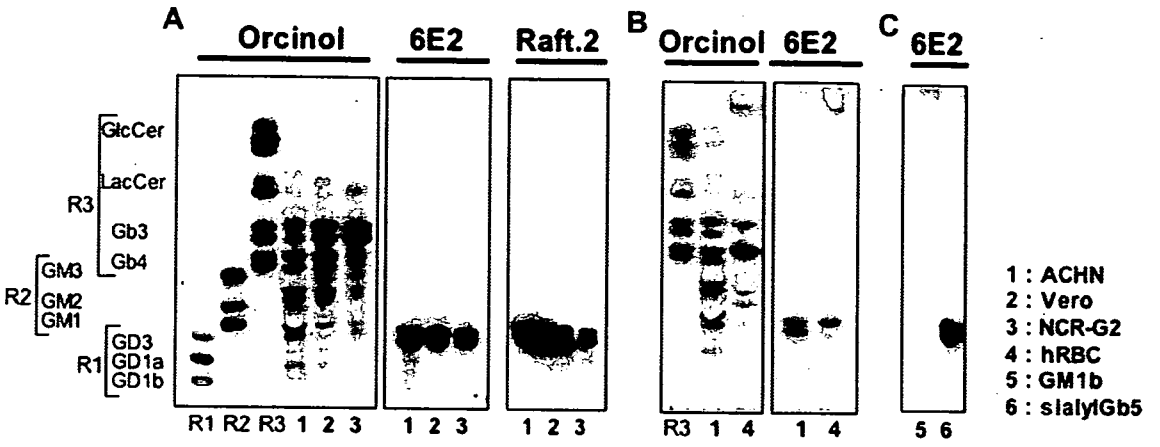

Fig. 1. TLC immunostaining of GSLs prepared from cultured cells and hRBCs. GSLs extracted from cultured cells and hRBCs or purified GSLs were separated by TLC in a solvent system of chloroform/methanol/water containing 0.2% $CaCl_2$ (5:4:1, v/v/v). Plates were chemically stained with orcinol-sulfurnic acid or were immunostained with 6E2 and Raft.2. Lane 1, ACHN; Lane 2, Vero; Lane 3, NCR-G2; Lane 4, hRBCs; Lane 5, GM1b; Lane 6, sialylGb5. Reference markers used were disialosyl gangliosides of GD3, GD1a, and GD1b (R1), monosialosyl gangliosides of GM3, GM2, and GM1 (R2), and neutral GSLs of GlcCer, LacCer, Gb3, and Gb4 (R3). The nomenclature for GSLs follows the recommendations [11] of the IUB, and the ganglioside nomenclature of Svennerholm [12] was used.

Mab. The 80 kDa protein might be associated with sialylGb5 in NCR-G3 cells and thus co-immunoprecipitated by 6E2.

Comparison of reactivity to sialyl Gb5 between 6E2 and MC813-70

MC813-70 established by immunizing with human EC cell lines has been most widely used as an anti-SSEA-4 antibody (mouse IgG_3, κ) [14]. Therefore we compared the reactivities of the Mabs 6E2 and MC813-70 by flow cytometry and dot-blot immunostaining. The fluorescence intensity obtained with 6E2 was stronger than that with MC813-70 in each cell line and hRBCs (Fig. 2A). A recent flow cytometric study showed that MC813-70 strongly stains hRBCs, but other anti-sialyGb5 Mabs do not [15]. However, our data indicate that 6E2 is more reactive than MC813-70. Next we compared the reactivity of the two

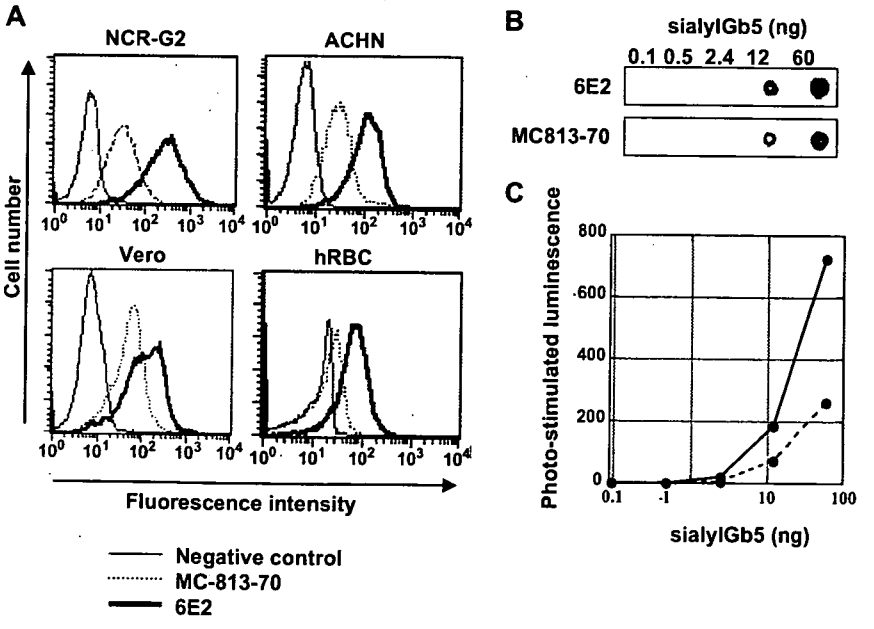

Fig. 2. Reactivity of 6E2 and MC813-70 with sialylGb5. (A) Flow cytometric analysis of SSEA-4-positive cells with 6E2. NCR-G2 cells, ACHN cells, Vero cells, and hRBCs were stained with 6E2 (bold line) or MC813-70 (dotted line) and with a FITC-conjugated secondary antibody and analyzed by flow cytometry. (B) An image of the dot-blot immunostaining of sialylGb5 obtained with a LAS-1000 luminescent imaging analyzer. (C) Measurement of antibodies bound (6E2: solid line, MC813-70: broken line).

Mabs with that of sialylGb5 by dot-blot immunostaining. Serially diluted sialylGb5 was dot-blotted onto a PVDF membrane, and the membrane was immunostained with the two Mabs. Both 6E2 and MC813-70 bound to more than 12 ng of sialylGb5, but the signals induced by 6E2 were stronger than those induced by MC813-70 (Fig. 2B,C). Thus, in addition to the flow cytometric analysis, the reactivity of 6E2 with sialylGb5 was stronger than that of MC813-70 by dot-blot immunostaining.

SSEA-4 Immunostaining of cynomolgus monkey ES cells

To confirm whether Mab 6E2 reacts with SSEA-4 on monkey ES cells, we performed an indirect immunofluorescence staining of cynomolgus monkey ES cells with Mab 6E2 and MC813-70. Mab 6E2 reacted with monkey ES cells (Fig. 3A) as well as MC-813-70 did (Fig. 3B). No difference in staining patterns of SSEA-4 between the two Mabs was observed. Mab 6E2 certainly stained SSEA-4 on monkey ES cells.

SSEA-4 immunostaining of "living" mouse preimplantation embryos without fixation

During early embryogenesis in mice, SSEA-4 had been reported to be expressed in fertilized eggs with levels gradually increasing to the morula stage and then decreasing [5]. Thus we examined the expression and distribution of SSEA-4 in preimplantation mouse embryos by immunostaining with both 6E2 and MC813-70. Both Mabs evenly stained the whole surface membranes of fixed mouse embryos, and no difference in staining pattern between the two was observed (data not shown). In order to perform a time-course of SSEA-4 distribution in a viable state, we performed immunostaining of preimplantation embryos without fixation.

3D-images of the 6E2 staining pattern obtained by confocal laser scanning microscopic observation clearly showed the localization of SSEA-4 on mouse preimplantation embryos. Two-cell embryos showed patches of SSEA-4 over the whole surface membrane with some accumulation at the interface between blastomeres (Fig. 4A). In 8-cell embryos, the amount accumulated at interfaces was further increased, as if planer membranes separate each blastomere, and some large patches were internalized but others were left on the surface membranes (Fig. 4B). The amount of SSEA-4 concentrated at the interfaces in morula was not as significant as in 8-cell embryos but still clearly observed and some patches were internalized (Fig. 4C).

2D-images of embryos stained with 6E2 showed a marked accumulation of SSEA-4 at the interfaces between blastomeres (Fig. 4D–F). These results suggest that sialylGb5 actively moves during development and tends to accumulate where blastomeres come into contact with each other.

Interestingly, however, the staining pattern of SSEA-4 using MC813-70 was different from that using 6E2. MC813-70 evenly stained the surface and the interface between blastomeres of 2-cell embryos with patches (Fig. 4G), and the amount of SSEA-4 at interfaces was not significant (Fig. 4J). In 8-cell embryos, there were patches of SSEA-4 in the central area of the outer surface of each blastomere (Fig. 4H, indicated by arrows), but the 2D-image showed that clustering also occurred at surfaces facing blastocoels (Fig. 4K, indicated by arrowheads). In morula embryos, SSEA-4 was distributed on the surface in patches and was enriched at the boundaries between blastomeres on the outer surface (Fig. 4I,L).

It remains unclear why the pattern of staining of mouse preimplantation embryos differs between 6E2 and MC813-70. The composition of fatty acids in GSLs influences the binding of antibodies [16,17] or bacterial toxins [18]. SialylGb5 recognized by the two MAbs might differ in composition of fatty acids, resulting in different immunostaining patterns. It was reported that the clustering of sialylGb5 by a Mab induces the activation of sialylGb5-associated kinases in raft microdomains of human mammary carcinoma cells, leading to downstream signaling [19,20]. The clustering of sialyGb5 by 6E2 on preimplantation mouse embryos may also induce the activation of some kinases, followed by downstream signaling. Recently, Comisky et al. suggested that lipid rafts and their associated molecules are spatiotemporally positioned to play a critical role in preimplantation developmental events [21]. The patches or clusters of sialylGb5 shown in our study suggest the presence of lipid rafts containing sialylGb5 on mouse embryos.

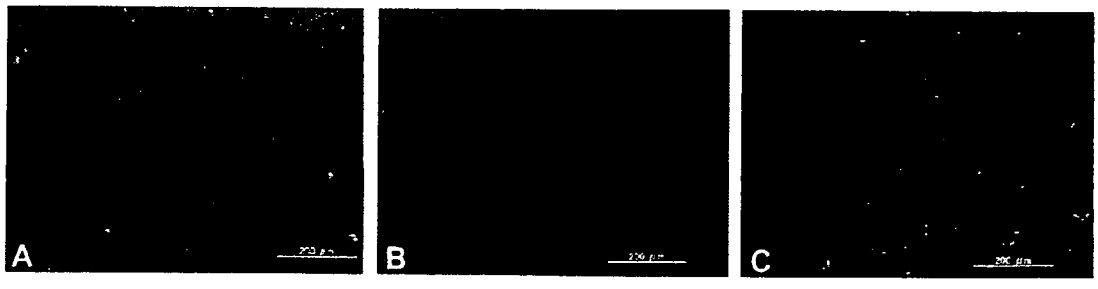

Fig. 3. Indirect immunostaining of cynomolgus monkey ES cell line CMK-6 with 6E2 and MC813-70. The CMK-6 cells were stained with 6E2 (A), MC813-70 (B), or isotype-matched mouse IgG (C), and visualized with secondary antibodies (green), followed by counterstaining of nuclei with DAPI (blue). Scale bars = 200 μm.

