was considered to indicate statistical significance.

3. Results

3.1. Expression of Nnat, Inv, and CK1 in the normal and psoriatic epidermis

We first compared the expression of Nnat, Inv, and CK1 in the epidermis of normal skin, psoriasis, chronic eczema and lichen planus. In the normal skin, Nnat expression was focal and weak in the basal to suprabasal layers. However, its expression was markedly augmented in the acanthotic epidermis in all of the psoriasis specimens (Figure 1). The overexpression of Nnat was also evident in lichen planus but to a less extent in chronic eczema (Figure 1). Inv expression was evident in the upper epidermis of the normal skin, and was augmented in the hyperplastic epidermis of psoriasis, chronic eczema and lichen planus (Figure 1). Suprabasal CK1 expression was confirmed in all normal skin, psoriasis, chronic eczema and lichen planus samples (Figure 1). As Nnat is involved in insulin secretion [6,7] and psoriasis is associated with metabolic syndrome [12, 13], we compared the immunostaining intensity of Nnat among patients with or without metabolic syndrome, but no apparent difference was noted.

We next examined the expression of Nnat, Inv, and CK1 in cultured NHEKs by performing immunofluorescence staining. We identified the expression of Nnat, Inv, and CK1 in the cytoplasm of NHEKs in vitro. Weak to moderate expression of Nnat and CK1 was variably observed in most NHEKs, whereas Inv expression was confined to large, differentiated keratinocytes (Figure 2, left panel). Overlay double immunofluorescence study with the Nnat and Inv expression (Figure 2, middle panel) or with the Nnat and CK1 expression (Figure2, right panel) revealed that only a part of Nnat colocalized with Inv or CK1, respectively.

3.2. Coordinated up-regulation of Nnat and Inv, but not CK1, during keratinocyte differentiation

The keratinocyte differentiation, as assessed by the up-regulation of Inv and CK1 expression, is

induced by elevated extracellular Ca^{++} or PMA treatment [14, 15]. We examined the effects of graded extracellular Ca^{++} concentration on Nnat, Inv, and CK1 expression at the protein level. As expected, a high concentration of Ca^{++} (1.2 mM) induced the up-regulation of Inv and CK1 expression compared to a low Ca^{++} (0.03 mM) environment (Figure 3A). Compared to Inv expression, CK1 up-regulation was very slight. Nnat expression was also significantly enhanced along with keratinocyte differentiation that was triggered by a high Ca^{++} condition (Figure 3A).

NHEKs that were treated with 10 ng/ml PMA also overproduced Inv and Nnat. However, PMA did not affect CK1 expression (Figure 3B). Cell-cell contact in the confluent condition (high cell density) is known to augment Inv expression but not keratin expression [11]. We next investigated the expression of Inv, CK1, and Nnat under a graded cell confluency condition. As shown in Figure 3C, the expression of Inv and Nnat was significantly up-regulated in a cell density-dependent manner, whereas the effect on CK1 expression was negligible. These data indicated that the expression of Nnat was coordinately regulated with Inv, but not with CK1, during keratinocyte differentiation.

In order to elucidate the role of Nnat on the Inv expression, we next knockdowned Nnat by transfecting Si-Nnat. The Si-Nnat treatment successfully knockdowned the Nnat mRNA levels compared to Si-control transfection (Figure 4A). As shown in Figure 4B, the Si-Nnat treatment specifically down-regulated the Inv, but not CK1, expression of NHEKs.

3.3 Nnat-transfected NHEKs overproduce Inv but not CK1

In order to further elucidate the influence of Nnat on Inv and CK1 expression, we developed Nnat-transfected NHEKs and empty vector-treated NHEKs (mock-NHEKs), and compared their Inv and CK1 expression. The Nnat-transfected NHEKs successfully overproduced Nnat at mRNA (Figure 5A) and protein (Figure 5B) levels.

In the Nnat-transfected NHEKs, the protein levels of Inv were significantly up-regulated.

