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Recombinant human laminin isoforms can support the undifferentiated growth of human embryonic stem cells

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ABSTRACT

Human embryonic stem cells (hESCs) are thought to be a promising cell source for cell transplantation therapy. For such a clinical application, the hESCs should be manipulated using appropriate and qualified materials. In this study, we examined the efficacy of recombinant human laminin (rhLM) isoforms on the undifferentiated growth of hESCs. We first determined the major integrins expressed on the hESCs to reveal the preference of the hESCs for rhLMs, and found that the hESCs mainly expressed integrin α6β1, which binds predominantly to laminin-111, -332 and -511/-521. When the hESCs were seeded onto rhLMs, the cells indeed adhered markedly to rhLM-332, and to rhLM-511 and rhLM-111 to a lesser extent. The hESCs proliferated on these three rhLMs for several passages while preserving their pluripotency. These results show that rhLM-111, -332, and -511 are good substrates to expand undifferentiated hESCs due to their high affinity to integrin α6β1 expressed on hESCs.

Human embryonic stem cells (hESCs) are pluripotent cells derived from the inner cell mass (ICM) of blastocysts [1]. Because hESCs have a potential to differentiate into cells and tissues of all three embryonic germ layers, they are expected to be a promising cell source for the clinical application of cell transplantation therapy. Although hESCs should be cultured under defined and preferably animal-free conditions for such purposes, hESCs are usually maintained by culturing on mouse embryonic fibroblast (MEF) feeders. Alternatively, hESCs can be maintained on a thin layer of Matrigel™, produced from mouse EHS tumor, in combination with conditioned medium of MEF (CM-MEF) [2]. Several animal-free and/or defined media for the feeder-free culture of hESCs, which can avoid the use of MEF or CM-MEF, have been reported [3]. However, most of these media still require using Matrigel as a basal scaffold on the surface of the culture vessels. Animal derivatives should be replaced, not only from the medium components, but from the supportive substrate, as well. However, animal-free substrates suitable for hESC culture need to be developed. As alternative methods to exclude animal-derived materials from the culture substrate, it has been shown that the use of human fibroblasts derived from hESCs [4] and bone marrow [5] as a feeder layer is effective to maintain the hESCs. However, the use of human fibroblasts may suffer from either the need for laborious quality control of the individual lots or the risk of contamination by human pathogens such as hepatitis virus or human immunodeficiency virus. Therefore, it is still necessary to seek defined substrates that have consistent quality and are free from potential contamination by pathogens.

In this study, we examined recombinant human laminin (rhLM) isoforms as candidates for the scaffold to culture hESCs. Although the laminins derived from mouse EHS tumor [2,6] and human placenta [7,8] could be used as a substrate for the cultivation of hESCs equally well as Matrigel, these laminins are restricted for use because of their animal derivation or limited production. In contrast, the rhLMs have the advantage of being an abundantly available

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and well-characterized human-origin protein produced by an *in vitro* system. Laminin, which is a major component of Matrigel [9], plays a key role in cellular adhesion and consists of three distinct subunits from α1 to 5, β1 to 3, and γ1 to 3 [10]. At least 15 laminin isoforms have been identified to date [10], and the expression of these isoforms is spatiotemporally regulated during embryonic development [11], thereby exerting their distinctive functions in different cell types through interactions with different types of cell surface receptor integrins. Integrin is a heterodimer consisting of two distinct chains from 18α and 8β in human, and 24 unique integrin isoforms including four laminin-binding types (α3β1, α6β1, α6β4, and α7β1) are generated [12]. Because the combination with laminins and integrins is robustly restricted, it is an efficient way to determine the integrins expressed on hESCs for the prediction of the laminin isoforms to which they adhere. Therefore, we first analyzed the type of integrins expressed in hESCs to gain an insight into the potential use of rhLMs in the cultivation of hESCs. We then assessed the abilities of a panel of rhLMs to support the cultivation of hESCs. We herein present our finding that a subset of laminin isoforms recognized by the integrins expressed on hESCs can be used as a substrate for the expansion of undifferentiated hESCs.

Materials and methods

Quantitative polymerase chain reaction (qPCR). Total RNAs were isolated using an RNeasy Mini Kit (QIAGEN) and reverse-transcribed using an Omniscript RT Kit (QIAGEN) according to the manufacturer's instructions. The qPCR was performed with the SYBR Green PCR Master Mix (Applied Biosystems) using the ABI 7000 real-time PCR system (Applied Biosystems). The primer sets for the individual laminin and integrin subunits used in this experiment are summarized in the Supplemental Tables. Some primer sequences were obtained from the on-line public database, PrimerBank [13]. The amplification levels of the individual genes were normalized to those of the standard cDNAs encoding individual laminin or integrin subunits and expressed as copy numbers per 5 ng of RNA used for reverse transcription. All experiments were performed in triplicate and the results are expressed as means ± SD.