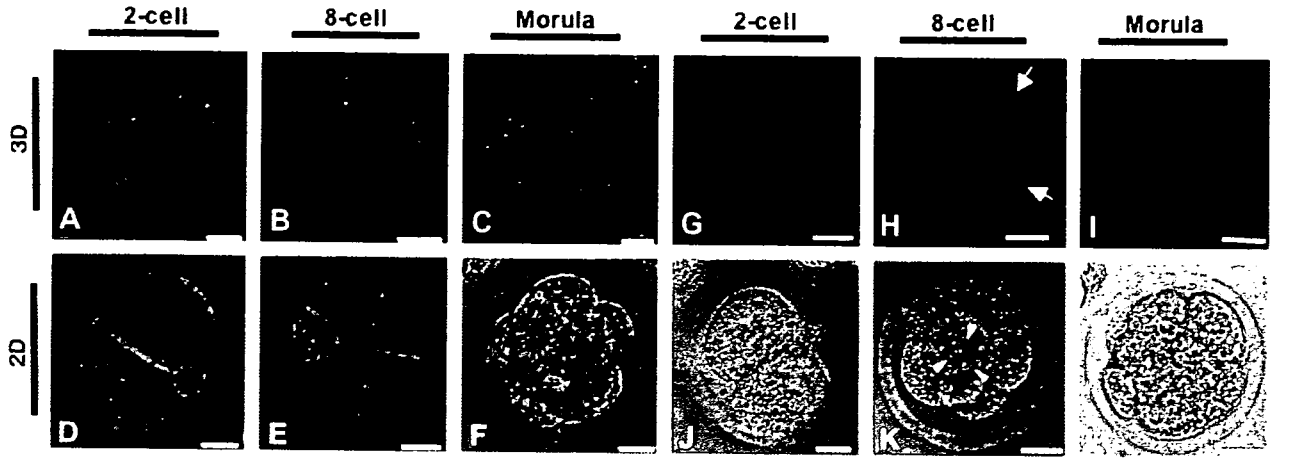

Fig. 4. Immunostaining of SSEA-4 on mouse preimplantation embryos with 6E2 and MC813-70. The embryos at the 2-cell (A, D, G, J), the 8-cell (B, E, H, K), and the morula (C, F, I, L) stages were stained with 6E2 (green) or MC813-70 (red). The panels designated 3D (A, B, C, G, H, I) are three-dimensional images reconstructed by stacking optical slice images using LSM software and the panels designated 2D (D, E, F, J, K, L) are an overlay of a fluorescent image and a differential interference contrast micrograph. Scale bars = 20 μm.

6E2 has high affinity for sialylGb5 and can be effectively conjugated with fluorescence reagents, leading to excellent staining of SSEA-4 in the surface membrane of "living" mouse preimplantation embryos. 6E2 should be of use for research into lipid rafts in early development and of great advantage for the characterization of ES cells and EC cells.

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Eye-open at birth phenotype with reduced keratinocyte motility in LGR4 null mice

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Abstract **We observed a consistent eye-open at birth (EOB) phenotype in mouse pups homozygous for a leucine-rich repeat containing G-protein coupled receptor 4 (*Lgr4*) allele deleting the whole transmembrane domain coding region. An in vitro wound-healing scratch assay showed notably reduced keratinocyte motility in the null mice. Phalloidin staining of F-actin in the eyelid epidermis was also reduced. We also generated keratinocyte-specific *Lgr4* deficient mice, circumventing the embryonic/neonatal lethality and kidney abnormalities. Most of the conditional *Lgr4* knockout mice showed the EOB phenotype. Thus, *Lgr4* might be a novel gene class regulating cell motility.**

Keywords: LGR4; GPR48; GPCR; Gene deletion mice; EOB; Keratinocyte

1. Introduction

Lgr4 (leucine-rich repeat containing G-protein coupled receptor 4) is one of the genes identified as novel G-protein coupled receptors (GPCRs) [1,2], designated *Lgr4–Lgr8*, from an EST database with high homology to glycoprotein hormone receptors including follicle-stimulating hormone receptor (FSHR) [3], luteinizing hormone/chorionic gonadotropin receptor (LH/CGR) [4,5] and thyroid-stimulating hormone receptor (TSHR) [6].

As *Lgr4* shows high homology with FSHR, LHR and THSR, this receptor has been thought to be involved in reproductive systems. Mazerbourg et al. reported the generation of *Lgr4* gene-interrupted mice using a gene-trapped ES cell line [7], in which the expression of *Lgr4* is severely attenuated by the insertion of the β-geo gene in an enhancer trap procedure [8]. They described the neonatal lethality of the null mice, but not the cause. Previously, we generated similar *Lgr4* knockout mice by completely removing exon18, which encodes the whole transmembrane domain of *Lgr4*, in order to eliminate the chance of receptor fragment localizing at the membrane or transmitting downstream signals [9]. In those *Lgr4* knockout mice, gross hypomorphic phenotypes developed in multiple tissues and organs, and the null mice showed hypoplastic kidneys with an increased concentration of plasm creatinine, which was strongly suspected to be the cause of the neonatal/embryonic lethality.

Recently, Mendive et al. as well as Hoshii et al. reported *Lgr4* gene-trap lines exhibiting defective postnatal development of the male reproductive tract [10,11], again in contrast to the embryonic/neonatal lethality seen in our *Lgr4* knockout mice on a 129Ola × C57BL/6 hybrid background [9]. In our *Lgr4* knockout mice it appeared that complete loss of the *Lgr4* gene would induce complete embryonic/neonatal lethality. As reported in our first paper, the typical phenotype of the *Lgr4* null mutants other than the kidney aberration was eye open at birth (EOB) with 100% penetrancy, strongly suggesting reduced keratinocyte proliferation and motility. Several studies have reported a strong relationship between a reduction in keratinocyte proliferation/motility and the EOB phenotype [12,13], and it has been suggested that *Lgr4* is essential for organ development and cancer cell invasion [10,11,14]. Additionally, a close relationship between cancer cell invasion and cell motility was reported, and it is well known that organ development requires cell motility [15,16]. We therefore suspected the existence of a close relationship between *Lgr4* and cell motility, and considered that common mechanisms might cause EOB and the other abnormalities observed in *Lgr4* null mice. In the present study we focused on the EOB phenotype in relation to the keratinocyte motility of the null mice.

We provide evidence that *Lgr4* has a role in keratinocyte motility. In addition, we succeeded in dissociating the EOB phenotype, first observed in all conventional *Lgr4* KO mice, from the kidney lesions and lethality using conditional *Lgr4* knockout mice crossed with K5-Cre transgenic mice.

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Abbreviations: LGR, leucine-rich repeat containing G-protein coupled receptor; GPCR, G-protein coupled receptor; FSHR, follicle-stimulating hormone receptor; LH/CGR, luteinizing hormone/chorionic gonadotropin receptor; TSHR, thyroid-stimulating hormone receptor; EOB, eye-open at birth; Arbp, acidic ribosomal phosphoprotein PO

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2. Materials and methods

2.1. Histology

For the eyelid histology, the heads of the mice were fixed in 4% paraformaldehyde overnight. After dehydration, they were embedded in paraffin. Paraffin blocks were sectioned at 2–5 μm thickness and stained with H&E (hematoxylin–eosin) and Phalloidin-TRITC (tetramethyl rhodamine isothiocyanate) using standard procedures.

2.2. In vitro apoptosis cell test

Sections from around the eyelids of E15.5 mice were used for the detection of apoptotic cells. Samples were processed according to the protocol of In Situ Cell Death Detection Kit (Roche, Japan).

2.3. Keratinocyte primary culture

Primary keratinocytes were isolated from neonatal mice. The epidermis was separated from the dermis with 0.8 U/ml dispase (Roche) overnight at 4 °C. Keratinocytes were dissociated by trypsin for 5 min at 34 °C and plated onto dishes precoated with collagen type I. The cells were cultured in minimum essential medium supplemented with 4% Chelex (Bio-Rad, Hercules, CA)-treated fetal calf serum epidermal growth factor (10 ng/ml; Gibco BRL), and 0.05 mM $CaCl_2$ at 34 °C in an 8% CO_2 incubator. Under these conditions, keratinocytes are maintained in an immature state characterized by active proliferation. For all the experiments, the cells were used one week after plating.

2.4. In vitro migration assay

Keratinocytes derived from each genotype of neonatal mice were cultured until confluent. After scratching with plastic tips, the distance that the keratinocyte migrated was measured every 3 h. Each sample was counted at 16 points and the average distance was calculated.

2.5. Quantitative RT-PCR

Messenger RNA derived from wild-type and *Lgr4* null mice keratinocyte was subjected to cDNA synthesis by standard procedures. Quantitative RT-PCR assays were performed with the DNA Engine Opticon System (MJ Research, Japan) with a cycling profile as follows: at 95 °C for 2 min, 39 cycles at 95 °C for 5 s, at 61.4 °C for 30 s, and at 72 °C for 30 s. The genes and primer sets are shown in the supplementary material.

2.6. Generation of keratinocyte specific Lgr4 deficient mice

To generate *Lgr4* fx/fx mice without frt-Neo-frt cassette, an initially targeted *Lgr4* mutant [9] was mated with Flp deleter [17]. Mice with the keratinocyte-specific *Lgr4* deletion were generated by breeding keratin5-Cre (K5-Cre); *Lgr4* +/− mice with *Lgr4* fx/fx mice [18]. The genetic backgrounds were C57Bl/6 × 129Ola for *Lgr4* +/− and *Lgr4* fx/fx, and C57Bl/6 × C3H for K5-Cre. Primers for *Lgr4* genotyping are shown in the supplementary material.

2.7. Statistical evaluation

All experimental data are expressed as mean S.E.M. Statistical comparisons in all the physiological and laboratory data were made among the genotype groups using ANOVA followed by Student's *t*-test for individual comparisons. *P* values of <0.05 were considered significant.

3. Results

3.1. Lgr4 null mice show morphological abnormalities in the eyelids at E15.5

Lgr4 null mice showed some gross abnormalities. In wild-type mice during the embryonic stages E15.5 through E16.5, epithelial cells extended to the center of the eyes and finally became fused. The mice were then born with their eyelids fused, and the eyelids opened gradually by 12–14 days after birth. However, *Lgr4* null mice showed the EOB phenotype with 100% penetrance (Fig. 1A). Histological analysis of the wild-type mice followed by immunostaining showed a high expression level of *Lgr4* at the protruding tips of the eyelids and in the epithelial cells of the cornea at E15.5, but no expression was detected in the same areas of the null mice (Fig. 1B). As shown in Fig. 1C right, the eyelid closure was impaired in the null mice as compared to the heterozygous mice (Fig. 1C, left).