However, CK1 expression was not affected by Nnat gene transfection (Figure 6A). The marked augmentation of Inv was also confirmed using immunofluorescence, where the expression levels of CK1 in the Nnat-transfected NHEKs were comparable to those in the mock-NHEKs (Figure 6B).

Since Inv and CK1 molecules are early (suprabasal layer) epidermal differentiation molecules, we next wanted to address the effect of Nnat on the expression of late-phase (granular layer level) epidermal differentiation molecules such as filaggrin, loricrin and S100A7 [16,17]. Of note, the effects of Nnat-transfection on the expression of filaggrin, loricrin and S100A7 were marginal like the CK1 expression, and only the Inv expression was specifically augmented in the Nnat-transfected NHEKs (Figure 6C).

3.4. Graded concentrations of glucose do not affect the expression of Nnat, Inv, and CK1

In pancreatic β-cells, a high glucose condition has been shown to be an efficient stimulator of Nnat expression [7]. We examined the effects of extracellular graded concentrations of glucose on the expression of Nnat, Inv, and CK1. As shown in Figure 7, varying the concentration of glucose did not affect the protein levels of these three molecules.

4. Discussion

The biological function of neuronatin is still poorly understood. Originally, it was believed to be a brain-specific developmental gene associated with neuronal differentiation [1,3,18,19]. However, it is also expressed in non-neuronal tissues such as the pituitary glands, lung, adrenal glands, uterus, skeletal muscles, ovaries, and pancreas [4,5]. The expression of neuronatin is higher in the aortic endothelium of diabetic and obese mice, while lower in several appetite regulatory hypothalamic nuclei during fasting [20,21]. There is also a significant association between SNPs in the human neuronatin gene and severe childhood and adult obesity [21].

Recent studies have showed its involvement in adipocyte differentiation and glucose-mediated insulin secretion from pancreatic β-cells [5,6,7]. A plausible relationship between Nnat and cytoplasmic Ca^{++} regulation is depicted for neural tissue. In mature hippocampal neurons, Nnat is localized somatodendritically. Hippocampal neurons that are transfected with the Nnat gene show higher dendritic Ca^{++} levels than in those transfected with an empty vector [22]. Lin et al. demonstrated that Nnat is indispensable for initiating neural induction through increasing intracellular Ca^{++} by using a neural developmental model from mammalian embryonic stem cells [23].

Our previous immunohistochemical studies revealed that Nnat is weakly and focally expressed in basal keratinocytes with intense staining in the sebaceous, eccrine, and apocrine glands [8]. As keratinocyte differentiation is critically regulated by intracellular Ca^{++} levels [15,24], we hypothesized that Nnat might be involved during keratinocyte differentiation. In this study, we demonstrated for the first time that cultured epidermal keratinocytes indeed expressed Nnat in vitro. Nnat expression was augmented in the acanthotic epidermis of psoriasis, chronic eczema and lichen planus. However, Nnat expression was closely associated with Inv, but not CK1, expression during keratinocyte differentiation, which was also confirmed by Nnat knockdown experiment. Keratinocytes that over-express Nnat exhibited significantly high Inv expression but not late-phase differentiation markers such as filaggrin, loricrin and S100A7 expression, suggesting its particular association with Inv, an early keratinoctyte differentiation marker.

Nnat is a maternally imprinted gene that is located on mouse chromosome 2 and human chromosome 20, and encodes a membrane protein in the endoplasmic reticulum (ER) [19,25]. Non-neuronal data from pancreatic β- and 3T3-L1 cells show that Nnat actually resides in the ER [5,7]. Recent co-immunoprecipitation assay shows that in the neuronal cells Nnat