Preparation of culture vessels coated with recombinant laminins. Five recombinant human laminin isoforms, rhLM-111, -211, -332, -411, and -511 were produced using the Free Style™ 293 Expression System (Invitrogen) and purified from conditioned medium as described previously [14,15]. The rhLMs were filtered through the 0.45-μm pores of Ultrafree-CL Centrifugal Filter Units (MILLIPORE) for sterile usage. The rhLMs were used to coat the culture vessels after dilution by PBS to 1 μg/cm^2 and were incubated for 3 h at 37 °C. As a control, culture vessels were also coated with Matrigel (1:40 dilution; 20 μg/cm^2) (BD Biosciences) diluted in DMEM/F12 for 3 h at 37 °C.

Cell culture. The hESC lines, KhES-1, KhES-2, and KhES-3, were normally maintained as described previously [16]. The cultivation of the hESCs on the laminin-coated vessels was performed using CM-MEF. CM-MEF was prepared as described by Xu et al. [2] with the addition of 5 ng/ml of FGF-2. The cells were detached every 3–5 days with 10 mg/ml collagenase IV (GIBCO) in DMEM/F12 (SIGMA) combined with dispersion by a cell scraper, and were seeded onto fresh vessels after a threefold dilution.

Growth rate analysis. Two hESC lines, KhES-1, and KhES-3 (2×10^5 cells), were seeded onto 12-well plates coated with different laminin isoforms. They were cultivated in CM-MEF and dissociated with 0.05% trypsin/1 mM EDTA every 24 h for cell counting. The cell counts were determined as the average of four wells in three independent assays. The statistical analysis was done by Student's *t*-test.

Alkaline phosphatase The cells were fixed with 4% formaldehyde in PBS for 15 min and treated with the Vector Blue Alkaline Phosphatase Substrate Kit III (VECTOR LABORATORIES) dissolved in 100 mM Tris–HCl buffer (pH 8.3) for 45 min. The staining and cell morphology were recorded using a phase contrast microscope.

Karyotype analysis. The karyotypes were analyzed by the G-banding staining method. The hESCs were treated with 100 ng/ml colcemid (GIBCO). After treatment with the hypotonic solution, the cells were spread onto glass slides and stained with Giemsa's solution. At least 50 chromosome spreads were analyzed using the Ikaros Karyotyping System (META system).

Flow cytometry. The hESCs were dissociated with 0.05% trypsin/1 mM EDTA and fixed with 1% formaldehyde in PBS. They were incubated for 30 min at 4 °C with the antibodies against stage-specific embryonic antigen (SSEA)-4 (CHEMICON), tumor rejection antigen (TRA)-1-60 (CHEMICON), and TRA-1-81 (CHEMICON), followed by incubation with Alexa344-conjugated secondary antibody (Santa Cruz). For the flow cytometric analysis using anti-Oct-4 antibody, the cells were permeabilized with SPB buffer (1 mg/ml Saponin and 1% BSA in PBS) and treated with the antibody in SPB buffer. The flow cytometric analysis was performed using the FACSCalibur HG™ flow cytometer and Cell Quest Pro software program (BD Biosciences).

Differentiation assay. The hESCs cultured on rhLMs were seeded onto Petri dish hESC culture medium without FGF-2. The cells were cultured in suspension for 10 days to form embryoid bodies, followed by extraction of RNA for determination of the expression levels of differentiation markers by semi-quantitative PCR. The primer sequences used were described by Tsuneyoshi et al. [17].

Immunofluorescence staining. The monoclonal antibodies against human laminin β2 (Abcam) and γ1 (Millpore) were purchased. The other monoclonal antibodies against the human laminin subunits were generated as described elsewhere [18,19]. The hESCs cultivated on a MEF feeder on multi-well culture slides (FALCON) were fixed with acetone for 10 min at −20 °C. The cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min and treated with blocking buffer (2% BSA in PBS) for 15 min at room temperature. They were incubated with the primary antibody overnight at 4 °C, and then incubated with the secondary antibody for 30 min at room temperature. Finally, they were embedded in Perma Fluor Aqueous Mounting Medium (Japan Turner) and photographed using the LSM5 PASCAL confocal laser scanning microscope (Zeiss).