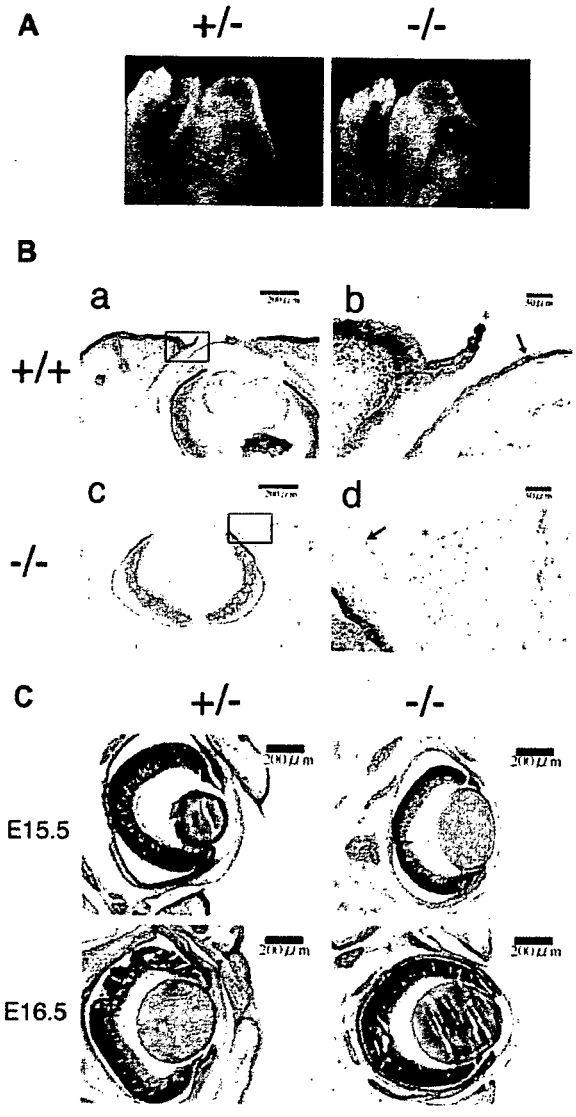

Fig. 1. *Lgr4* deficiency causes impairment in embryonic eyelid closure. (A) Left panel and right panel show morphology around eyes of *Lgr4* heterozygous (*Lgr4* +/−) and *Lgr4* null mice, respectively at postnatal day 0. (B) Eyelid sections prepared from E 15.5 wild-type (a, b) or null (c, d) mouse fetuses were immunostained with rabbit anti-LGR4 antibody (ab 12576, Abcam, USA), followed by HRP-conjugated second antibody. Asterisks show epithelial cell layer at the protruding tips of the growing eyelids, and arrows show the corneal epithelium layer. (C) Histological analysis (H&E staining) of E15.5 and E16.5 embryos.

3.2. Proliferation and motility of keratinocytes from Lgr4 null mice

EOB has been reported as a typical phenotype reflecting keratinocyte proliferation/motility, which we then measured. No decrease in proliferating cells was detected in the eyelid epi-

thelium of the null mutants at E15.5 (data not shown). Since a reduction of the cytoplasmic accumulation of F-actin was observed with the EOB phenotype in some gene knockout studies [19,20], we stained the eyelid sections with phalloidin and found that there was less filamentous accumulation of F-actin at the margin of the eyelid epithelium, as shown in Fig. 2A. Furthermore, we examined the extent of keratinocyte proliferation in vitro using the incorporation of BrdU, but no significant difference between the wild-type and the *Lgr4* null mice was observed (date not shown). Next, the keratinocyte motility was measured by an in vitro migration assay. The lack of *Lgr4* was related to a reduction of the keratinocyte motility, and we observed a significant delay in healing 3 hours after scratching in the cells from the null mice (Fig. 2B and C). To further analyze other possible mechanisms responsible for EOB in addition to the reduced motility of the null mice, wild-type and *Lgr4* null fetuses at E15.5 were subjected to TUNEL assay to examine the extent of cell apoptosis around the eyelid tissues. However, neirther types of embryos showed any apoptotic cells (Fig. 2D).

These results suggest that the EOB observed in the null mice was not induced by an enhancement of apoptosis, and provide further evidence that *Lgr4* is a critical regulator for keratinocyte motility in the epidermal tissue of eyelids.

3.3. Quantitative RT-PCR of EOB related genes in keratinocytes from Lgr4 null mice

The disruption of *EGFR*, *EGF*, *TGF-α*, *ADAM17*, *c-jun*, *ActivinA* and *ActivinB* genes has been reported to cause EOB. We measured the expression levels of these genes in mRNA prepared from wild-type and *Lgr4* null keratinocytes, but did not detect any significant differences in their expression levels (Fig. 3). In addition, the expression level of EGFR around the eyelid at E15.5 in the null mutants was normal (Supplementary Fig. S1). The phosphorylation of EGFR around the eyelid at E15.5 was examined in wild-type and *Lgr4* null mice by immunostaining, but no significant difference was observed (date not shown). The phosphorylation of ERK and JNK of the same samples also showed no difference (Supplementary Fig.S1).

3.4. Generation of keratinocyte-specific Lgr4 deficient mice

As mentioned above, the EOB phenotype observed in *Lgr4* null mice was suspected to be closely related to reduced keratinocyte motility. We previously reported that *Lgr4* null mice showed renal hypoplasia [9]. To separate the EOB phenotype from the renal hypoplasia observed in *Lgr4* null mice, we additionally generated keratinocyte-specific *Lgr4* deficient mice (*Lgr4* conditional knockout mice) as described in Section 2

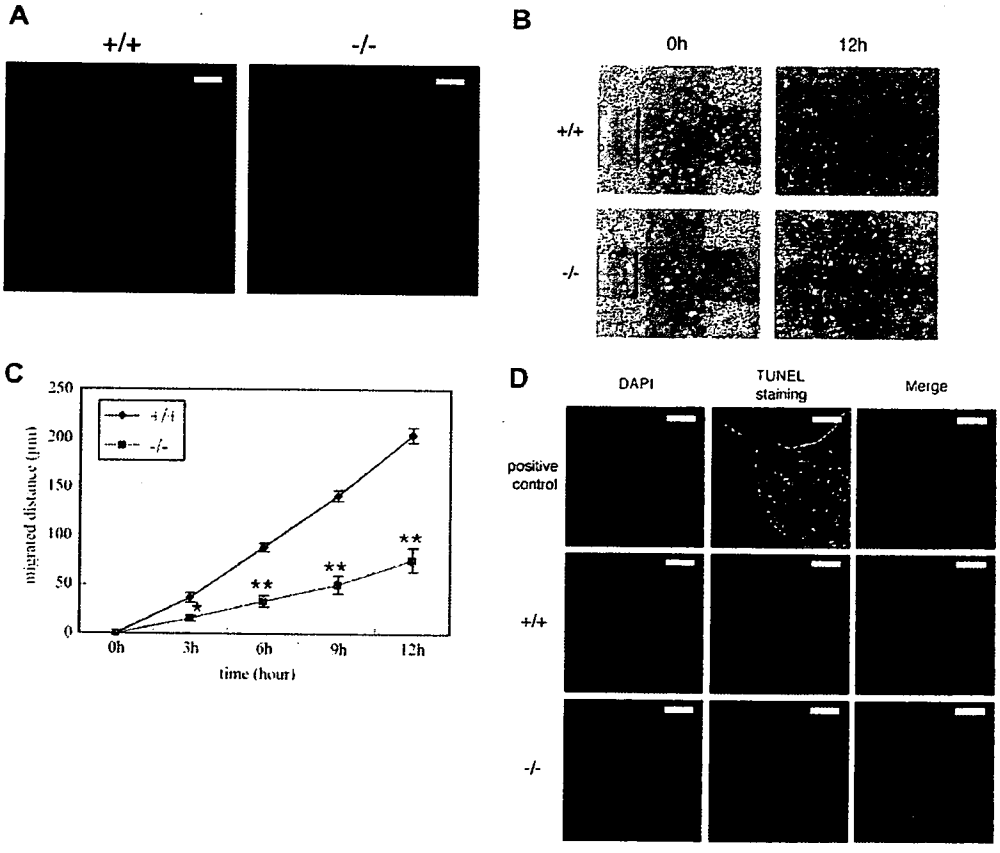

Fig. 2. EOB phenotype and impaired keratinocyte motility. (A) Coronal eye sections of E15.5 wild-type (*Lgr4* +/+) and null fetuses were stained with phalloidin-TRITC. Scale bar: 30 μm. (B) Keratinocyte motility was assessed by in vitro wound-healing scratch assay, and values representing the mean (S.E.M.; vertical bar) of 16 independent wounds are shown by a linegraph (C). (*; $P < 0.005$, **; $P < 0.001$) (D) Neither wild-type nor *Lgr4* null E15.5 fetuses showed apoptotic cells in the eyelids. Positive control is tissue treated with DNase I. Scale bar: 60 μm.

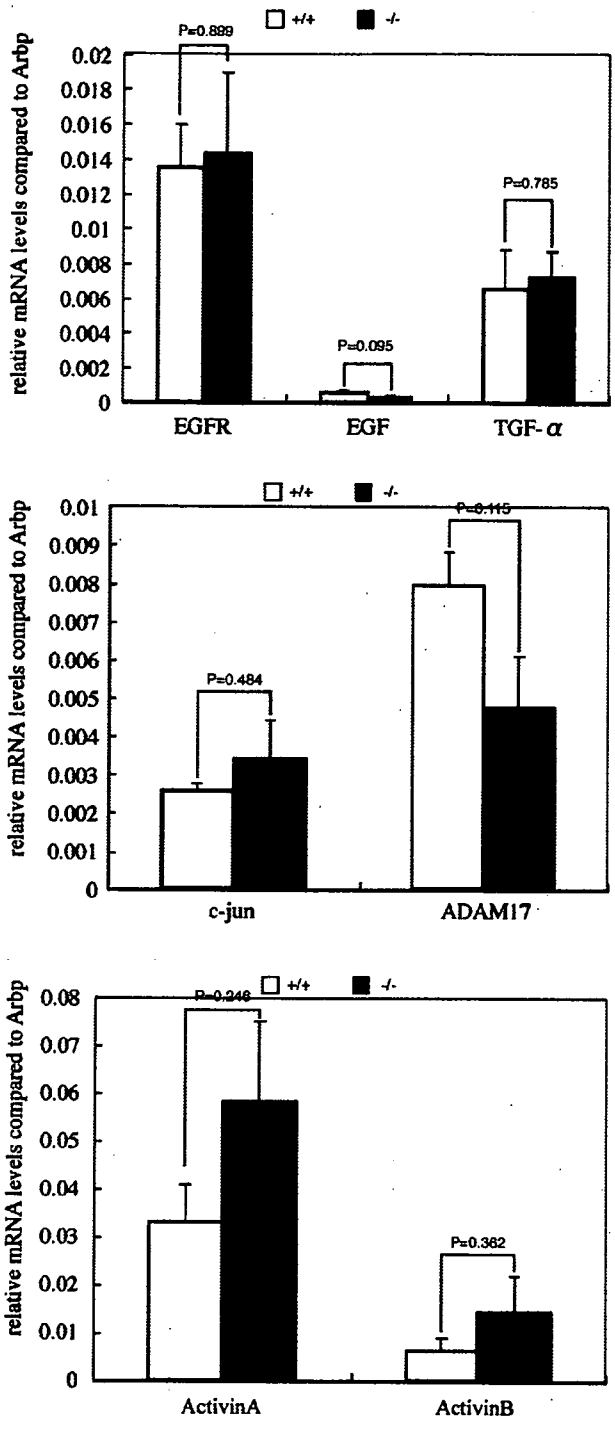

Fig. 3. Quantitative RT-PCR for cell motility-related genes. EGFR, EGF, TGF-α, c-jun, ADAM17, ActivinA and ActivinB mRNA levels in keratinocyte derived from *Lgr4* wild-type and null mice (P0) were quantified (all bars; $n = 3$). Error bars represent ± S.E.M.