co-localizes with sarcoplasmic/endoplasmic reticulum Ca^{++} ATPase isoform 2 (SERCA2), which is an adenosine triphosphate-dependent calcium pump that transfers calcium from the cytosol to the calcium-rich lumen of the ER. In addition, Nnat is likely to increase the cytosolic Ca^{++} level by antagonizing the function of the SERCA2 pump [22,23]. SERCA2 influences the formation of calcium oscillations, regulates the return to resting cytoplasmic calcium concentrations after signal-induced ER calcium mobilization, and maintains the calcium-rich environment of the ER lumen [24]. The importance of SERCA2, which is encoded by the *ATP2A2* gene, in terms of keratinocyte homeostasis and differentiation has been highlighted in Darier's disease, which is a genodermatosis with haploinsufficiency of the gene [24,26]. It is possible to speculate that Nnat may crosstalk with SERCA2 in the human keratinocytes.

Neuronatin expression is regulated in different metabolic states and its mutation is associated with obesity [20,21]. The relationship between Nnat and glucose metabolism has been recently documented in Lafora disease, which is an inherited form of progressive myoclonus epilepsy. It is caused by mutations in either the EPM2A gene, which encodes the protein phosphatase laforin, or the NHLRC1 gene, which encodes the ubiquitin ligase malin. The malin–laforin complex induces the proteosomal degradation of protein targeting to glycogen. Loss of function of the malin–laforin complex reduces the degradation of Nnat, which results in the accumulation of Nnat in the cytoplasm [27]. The accumulated Nnat is likely to accelerate glycogen synthesis, thereby leading to the formation of characteristic PAS-positive glycogen inclusion bodies that are called Lafora bodies. Lafora bodies, which are an important diagnostic feature, are predominantly detected in cutaneous eccrine glands where the expression of Nnat is normally abundant [8,27]. In addition, high glucose concentration that is sensed by pancreatic β-cell induces neuronatin-mediated insulin secretion and apoptosis [7]. However, in the present study, the graded concentrations of glucose did not affect the expression of Nnat, Inv, and CK1 under

our experimental condition. In addition, comorbidity of metabolic syndrome with psoriasis, chronic eczema and lichen planus did not affect the expression pattern of Nnat in the acanthotic epidermis.

In neuronal tissue, Nnat seems to augment tumor cell growth. The expression of Nnat promotes medulloblastoma growth [28]. In glioblastoma multiforme, Nnat expression is exclusively confined to tumor stem cells and is associated with a significant increase in cellular proliferation. Moreover, over-expressed Nnat is an independent risk factor for decreased patient survival [29]. Our previous results support this observation because cutaneous malignant peripheral nerve sheath tumors harbor abundant Nnat proteins in the cytoplasm, whereas its presence is negligible or only weakly observed in benign neurofibromas [9]. In keratinocytes, however, this is not the case, because the abundant expression of Nnat has been demonstrated in benign and malignant sweat gland tumors [8]. The intensity of Nnat expression is also comparable among benign and malignant epidermal tumors (our unpublished data). The acanthotic epidermis of psoriasis, chronic eczema and lichen planus also showed the enhanced expression of Nnat in the present study. These findings indicate that Nnat is more closely related to differentiation than tumorigenesis in keratinocytes.

To our knowledge, this is the first report on the relationship of Nnat to the early epidermal keratinocyte differentiation.

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Figure Legends

Figure 1 Immunohistological localization of neuronatin (Nnat), involucrin (Inv), and cytokeratin1 (CK1) in normal skin (N=10), psoriasis (N=10), chronic eczema (N=10) and lichen planus (N=10). Black bar indicates 100μm.

Figure 2 Immunofluorescent visualization of neuronatin (Nnat), involucrin (Inv), and cytokeratin1 (CK1) in cultured NHEKs

The expression of Nnat, Inv, and CK1 was detected in NHEKs by performing an immunofluorescence study. Normal rabbit IgG served as a negative control. The nuclei were stained with DAPI (left panel). Overlay double immunofluorescence study was shown with the Nnat and Inv expression (middle panel) and with the Nnat and CK1 expression (right panel).