Results

Determination of integrin isoforms expressed on hESCs

To examine the preference of the laminin isoforms as a substratum for hESCs, we analyzed the expression of the integrin subunits in the hESCs by qPCR, mainly focusing on the laminin-binding types. In addition to all α subunits which constitute the laminin-binding type (α3, α6, and α7), the α subunits for the collagen-binding type (α1 and α2) and the RGD-binding type (α4, α5, α8, and αV) were examined. As shown in Fig. 1, the α6 chain was expressed most abundantly in all three hESCs. The α3 and α7 chains were also expressed in three hESCs, particularly at relatively high levels in KhES-2 cells. Between the two β chains of the laminin-binding type, the β1 but not β4 chain was detected. Together with these results, laminin-binding integrin α6β1 was the major type of integrin expressed in hESCs. Integrin α6β1 has been shown to bind preferentially to laminin-111, -332, and -511 [20], leading to the idea that hESCs adhere to the substrate via interaction with at least one of these laminin isoforms.

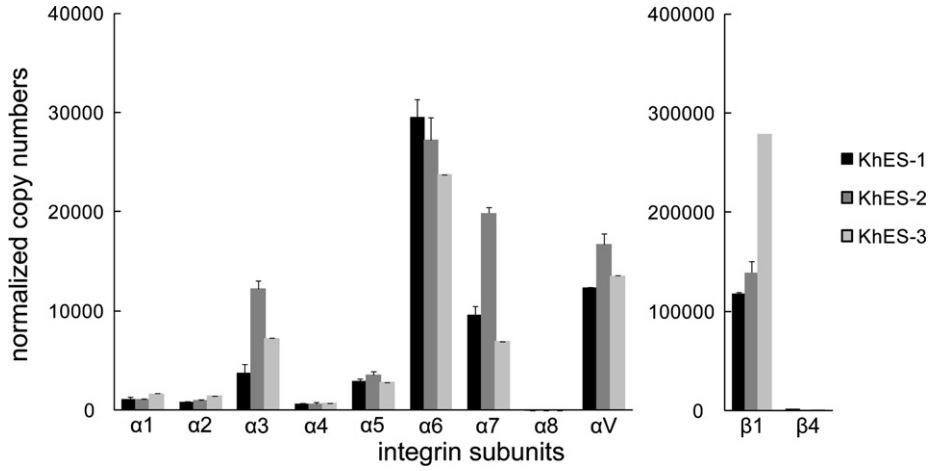

Fig. 1. Determination of integrin isoforms expressed on hESCs. Total RNA of three hESCs on MEF feeder was used for the qPCR analysis. The copy numbers were normalized to qPCR reactions using plasmids which have cDNA of each integrin subunit as templates.

Cultivation of hESCs on rhLMs

We next evaluated the initial plating efficiency and the growth rates of hESCs on various rhLM isoforms. Because integrin α6β1 was predominantly expressed in the hESCs, rhLM-111, -332, and -511 were examined. rhLM-211 and -411 were also examined to include laminin isoforms that have affinity to other laminin-binding integrin isoforms α3β1 and α7β1, which are expressed in hESCs less abundantly than α6β1. When KhES-1 cells were seeded onto rhLMs with CM-MEF, the cells adhered well on rhLM-332 (90% plating efficiency relative to that on Matrigel). KhES-1 cells also adhered on rhLM-511 (30%) and rhLM-111 (20%), while they barely adhered on rhLM-211 and rhLM-411. The cells on rhLM-332, -511, and -111 exhibited logarithmic growth in CM-MEF, while the cells on rhLM-211 and rhLM-411 did not show any significant growth (Fig. 2A). When cells of another hESC line, KhES-3, were seeded on rhLMs, the cells adhered well on rhLM-332 (62%), rhLM-511 (54%), and rhLM-111 (18%) (Fig. 2B) and showed growth equal to that on Matrigel. Unlike the KhES-1 cells, the KhES-3 cells adhered to rhLM-211 (7.3%) and rhLM-411 (4.7%), and they showed slower growth on these rhLMs. Although the KhES-1 and KhES-3 cells showed slight difference in their competency to adhere and proliferate on different rhLMs, rhLM-332 was found to be the most efficient substrate for the *in vitro* cultivation of hESCs. rhLM-511 and rhLM-111 were also capable of supporting hESC cultivation with less potency in comparison to rhLM-332.