(Supplementary Fig. S2A and B). The keratinocyte-specific deletion of *Lgr4* gene in *Lgr4* conditional knockout mice was confirmed by RT-PCR (Fig. 4A). The *Lgr4* conditional knockout mice were spared the embryonic/neonatal lethality (data not shown) and almost all of them showed the EOB phenotype (Fig. 4B and C). The body size and weight of several organs including the kidneys and liver were all reduced in *Lgr4* null mice [9], whereas those of the conditional knockout mice were all normal (Fig. 4D and E).

4. Discussion

In this report, we demonstrated an abnormality in the motility of keratinocytes and the EOB phenotype in *Lgr4* null mice. It is known that, during normal mouse development, the epithelial cells on the protruding tips of both the upper and lower eyelids migrate along the surface of the cornea and fuse with each other by E16.5 [12]. This suggests that EOB could be due to a defect in prenatal eyelid extension. EOB has been reported as a typical phenotype in mutant mice lacking several genes affecting epithelial cell proliferation and motility [12,13]. The genes whose deletion results in EOB include transcription factors controlling cell proliferation (c-jun [21,22]), growth factors (FGF10 [23], HB-EGF [20,24], TGF-α [25], ActivinB [26]) and their receptors (EGFR [27]), and related cytoplasmic factors functioning in signal transduction pathways (MEKK1 [28,29], JNK [30]). Xia et al. reviewed the signaling pathways required for embryonic eyelid closure in normal developmental stages and classified these into two major signaling pathways, TGFβ/activin-MEKK1-JNK/p38 and TGF-α/EGFR-ERK [13]. Our data strongly suggest that *Lgr4* plays a critical role in regulating the formation of eyelids in the embryonic stage and that it contributes to epithelial cell motility. In addition, epidermal wound-healing activity [21,27] and tumorigenesis require epithelial cell motility [29]. Although the downstream signaling mechanisms of *Lgr4* are not clear, we speculate that a novel signaling pathway exists in keratinocytes to regulate the cell motility.

We generated *Lgr4* conditional knockout mice that were spared the embryonic/neonatal lethality, reduced body weight and renal hypoplasia observed in *Lgr4* null mice, and were born with a mendelian distribution. However, almost all of these mice showed the EOB phenotype. These results strongly suggest that the keratinocyte aberration shown by *Lgr4* null mice was not induced by renal hypoplasia.

The observed impairment in keratinocyte motility may suggest that *Lgr4* controls the cell motility of keratinocytes. In this context, elucidating the precise role of *Lgr4* along with its cognate ligand would advance the knowledge of the epithelial cell motility mechanism. Interestingly, one of our *Lgr4* null mice survived for more than 40 days, showing turbid corneas in addition to various defects. Microscopic observation of the cornea showed many stripes (data not shown), and we presume that the degeneration of the cornea, including wounds, resulted from excoriation by the floor material in the cage because of the EOB. However, immunostaining of the eye tissue clearly showed positive staining at the cornea in the wild-type mice in addition to the signal at the protruding tips of the eyelid (Fig. 1B). This result also suggests the possibility that the turbid cornea observed in null mice might be caused by a deficiency of the *Lgr4* gene. Therefore, a cornea-specific deletion of *Lgr4* will be required to study further the function of *Lgr4* in the development, growth and maintenance of the cornea tissue. To our knowledge, there are no published reports of EOB caused by a deficiency of GPCR genes. In addition, in the other reports on the generation of *Lgr4* mutants (by insertion of a gene cassette carrying a splice acceptor into to an intron of

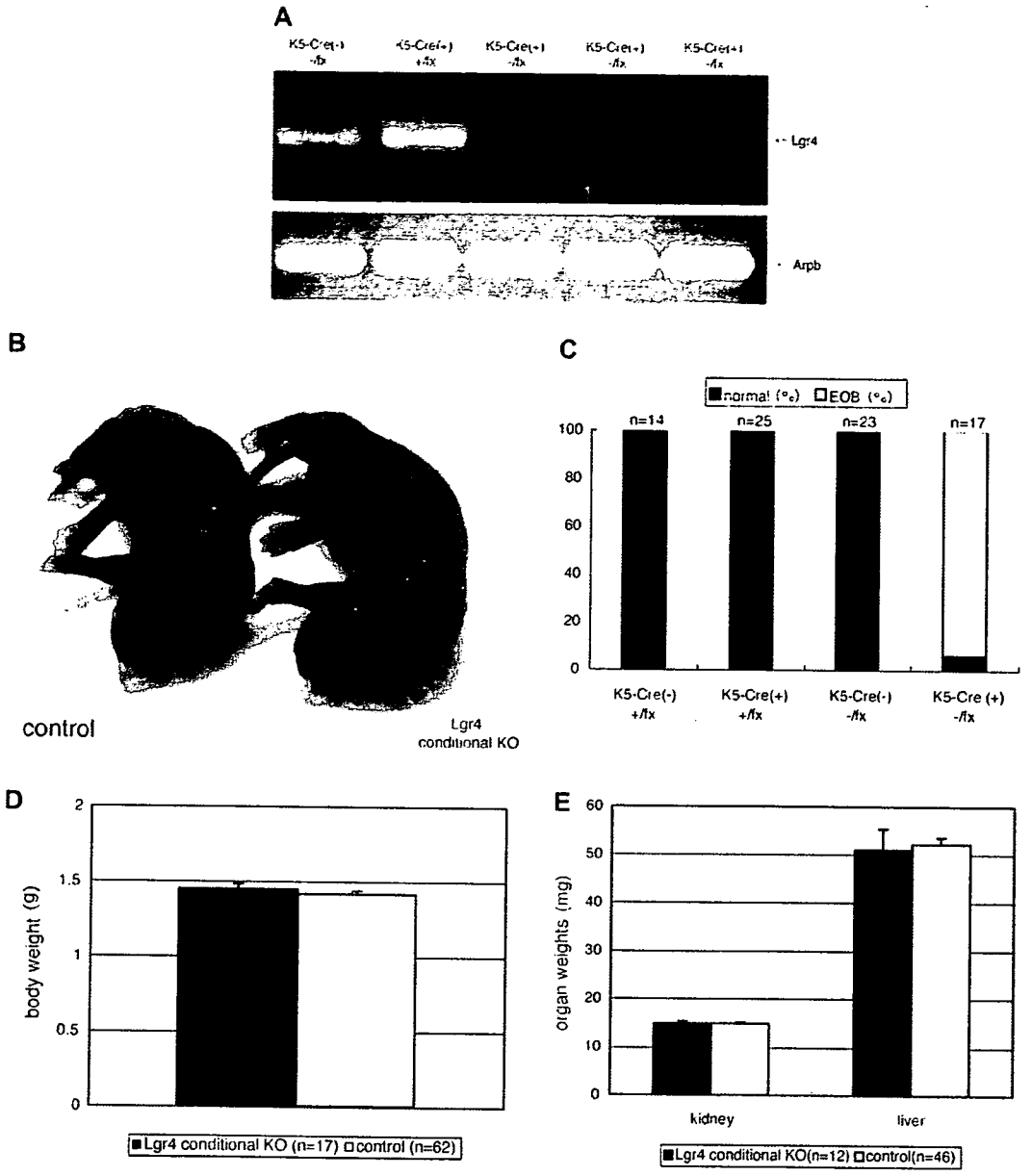

Fig. 4. Characteristics of *Lgr4* conditional knockout mice. (A) RT-PCR for *Lgr4* showed no expression of *Lgr4* mRNA in keratinocytes. Primer sets are shown in the supplementary material. (B) *Lgr4* conditional knockout mice show the typical EOB phenotype. (C) As in the case of *Lgr4* null mice, almost all of the *Lgr4* conditional knockout mice show the EOB phenotype. The body weight (D) and the organ weight of the liver and kidneys (E) of *Lgr4* conditional knockout mice are all within normal ranges.

the gene) on several genetic backgrounds [8,10,11], the EOB phenotype of the null mice was not described except in one abstract for a poster presentation [31]. Nevertheless, we predicted that *Lgr4* and its unidentified ligand(s) would likely be related to regulatory mechanisms controlling the development and maintenance of epidermal tissue including that of the eyelid.

In conclusion, we generated two mouse models, one with a complete deletion of the *Lgr4* function and another with a conditional *Lgr4* knockout, and provide evidence that *Lgr4* is involved in keratinocyte motility affecting eyelid formation. Adult conditional *Lgr4* knockout mice will be a useful tool for examining the *Lgr4* function in keratinocytes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.08.064.

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Eye-open at birth phenotype with reduced keratinocyte motility in LGR4 null mice

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Abstract **We observed a consistent eye-open at birth (EOB) phenotype in mouse pups homozygous for a leucine-rich repeat containing G-protein coupled receptor 4 (*Lgr4*) allele deleting the whole transmembrane domain coding region. An in vitro wound-healing scratch assay showed notably reduced keratinocyte motility in the null mice. Phalloidin staining of F-actin in the eyelid epidermis was also reduced. We also generated keratinocyte-specific *Lgr4* deficient mice, circumventing the embryonic/neonatal lethality and kidney abnormalities. Most of the conditional *Lgr4* knockout mice showed the EOB phenotype. Thus, *Lgr4* might be a novel gene class regulating cell motility.**

Keywords: LGR4; GPR48; GPCR; Gene deletion mice; EOB; Keratinocyte

1. Introduction

Lgr4 (leucine-rich repeat containing G-protein coupled receptor 4) is one of the genes identified as novel G-protein coupled receptors (GPCRs) [1,2], designated *Lgr4–Lgr8*, from an EST database with high homology to glycoprotein hormone receptors including follicle-stimulating hormone receptor (FSHR) [3], luteinizing hormone/chorionic gonadotropin receptor (LH/CGR) [4,5] and thyroid-stimulating hormone receptor (TSHR) [6].

As *Lgr4* shows high homology with FSHR, LHR and THSR, this receptor has been thought to be involved in reproductive systems. Mazerbourg et al. reported the generation of *Lgr4* gene-interrupted mice using a gene-trapped ES cell line [7], in which the expression of *Lgr4* is severely attenuated by the insertion of the β-geo gene in an enhancer trap procedure [8]. They described the neonatal lethality of the null mice, but not the cause. Previously, we generated similar *Lgr4* knockout mice by completely removing exon18, which encodes the whole transmembrane domain of *Lgr4*, in order to eliminate the chance of receptor fragment localizing at the membrane or transmitting downstream signals [9]. In those *Lgr4* knockout mice, gross hypomorphic phenotypes developed in multiple tissues and organs, and the null mice showed hypoplastic kidneys with an increased concentration of plasm creatinine, which was strongly suspected to be the cause of the neonatal/embryonic lethality.