Figure 3 Protein expression neuronatin (Nnat), involucrin (Inv), and cytokeratin1 (CK1) under epidermal differentiation stimuli

The protein levels of Nnat, Inv, and CK1 were assessed by using Western blotting under three different epidermal differentiation conditions. A: graded concentrations of extracellular Ca^{++} levels; B: treatment with PMA (10 ng/ml); and C: graded confluency of cell density

Figure 4 Transfection of small interference (Si)-RNA

A: Transfection of Si-Nnat significantly knock-downed the mRNA expression of Nnat compared with Si-control-transfected NHEKs. B: The expression of Inv, but not CK1, was down-regulated in NHEKs transfected by Si-Nnat when compared with Si-control.

Figure 5 Establishment of neuronatin (Nnat)-transfected NHEKs

Nnat-transfected NHEKs expressed higher levels of Nnat at mRNA (A) and protein (B) levels compared to mock-NHEKs.

Figure 6 Expression of involucrin (Inv) and cytokeratin1 (CK1) in mock- and Nnat-transfected NHEKs

A: Western blot analysis showed the augmented expression of Inv but not CK1 in Nnat-transfected NHEKs. B: Immunofluorescent demonstration of Inv and CK1 in mock- and Nnat-transfected NHEKs. The nuclei were stained with DAPI. C: mRNA expressions of Inv, CK1, filaggrin, loricrin and S100A7 were compared between the Nnat-transfected and mock-NHEKs.

Figure 7 Expression of (Nnat), involucrin (Inv), and cytokeratin1 (CK1) in NHEKs that were treated with graded concentrations of extracellular glucose