hESCs cultured on rhLM isoforms maintains an undifferentiated state

To investigate whether hESCs retain an undifferentiated state during maintenance on the rhLM isoforms, we cultured hESCs on rhLM-111, -332, and -511 with CM-MEF for 10 passages and estimated the expression of undifferentiated markers. After 10 passages, the hESCs were grown on rhLM-111, rhLM-332, or rhLM-511 as compactly aggregated colonies which were similar to the undifferentiated colonies on Matrigel (Fig. 3A, upper panel). These colonies on three rhLMs positively stained for alkaline phosphatase activity, thus suggesting further support to their undifferentiated state (Fig. 3A, lower panel). To further confirm the undifferentiated state, we analyzed the expression of the undifferentiated markers SSEA-4, Oct-4, TRA-1-60, and TRA-1-81 by flow cytometry. The hESCs cultured on each of the rhLMs were positive for these markers (Fig. 3B). We also analyzed the expression levels of a panel of marker genes for both undifferentiated and differentiated states by RT-PCR. The expressions of the undifferentiated markers, Nanog, SOX2 and Oct4, were detected in the hESCs grown on rhLMs, while the differentiation markers including GATA6, Brachury, PAX6 and CG-α were barely detectable (Supplemental Fig. 1).

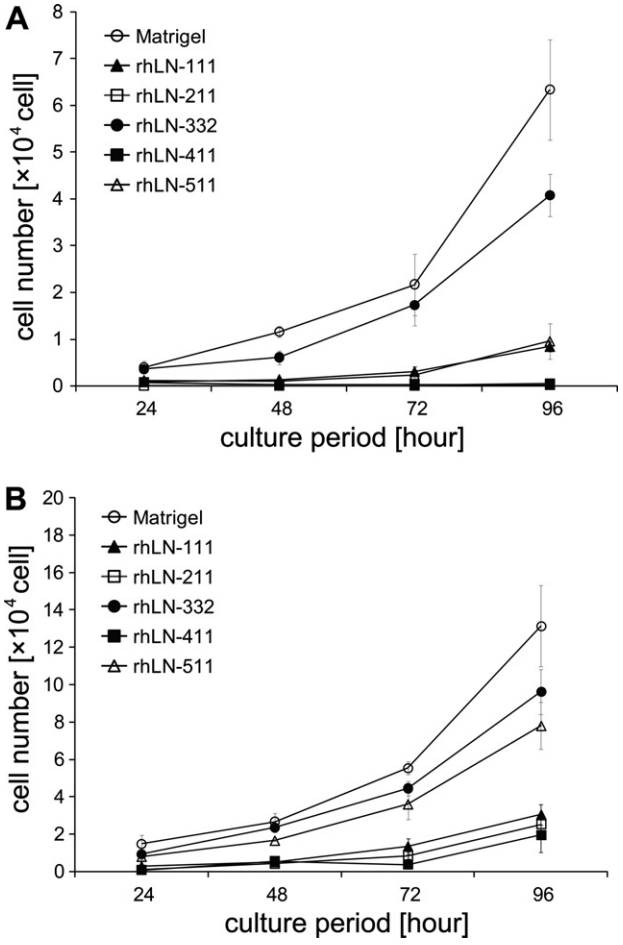

Fig. 2. Growth rate of hESCs on rhLM isoforms. Cells (2×10^5) of hESC lines, KhES-1 and KhES-3, were seeded in a multi-well plate coated with rhLM isoforms and cultured with CM-MEF. The cell number was counted every 24 h. Bars, standard deviation of $n = 4$ experiments. (A) The KhES-1 cell line and (B) the KhES-3 cell line. The plating efficiencies on Matrigel after 24 h were 2.0% (KhES-1) and 15% (KhES-3).