Recently, Mendive et al. as well as Hoshii et al. reported *Lgr4* gene-trap lines exhibiting defective postnatal development of the male reproductive tract [10,11], again in contrast to the embryonic/neonatal lethality seen in our *Lgr4* knockout mice on a 129Ola × C57BL/6 hybrid background [9]. In our *Lgr4* knockout mice it appeared that complete loss of the *Lgr4* gene would induce complete embryonic/neonatal lethality. As reported in our first paper, the typical phenotype of the *Lgr4* null mutants other than the kidney aberration was eye open at birth (EOB) with 100% penetrancy, strongly suggesting reduced keratinocyte proliferation and motility. Several studies have reported a strong relationship between a reduction in keratinocyte proliferation/motility and the EOB phenotype [12,13], and it has been suggested that *Lgr4* is essential for organ development and cancer cell invasion [10,11,14]. Additionally, a close relationship between cancer cell invasion and cell motility was reported, and it is well known that organ development requires cell motility [15,16]. We therefore suspected the existence of a close relationship between *Lgr4* and cell motility, and considered that common mechanisms might cause EOB and the other abnormalities observed in *Lgr4* null mice. In the present study we focused on the EOB phenotype in relation to the keratinocyte motility of the null mice.

We provide evidence that *Lgr4* has a role in keratinocyte motility. In addition, we succeeded in dissociating the EOB phenotype, first observed in all conventional *Lgr4* KO mice, from the kidney lesions and lethality using conditional *Lgr4* knockout mice crossed with K5-Cre transgenic mice.

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Abbreviations: LGR, leucine-rich repeat containing G-protein coupled receptor; GPCR, G-protein coupled receptor; FSHR, follicle-stimulating hormone receptor; LH/CGR, luteinizing hormone/chorionic gonadotropin receptor; TSHR, thyroid-stimulating hormone receptor; EOB, eye-open at birth; Arbp, acidic ribosomal phosphoprotein PO

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2. Materials and methods

2.1. Histology

For the eyelid histology, the heads of the mice were fixed in 4% paraformaldehyde overnight. After dehydration, they were embedded in paraffin. Paraffin blocks were sectioned at 2–5 μm thickness and stained with H&E (hematoxylin–eosin) and Phalloidin-TRITC (tetramethyl rhodamine isothiocyanate) using standard procedures.

2.2. In vitro apoptosis cell test

Sections from around the eyelids of E15.5 mice were used for the detection of apoptotic cells. Samples were processed according to the protocol of In Situ Cell Death Detection Kit (Roche, Japan).

2.3. Keratinocyte primary culture

Primary keratinocytes were isolated from neonatal mice. The epidermis was separated from the dermis with 0.8 U/ml dispase (Roche) overnight at 4 °C. Keratinocytes were dissociated by trypsin for 5 min at 34 °C and plated onto dishes precoated with collagen type I. The cells were cultured in minimum essential medium supplemented with 4% Chelex (Bio-Rad, Hercules, CA)-treated fetal calf serum epidermal growth factor (10 ng/ml; Gibco BRL), and 0.05 mM $CaCl_2$ at 34 °C in an 8% CO_2 incubator. Under these conditions, keratinocytes are maintained in an immature state characterized by active proliferation. For all the experiments, the cells were used one week after plating.

2.4. In vitro migration assay

Keratinocytes derived from each genotype of neonatal mice were cultured until confluent. After scratching with plastic tips, the distance that the keratinocyte migrated was measured every 3 h. Each sample was counted at 16 points and the average distance was calculated.

2.5. Quantitative RT-PCR

Messenger RNA derived from wild-type and *Lgr4* null mice keratinocyte was subjected to cDNA synthesis by standard procedures. Quantitative RT-PCR assays were performed with the DNA Engine Opticon System (MJ Research, Japan) with a cycling profile as follows: at 95 °C for 2 min, 39 cycles at 95 °C for 5 s, at 61.4 °C for 30 s, and at 72 °C for 30 s. The genes and primer sets are shown in the supplementary material.

2.6. Generation of keratinocyte specific Lgr4 deficient mice

To generate *Lgr4* fx/fx mice without frt-Neo-frt cassette, an initially targeted *Lgr4* mutant [9] was mated with Flp deleter [17]. Mice with the keratinocyte-specific *Lgr4* deletion were generated by breeding keratin5-Cre (K5-Cre); *Lgr4* +/− mice with *Lgr4* fx/fx mice [18]. The genetic backgrounds were C57Bl/6 × 129Ola for *Lgr4* +/− and *Lgr4* fx/fx, and C57Bl/6 × C3H for K5-Cre. Primers for *Lgr4* genotyping are shown in the supplementary material.

2.7. Statistical evaluation

All experimental data are expressed as mean S.E.M. Statistical comparisons in all the physiological and laboratory data were made among the genotype groups using ANOVA followed by Student's *t*-test for individual comparisons. *P* values of <0.05 were considered significant.

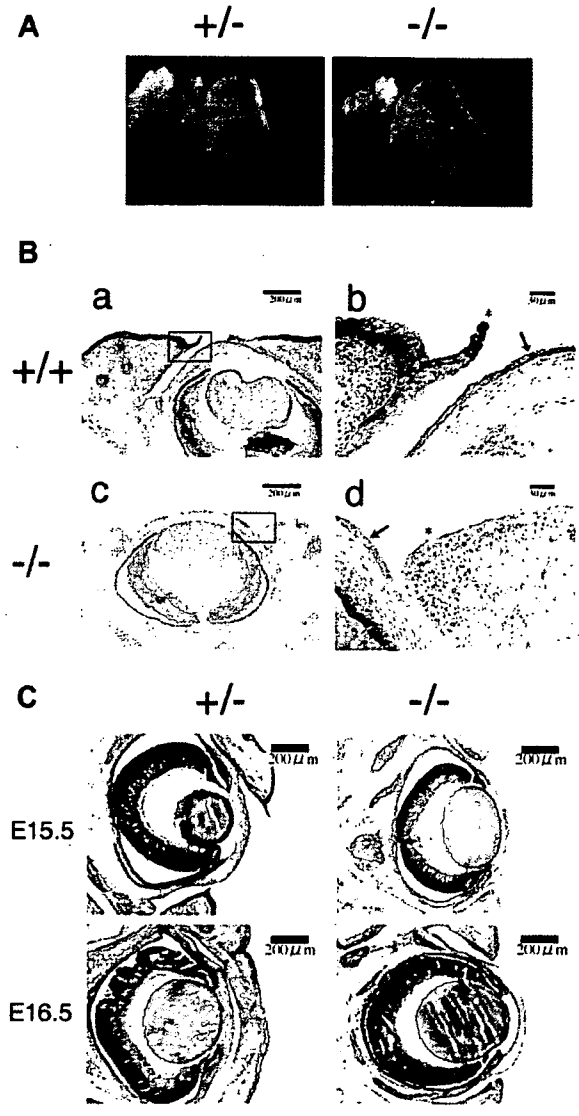

Fig. 1. *Lgr4* deficiency causes impairment in embryonic eyelid closure. (A) Left panel and right panel show morphology around eyes of *Lgr4* heterozygous (*Lgr4* +/−) and *Lgr4* null mice, respectively at postnatal day 0. (B) Eyelid sections prepared from E 15.5 wild-type (a, b) or null (c, d) mouse fetuses were immunostained with rabbit anti-LGR4 antibody (ab 12576, Abcam, USA), followed by HRP-conjugated second antibody. Asterisks show epithelial cell layer at the protruding tips of the growing eyelids, and arrows show the corneal epithelium layer. (C) Histological analysis (H&E staining) of E15.5 and E16.5 embryos.

3. Results

3.1. Lgr4 null mice show morphological abnormalities in the eyelids at E15.5

Lgr4 null mice showed some gross abnormalities. In wild-type mice during the embryonic stages E15.5 through E16.5, epithelial cells extended to the center of the eyes and finally became fused. The mice were then born with their eyelids fused, and the eyelids opened gradually by 12–14 days after birth. However, *Lgr4* null mice showed the EOB phenotype with 100% penetrance (Fig. 1A). Histological analysis of the wild-type mice followed by immunostaining showed a high expression level of *Lgr4* at the protruding tips of the eyelids and in the epithelial cells of the cornea at E15.5, but no expression was detected in the same areas of the null mice (Fig. 1B). As shown in Fig. 1C right, the eyelid closure was impaired in the null mice as compared to the heterozygous mice (Fig. 1C, left).

3.2. Proliferation and motility of keratinocytes from Lgr4 null mice

EOB has been reported as a typical phenotype reflecting keratinocyte proliferation/motility, which we then measured. No decrease in proliferating cells was detected in the eyelid epi-

thelium of the null mutants at E15.5 (data not shown). Since a reduction of the cytoplasmic accumulation of F-actin was observed with the EOB phenotype in some gene knockout studies [19,20], we stained the eyelid sections with phalloidin and found that there was less filamentous accumulation of F-actin at the margin of the eyelid epithelium, as shown in Fig. 2A. Furthermore, we examined the extent of keratinocyte proliferation in vitro using the incorporation of BrdU, but no significant difference between the wild-type and the *Lgr4* null mice was observed (date not shown). Next, the keratinocyte motility was measured by an in vitro migration assay. The lack of *Lgr4* was related to a reduction of the keratinocyte motility, and we observed a significant delay in healing 3 hours after scratching in the cells from the null mice (Fig. 2B and C). To further analyze other possible mechanisms responsible for EOB in addition to the reduced motility of the null mice, wild-type and *Lgr4* null fetuses at E15.5 were subjected to TUNEL assay to examine the extent of cell apoptosis around the eyelid tissues. However, neirther types of embryos showed any apoptotic cells (Fig. 2D).

These results suggest that the EOB observed in the null mice was not induced by an enhancement of apoptosis, and provide further evidence that *Lgr4* is a critical regulator for keratinocyte motility in the epidermal tissue of eyelids.

3.3. Quantitative RT-PCR of EOB related genes in keratinocytes from Lgr4 null mice

The disruption of *EGFR*, *EGF*, *TGF-α*, *ADAM17*, *c-jun*, *ActivinA* and *ActivinB* genes has been reported to cause EOB. We measured the expression levels of these genes in mRNA prepared from wild-type and *Lgr4* null keratinocytes, but did not detect any significant differences in their expression levels (Fig. 3). In addition, the expression level of EGFR around the eyelid at E15.5 in the null mutants was normal (Supplementary Fig. S1). The phosphorylation of EGFR around the eyelid at E15.5 was examined in wild-type and *Lgr4* null mice by immunostaining, but no significant difference was observed (date not shown). The phosphorylation of ERK and JNK of the same samples also showed no difference (Supplementary Fig.S1).