Figure 1

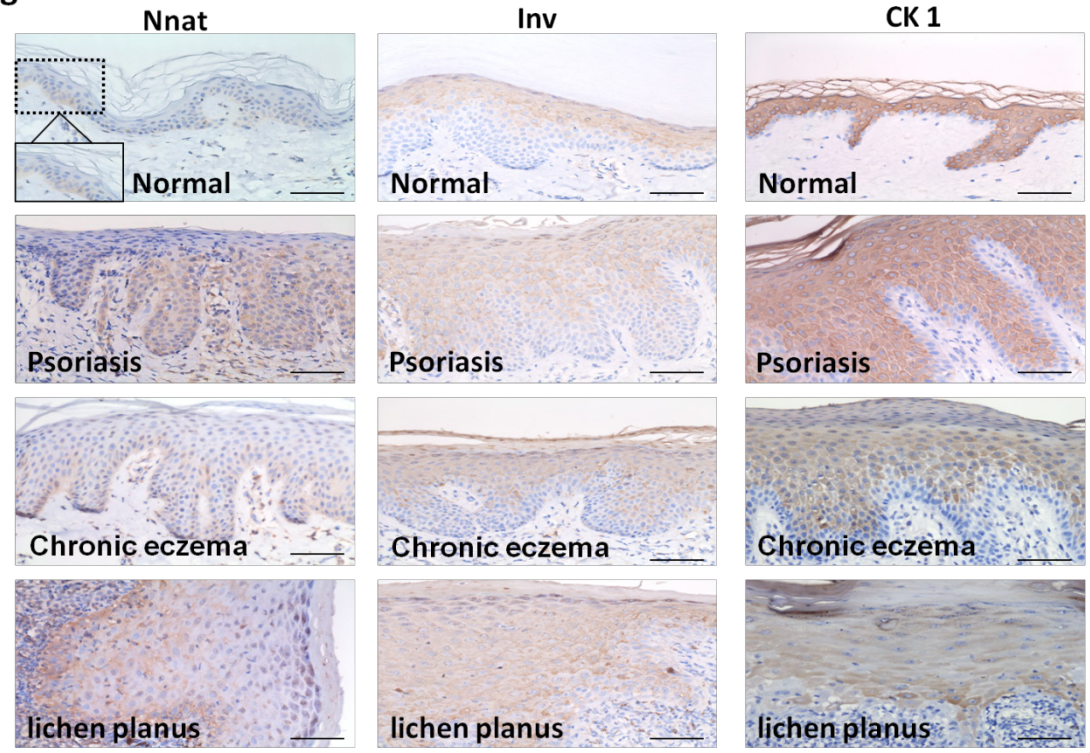


Figure 2

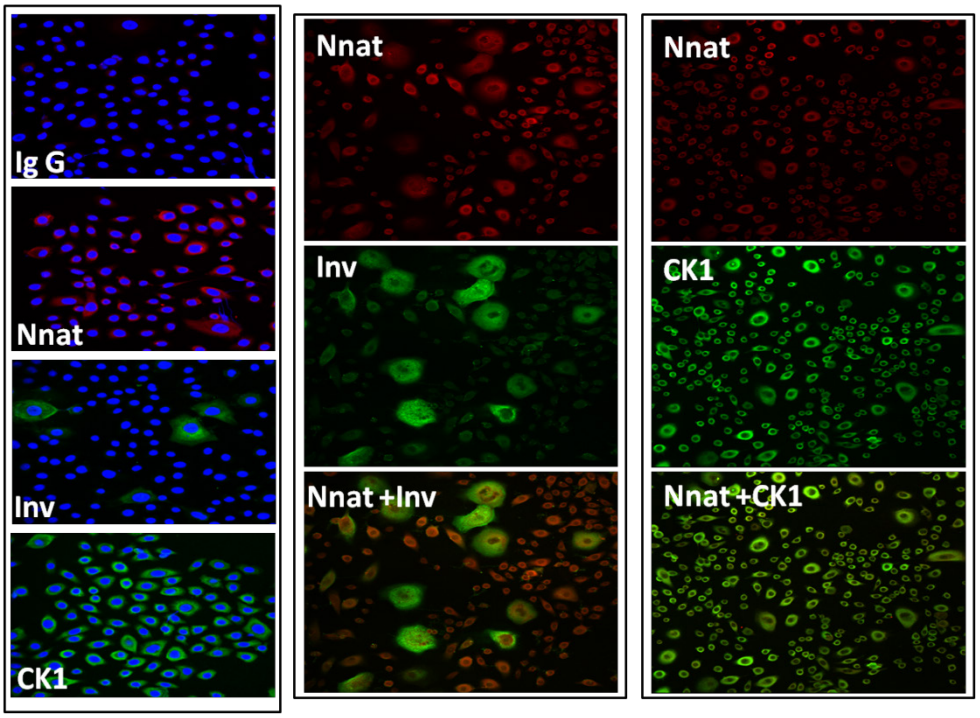


Figure 3

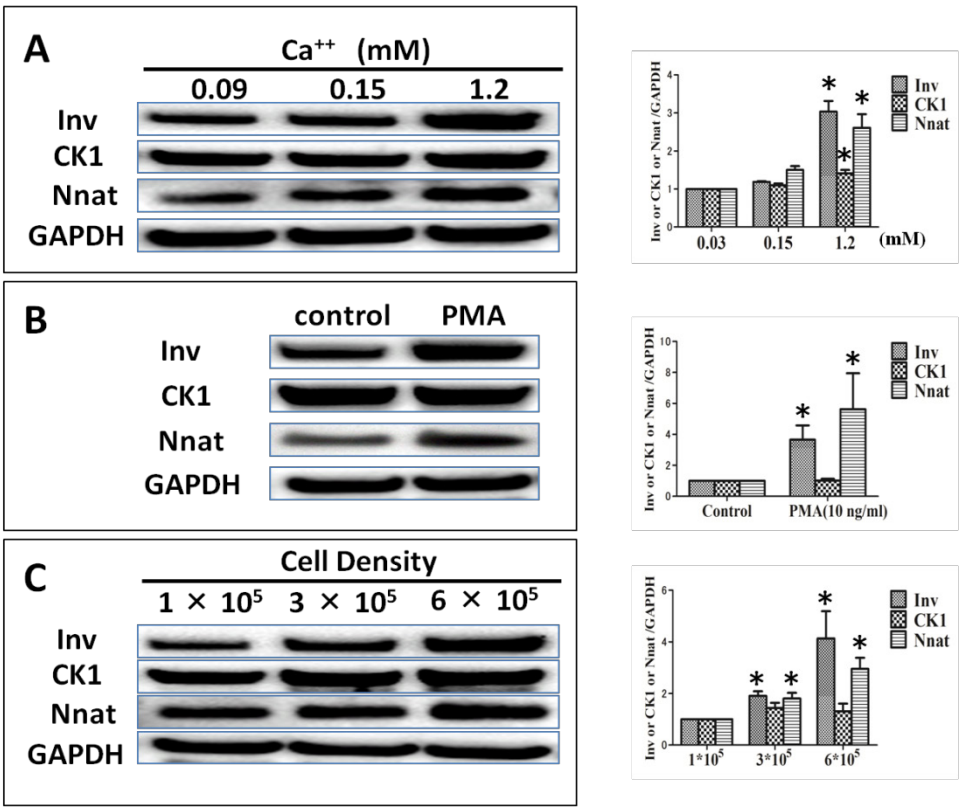


Figure 4

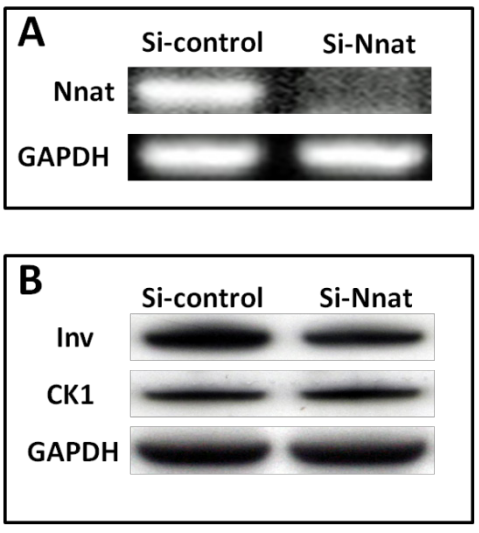


Figure 5

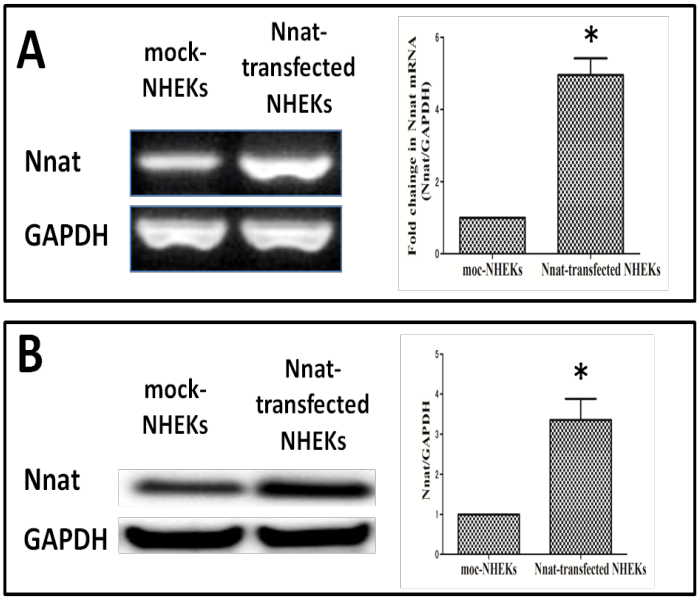


Figure 6

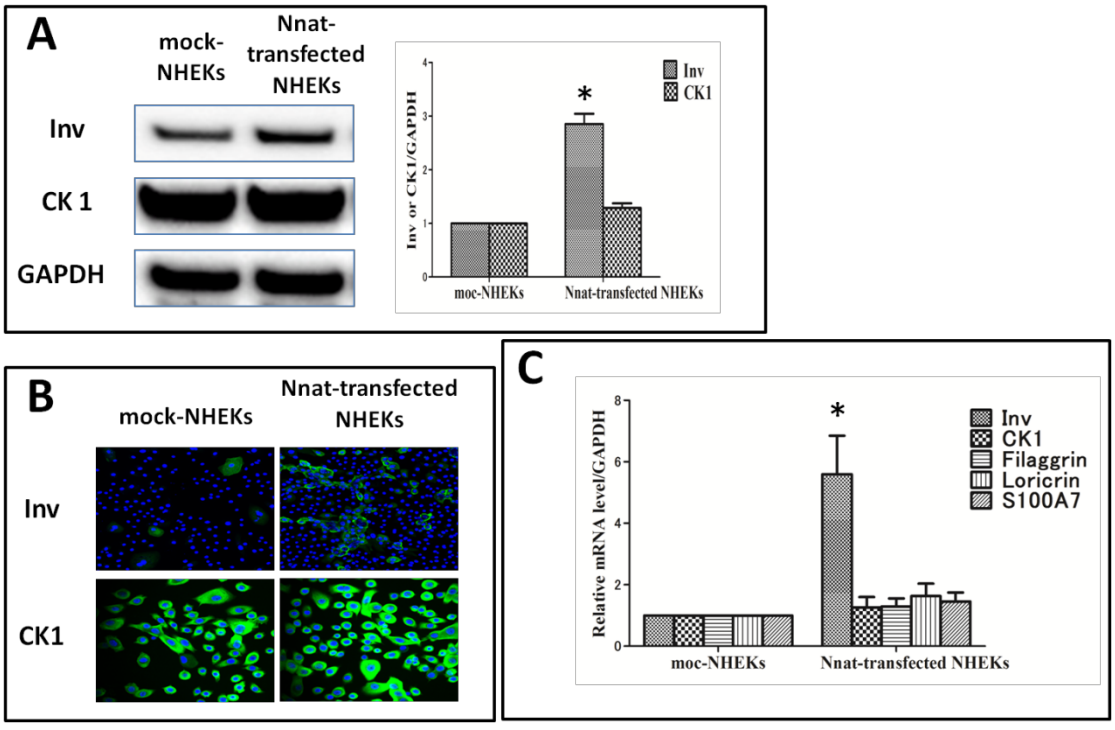