3.4. Generation of keratinocyte-specific Lgr4 deficient mice

As mentioned above, the EOB phenotype observed in *Lgr4* null mice was suspected to be closely related to reduced keratinocyte motility. We previously reported that *Lgr4* null mice showed renal hypoplasia [9]. To separate the EOB phenotype from the renal hypoplasia observed in *Lgr4* null mice, we additionally generated keratinocyte-specific *Lgr4* deficient mice (*Lgr4* conditional knockout mice) as described in Section 2

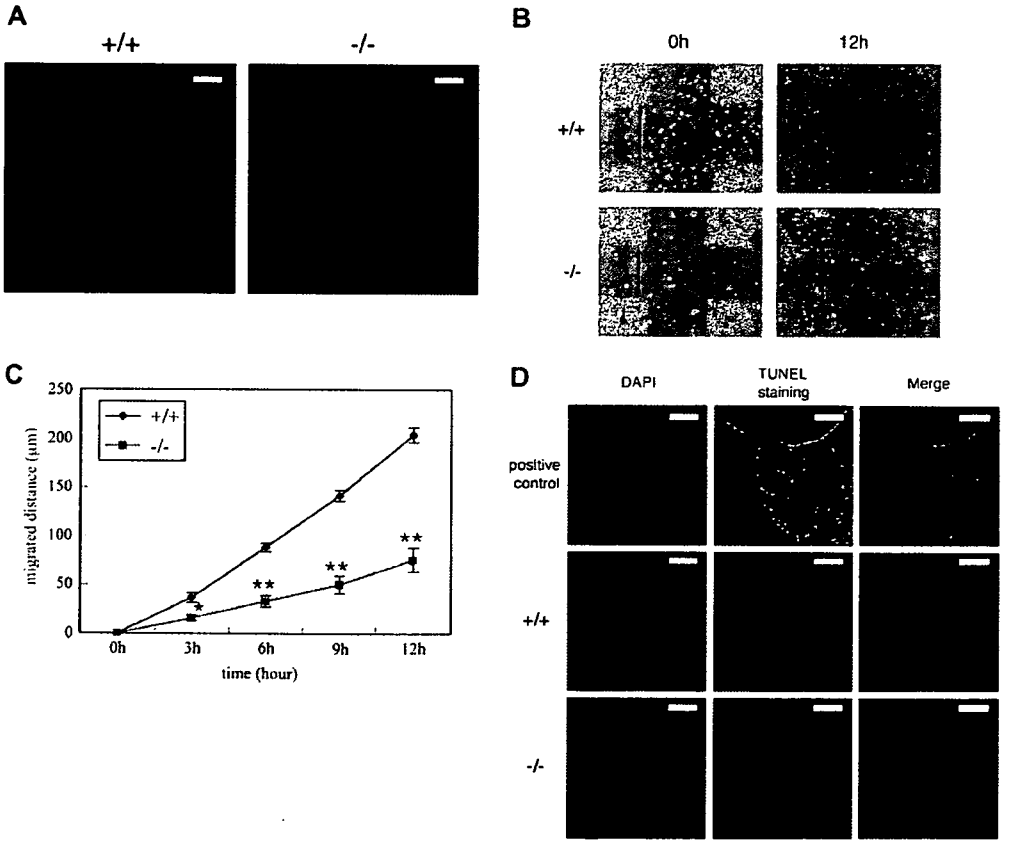

Fig. 2. EOB phenotype and impaired keratinocyte motility. (A) Coronal eye sections of E15.5 wild-type (*Lgr4* +/+) and null fetuses were stained with phalloidin-TRITC. Scale bar: 30 μm. (B) Keratinocyte motility was assessed by in vitro wound-healing scratch assay, and values representing the mean (S.E.M.; vertical bar) of 16 independent wounds are shown by a linegraph (C). (*; $P < 0.005$, **; $P < 0.001$) (D) Neither wild-type nor *Lgr4* null E15.5 fetuses showed apoptotic cells in the eyelids. Positive control is tissue treated with DNase I. Scale bar: 60 μm.

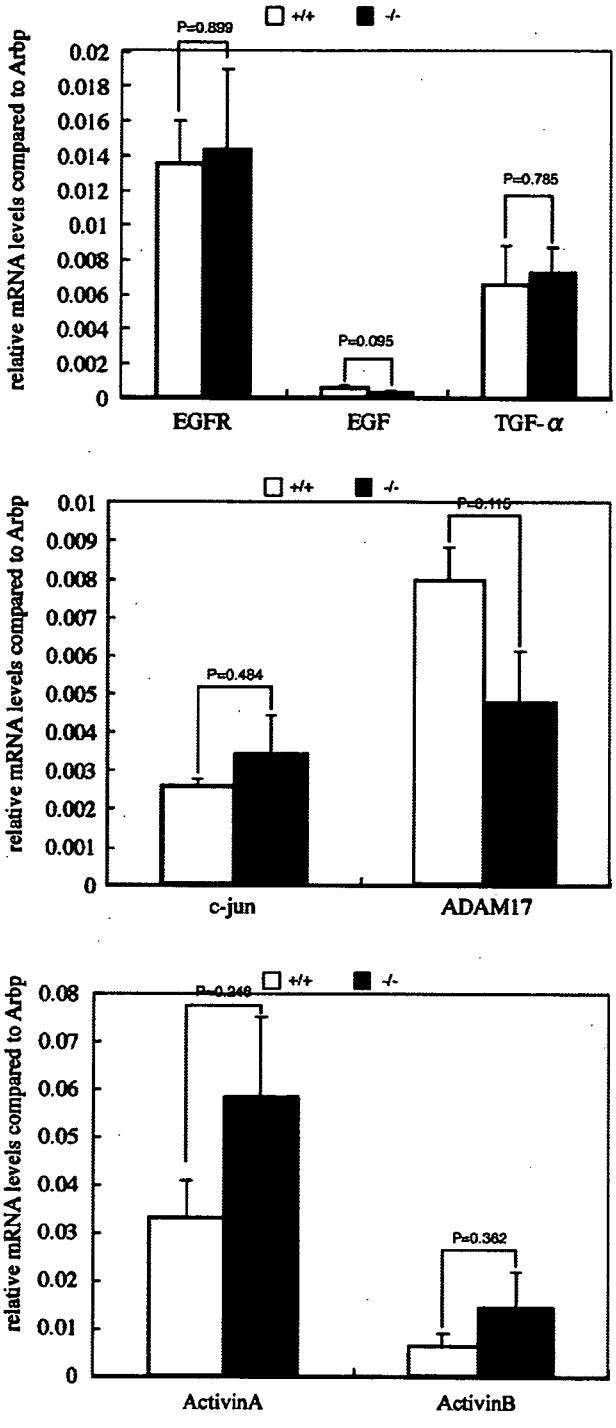

Fig. 3. Quantitative RT-PCR for cell motility-related genes. EGFR, EGF, TGF-α, c-jun, ADAM17, ActivinA and ActivinB mRNA levels in keratinocyte derived from *Lgr4* wild-type and null mice (P0) were quantified (all bars; $n = 3$). Error bars represent ± S.E.M.

(Supplementary Fig. S2A and B). The keratinocyte-specific deletion of *Lgr4* gene in *Lgr4* conditional knockout mice was confirmed by RT-PCR (Fig. 4A). The *Lgr4* conditional knockout mice were spared the embryonic/neonatal lethality (data not shown) and almost all of them showed the EOB phenotype (Fig. 4B and C). The body size and weight of several organs including the kidneys and liver were all reduced in *Lgr4* null mice [9], whereas those of the conditional knockout mice were all normal (Fig. 4D and E).

4. Discussion

In this report, we demonstrated an abnormality in the motility of keratinocytes and the EOB phenotype in *Lgr4* null mice. It is known that, during normal mouse development, the epithelial cells on the protruding tips of both the upper and lower eyelids migrate along the surface of the cornea and fuse with each other by E16.5 [12]. This suggests that EOB could be due to a defect in prenatal eyelid extension. EOB has been reported as a typical phenotype in mutant mice lacking several genes affecting epithelial cell proliferation and motility [12,13]. The genes whose deletion results in EOB include transcription factors controlling cell proliferation (c-jun [21,22]), growth factors (FGF10 [23], HB-EGF [20,24], TGF-α [25], ActivinB [26]) and their receptors (EGFR [27]), and related cytoplasmic factors functioning in signal transduction pathways (MEKK1 [28,29], JNK [30]). Xia et al. reviewed the signaling pathways required for embryonic eyelid closure in normal developmental stages and classified these into two major signaling pathways, TGFβ/activin-MEKK1-JNK/p38 and TGF-α/EGFR-ERK [13]. Our data strongly suggest that *Lgr4* plays a critical role in regulating the formation of eyelids in the embryonic stage and that it contributes to epithelial cell motility. In addition, epidermal wound-healing activity [21,27] and tumorigenesis require epithelial cell motility [29]. Although the downstream signaling mechanisms of *Lgr4* are not clear, we speculate that a novel signaling pathway exists in keratinocytes to regulate the cell motility.

We generated *Lgr4* conditional knockout mice that were spared the embryonic/neonatal lethality, reduced body weight and renal hypoplasia observed in *Lgr4* null mice, and were born with a mendelian distribution. However, almost all of these mice showed the EOB phenotype. These results strongly suggest that the keratinocyte aberration shown by *Lgr4* null mice was not induced by renal hypoplasia.

The observed impairment in keratinocyte motility may suggest that *Lgr4* controls the cell motility of keratinocytes. In this context, elucidating the precise role of *Lgr4* along with its cognate ligand would advance the knowledge of the epithelial cell motility mechanism. Interestingly, one of our *Lgr4* null mice survived for more than 40 days, showing turbid corneas in addition to various defects. Microscopic observation of the cornea showed many stripes (data not shown), and we presume that the degeneration of the cornea, including wounds, resulted from excoriation by the floor material in the cage because of the EOB. However, immunostaining of the eye tissue clearly showed positive staining at the cornea in the wild-type mice in addition to the signal at the protruding tips of the eyelid (Fig. 1B). This result also suggests the possibility that the turbid cornea observed in null mice might be caused by a deficiency of the *Lgr4* gene. Therefore, a cornea-specific deletion of *Lgr4* will be required to study further the function of *Lgr4* in the development, growth and maintenance of the cornea tissue. To our knowledge, there are no published reports of EOB caused by a deficiency of GPCR genes. In addition, in the other reports on the generation of *Lgr4* mutants (by insertion of a gene cassette carrying a splice acceptor into to an intron of