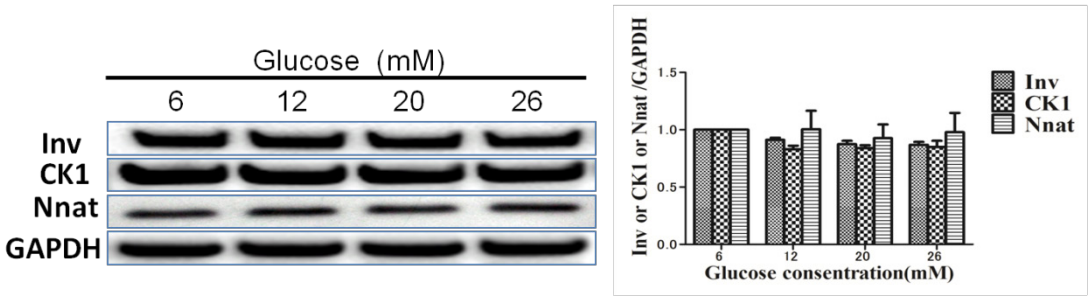


Figure 7


Glucose (mM)
6
12
20
26
Inv
CK1
Nnat
GAPDH
Inv or CK1 or Nnat /GAPDH
1.5
1.0
0.5
0.0
6
12
20
26
Glucose consentration(mM)
Inv
CK1
Nnat