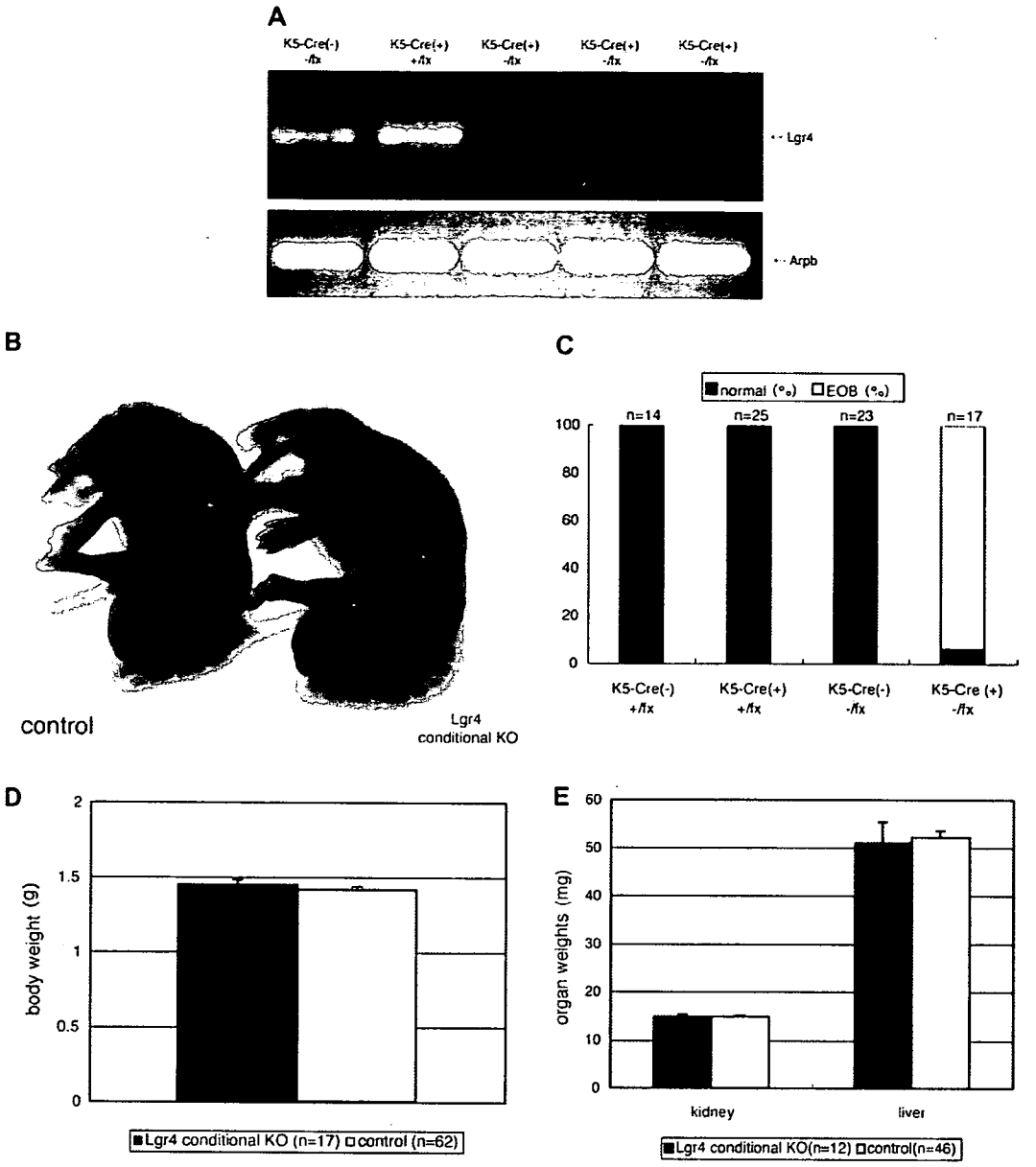

Fig. 4. Characteristics of *Lgr4* conditional knockout mice. (A) RT-PCR for *Lgr4* showed no expression of *Lgr4* mRNA in keratinocytes. Primer sets are shown in the supplementary material. (B) *Lgr4* conditional knockout mice show the typical EOB phenotype. (C) As in the case of *Lgr4* null mice, almost all of the *Lgr4* conditional knockout mice show the EOB phenotype. The body weight (D) and the organ weight of the liver and kidneys (E) of *Lgr4* conditional knockout mice are all within normal ranges.

the gene) on several genetic backgrounds [8,10,11], the EOB phenotype of the null mice was not described except in one abstract for a poster presentation [31]. Nevertheless, we predicted that *Lgr4* and its unidentified ligand(s) would likely be related to regulatory mechanisms controlling the development and maintenance of epidermal tissue including that of the eyelid.

In conclusion, we generated two mouse models, one with a complete deletion of the *Lgr4* function and another with a conditional *Lgr4* knockout, and provide evidence that *Lgr4* is involved in keratinocyte motility affecting eyelid formation. Adult conditional *Lgr4* knockout mice will be a useful tool for examining the *Lgr4* function in keratinocytes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.08.064.

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Study on the quality control of cell therapy products Determination of *N*-glycolylneuraminic acid incorporated into human cells by nano-flow liquid chromatography/Fourier transformation ion cyclotron mass spectrometry

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Abstract

N-Glycolylneuraminic acid (NeuGc), an acidic nine-carbon sugar, is produced in several animals, such as cattle and mice. Since human cells cannot synthesize NeuGc, it is considered to be immunogenic in humans. Recently, NeuGc contamination was reported in human embryonic stem cells cultured with xenogeneic serum and cells, suggesting that possibly NeuGc may harm the efficacy and safety of cell therapy products. Sialic acids have been determined by derivatization with 1,2-diamino-4,5-methylenedioxybenzene (DMB) followed by liquid chromatography/mass spectrometry (LC/MS) and liquid chromatography/tandem mass spectrometry (LC/MS/MS); however, the limited availability of cell therapy products requires more sensitive and specific methods for the quality test. Here we studied the use of nano-flow liquid chromatography/Fourier transformation ion cyclotron resonance mass spectrometry (nanoLC/FTMS) and nanoLC/MS/MS for NeuGc-specific determination at a low femtomole level. Using our method, we found NeuGc contamination of the human cell line (HL-60RG cells) cultured with human serum. Our method needs only 2.5×10^3 cells for one injection and would be applicable to the determination of NeuGc in cell therapy products.

Keywords: *N*-Glycolylneuraminic acid; Nano-flow liquid chromatography; Fourier transformation ion cyclotron mass spectrometry; Cell therapy products

1. Introduction

Sialic acids are a family of acidic nine-carbon sugars found in the non-reducing terminal of *N*-linked and *O*-linked oligosaccharides of glycoproteins and glycolipids [1,2]. There are more than 30 members with different substitutions on the amino group at carbon 5 and on hydroxyl groups at carbons 4, 7, 8 and 9 [2–8]. *N*-Glycolylneuraminic acid (NeuGc), a 5-*N*-glycolylated sialic acid, is produced in several animals, such as cattle, horses, mice and rats [9]. Since human cells cannot synthesize NeuGc due to mutation of the cytidine monophospho (CMP)-*N*-acetylneuraminic acid (NeuAc) hydroxylase gene [10,11], NeuGc is considered to be antigenic and to induce immunoreaction in humans [4,12,13].

Advances in biotechnology and cell culture techniques make it possible to administer human and animal cells directly to patients as cell therapy products. In cell therapy and tissue engineering, human embryonic stem (ES) cells are expected to be useful for the treatment of many diseases. Recently, it was reported that NeuGc is incorporated into ES cells from human and mouse feeder cells and cultivation media containing xenogeneic serum, such as fetal calf serum (FCS) [14,15]. Since NeuGc is a foreign component in humans, it is feared that NeuGc may harm the efficacy and safety of cell therapy products. To

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assess the adverse effects of NeuGc, it is necessary to quantify NeuGc in cell therapy products.

Sialic acids have been determined by labeling with 1,2-diamino-4,5-methylenedioxybenzene (DMB) followed by conventional high-performance liquid chromatography (HPLC) with fluorescent detection [16–20]. The femtomole level of sialic acid can be determined by fluorescent detection [19]. The use of liquid chromatography/mass spectrometry (LC/MS) and liquid chromatography/tandem mass spectrometry (LC/MS/MS) has more advantage in the identification of sialic acid species [18,20–22]. The derivatization of sialic acids with DMB has advantages of good separation of NeuGc from NeuAc in chromatography and enhancement of ionization efficiency in MS. However, more sensitive and specific methods are desired for the quality control of cell therapy products, since in many case only a low number of cell products, approximately 1×10^6 to 1×10^8, should be available for quality tests.

In this study, we studied the use of nano-flow liquid chromatography/Fourier transformation ion cyclotron resonance mass spectrometry (nanoLC/FTMS) and LC/MS/MS to achieve the sensitive and specific determination of NeuGc. The potential of the method for quality testing of cell therapy products was evaluated using substrain of human promyelocytic leukemia HL-60 cells (HL-60RG cells) as model cells. Using this method, we determined NeuGc in membrane fractions from HL-60RG cells cultured with FCS, human serum and serum-free medium.

2. Experimental

2.1. Materials

NeuGc and NeuAc were purchased from Nacalai Tesque (Kyoto, Japan). FCS and normal human serum were purchased from Dainippon Sumitomo Pharma (Osaka, Japan). RPMI1640 medium and ASF104 medium were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Ajinomoto (Tokyo, Japan), respectively.

2.2. Cell culture

Substrain of human promyelocytic leukemia HL-60 cells (HL-60RG cells, JCRB Cellbank, Osaka, Japan) was cultured in RPMI1640 medium supplemented with 10% FCS, 100 unit/ml of penicillin and 100 μg/ml of streptomycin under a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. HL-60RG cells were replaced at 2×10^5 cells/100 mm dish in RPMI1640 medium supplemented with 10% FCS or 10% normal human serum, and in serum-free ASF104 medium. The media were replaced four times, and semi-confluent growth cells were harvested.

2.3. Fractionation of the membrane fraction

The cells were washed in phosphate buffer saline (PBS) supplemented with protease inhibitors (protease inhibitor mix solution, Wako, Tokyo, Japan) three times. The washed cells (1×10^6) were suspended in 100 μl of 0.25 M sucrose/10 mM Tris–HCl buffer (pH 7.4) containing protease inhibitors, and sonicated at 4 °C for 30 s, two times (40W. Bioruptor UCW-201, Tosyoudenki, Kanagawa, Japan). After the nuclei were removed by centrifugation at 4 °C, 450 × *g* for 10 min, the mitochondria and lysosome fractions were removed by re-centrifugation at 4 °C, 20,000 × *g* for 10 min. The membrane fractions were precipitated by ultracentrifugation at 4 °C, 100,000 × *g* for 60 min. The membrane fractions were washed in 100 μl of 150 mM ammonium acetate buffer (pH 7.4) and recovered by re-ultracentrifugation.

2.4. Derivatization of NeuGc and NeuAc with DMB reagent

The membrane fractions were sonicated in 250 μl of H_2O and then incubated with 250 μl of 4 M acetic acid (final concentration, 2 M) at 80 °C for 3 h. The released sialic acids were passed though a solid-phase extraction cartridge (SepPak C-18, Waters, Milford, MA, USA) with 2 ml of H_2O, dried under vacuum, and resolved in 50 μl of H_2O. The solution was incubated with DMB according to the manufacturer's instruction (Takara, Tokyo, Japan), and the reaction mixture was applied on a solid-phase extraction cartridge (Envi-Carb C, Supelco, Bellefonte, PA, USA). After washing the cartridge with 2.5 ml of 5 mM ammonium acetate (pH 9.6) for desalting, the DMB-labeled sialic acids were eluted with 3 ml of 45% acetonitrile/5 mM ammonium acetate (pH 9.6). The collected fraction was freeze dried.

2.5. nanoLC/FTMS

DMB-labeled sialic acids were separated by HPLC using Paradigm MS4 (Michrom BioResource, Auburn, CA, USA) equipped with a reversed-phase C18 column (Magic C18, 50 mm × 0.1 mm, 3 μm, Michrom BioResource, Auburn, CA, USA). Elution was achieved using 0.1% formic acid/2% ace-

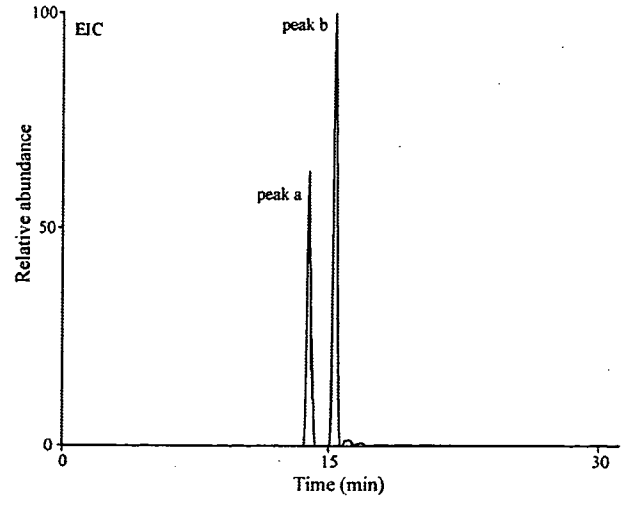

Fig. 1. EIC at *m/z* 426.13–426.17 and *m/z* 442.12–442.16 obtained by SIM (*m/z* 400–450) of DMB-NeuGc and DMB-NeuAc in the positive ion mode.

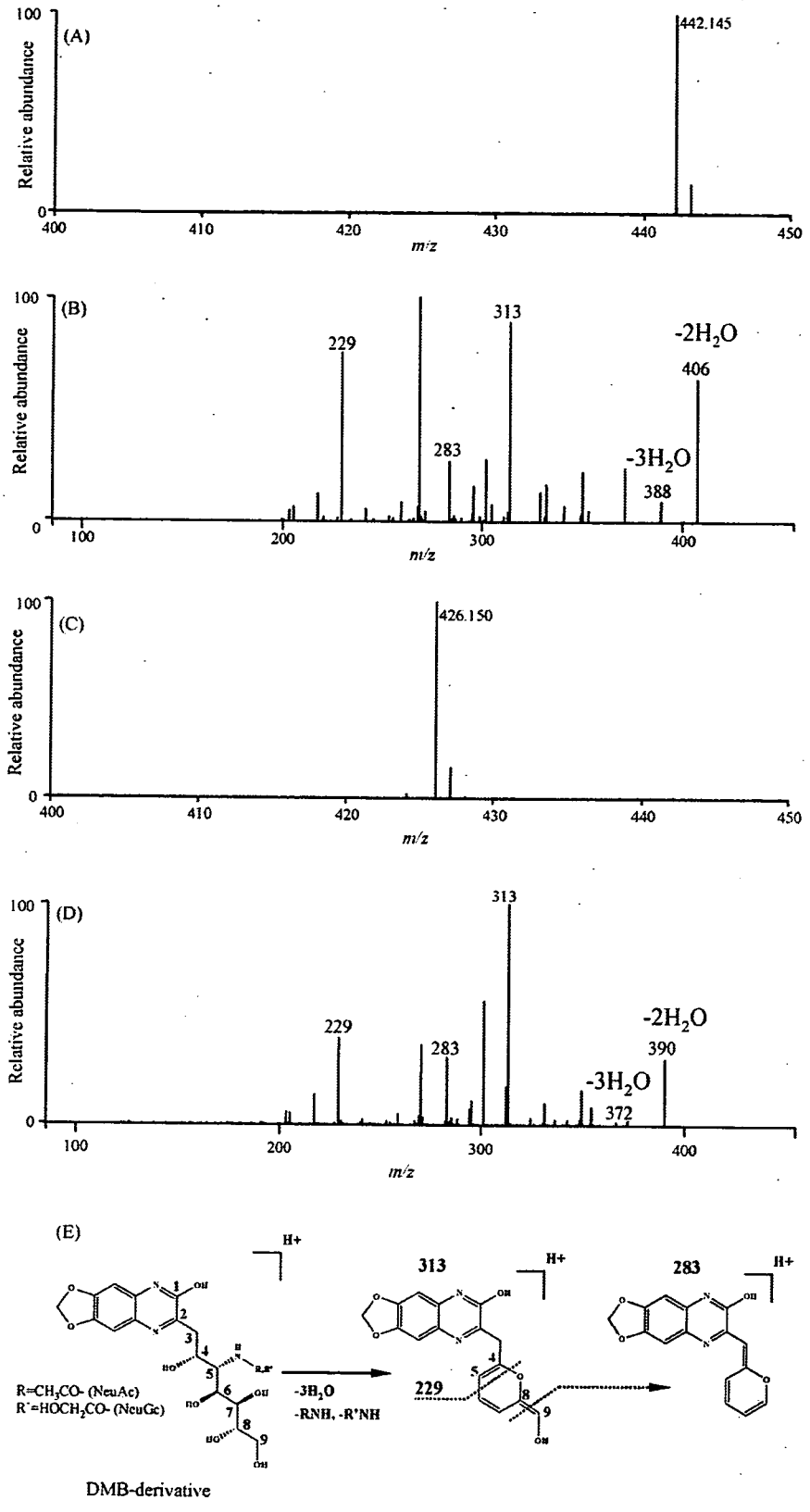

Fig. 2. (A) Typical MS spectrum of peak a. (B) MS/MS spectrum of $[M+H]^+$ (*m/z* 442.145) acquired from around peak a. (C) Typical MS spectrum of peak b. (D) MS/MS spectrum of $[M+H]^+$ (*m/z* 426.150) acquired from around peak b. (E) Fragmentation of DMB-NeuGc and DMB-NeuAc.

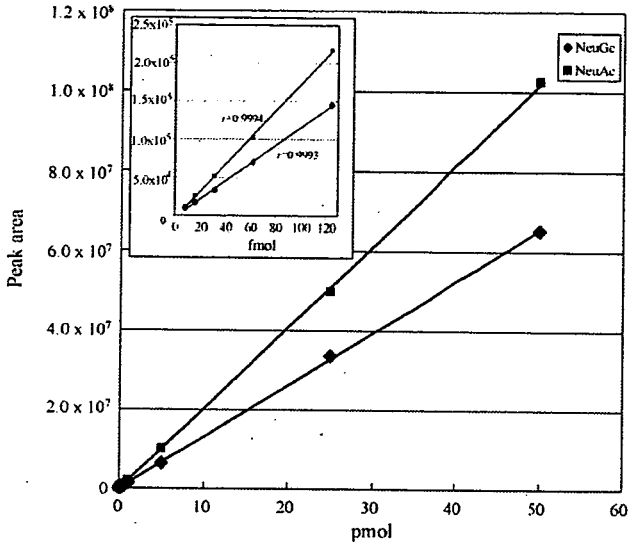

Fig. 3. Calibration curves of DMB-NeuGc ($r=0.9998$) and DMB-NeuAc ($r=0.9995$).

tonitrile (pump A) and 0.1% formic acid/80% acetonitrile (pump B) with a linear gradient of 10–90% of B in 30 min at a flow rate of 750 nl/min. On-line MS and MS/MS were performed using an Fourier transformation ion cyclotron resonance (FT)/ion trap (IT) type mass spectrometer (LTQ-FT, Thermo-Electron, San Jose, CA, USA) equipped with a nanoelectrospray ion source (AMR, Tokyo, Japan). DMB-NeuAc and DMB-NeuGc were determined by selected ion monitoring (SIM) in the positive ion mode. The analytical conditions were set to 200 °C for capillary temperature, 1800 eV spray voltage, *m/z* 400–450 scan range, and 35% collision energy. The automatic gain control (AGC) value, which is adjusted for the amount of imported ions for FTMS, was set to 5×10^4. Maximum injection times, which are the adjusted times of imported ions, for ITMS and FTMS, were set to 50 and 1250 ms, respectively.

2.6. Method validation

The linearity of the signal intensity peak area of DMB-NeuAc and DMB-NeuGc was assessed by injections of 0.0078–500 pmol DMB derivatives. Correlation coefficients were calibrated using a least-squares linear regression model. The detection limit (DL) and the quantification limit (QL) were calculated using the formulas $DL = 3.3 \times \sigma/slope$ (σ: average of noise on chromatograph) and $QL = 10 \times \sigma/slope$, respectively. Accuracy and precision were determined by measuring three samples, where NeuGc spiked at the concentration of 50 fmol to the membrane fraction of cells cultured in serum-free medium which contains no NeuGc before the derivatization of NeuGc with DMB. Accuracy was calculated by comparison of the mean peak area and the calibration curve. Precision was estimated by relative standard deviation (RSD) from three samples.

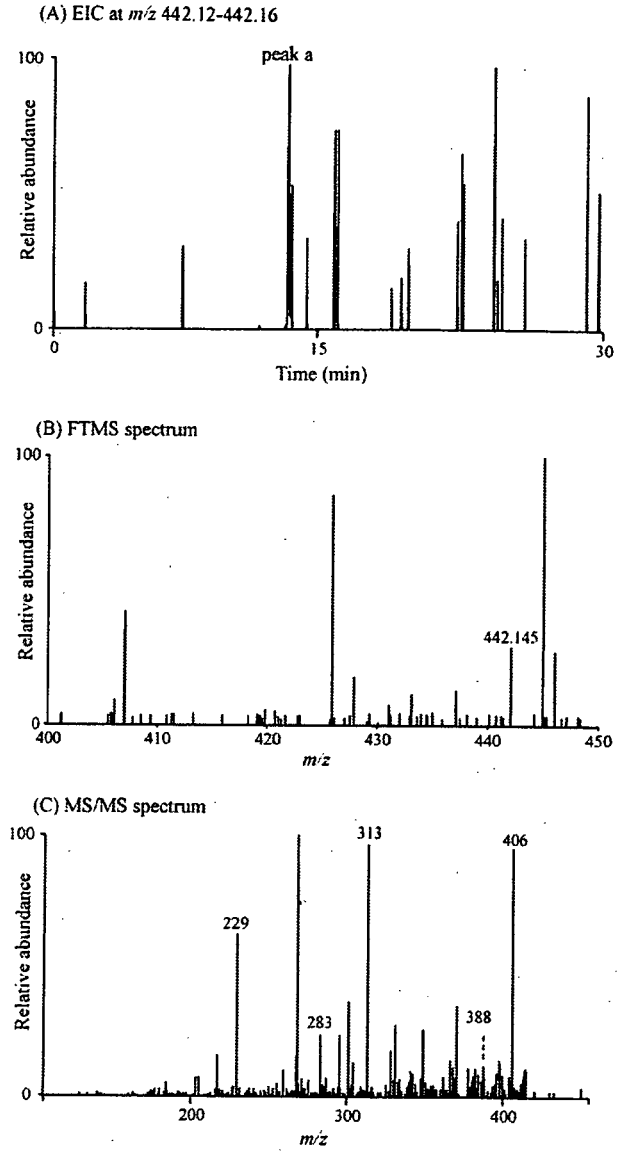

Fig. 4. Detection of DMB-NeuGc in the membrane fractions of HL-60RG cells (2.5×10^3) cultured with 10% FCS. (A) EIC at *m/z* 442.12–442.16 obtained by SIM. (B) Typical MS spectrum of peak a. (C) MS/MS spectrum of $[M+H]^+$ (*m/z* 442.145) acquired from around peak a.

3. Results and discussion

3.1. Analysis of NeuGc and NeuAc by nanoLC/FTMS

It was reported that DMB-NeuGc yielded its dehydrated ion (*m/z* 424) together with molecular ion (*m/z* 442) by MS in the positive ion mode [18,21]. To control the dehydration of molecular ion in the ion trap device, AGC value, which regulates the amount of ions trapped into ion trap device, was set to 5×10^4 (default value, 5×10^5). This value was also useful for the detection of molecular ion of DMB-NeuAc.

Using the AGC value at 5×10^4, SIM (*m/z* 400–450) was carried out in the positive ion mode. When a mix-