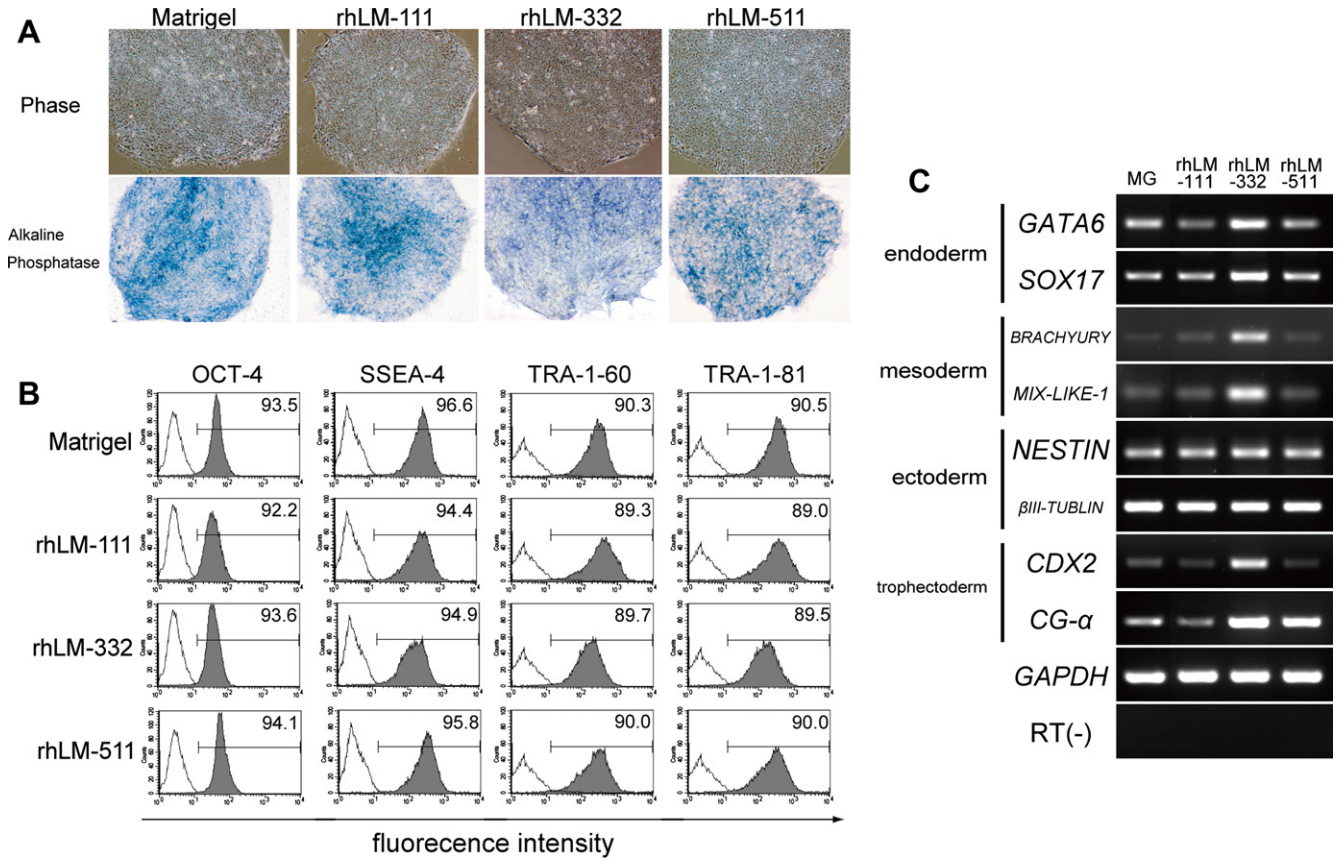

Fig. 3. Characterization of the undifferentiated state of hESCs cultured on rhLM isoforms for 10 passages. (A) Morphology of an hESC colony. Upper panels; phase contrast image. Lower panels; cells stained for alkaline phosphatase activity. (B) A flow cytometric analysis with the antibodies of undifferentiated markers. White area; negative control. Gray area; positive cells. The number in the panel shows the percentage of the positive cell population. (C) An RT-PCR analysis of the differentiation marker genes. Total RNA was extracted from day-10 embryoid bodies produced from cells after 10 passages on rhLM isoforms and used for the analysis. MG, Matrigel.

To evaluate the differentiation potency of the hESCs maintained on rhLMs, we induced differentiation of the hESCs by embryoid bodies (EBs) formation after a subculture on rhLMs and analyzed the expression of the differentiation markers for three germ layer cells by RT-PCR. The hESCs, after the subculture on rhLMs, formed EBs with indistinguishable morphology to EBs from cells cultured on Matrigel (data not shown). The RT-PCR analyses for EBs at 10 days showed the expression of GATA6, SOX17, NESTIN and βIII-tubulin at a similar level among the rhLMs. The expression of Brachury, MIX-LIKE-1, CDX2, and CG-α were also detected in all three EBs (Fig. 3C). These results indicated that the hESCs cultured on rhLMs retained pluripotency. A karyotype analysis of the hESCs at the 10th passage showed that they retained the normal karyotype on rhLMs (Supplemental Fig. 2). Together with these data, it was determined that hLM-111, -332, and -511 can stably maintain hESCs in the undifferentiated state.

Expression of laminin isoforms in hESCs

Finally, we examined the expression of laminin subunits in hESCs by immunostaining. The hESCs grown on the MEF feeder were stained with antibodies directed to individual laminin subunits, and immunofluorescence signals were detected only with antibodies against α5, β1, β2, and γ1 chains around the cell peripheries (Fig. 4A). These results were further confirmed by qPCR. Among five α subunit chains, α5 was most abundantly expressed in all three hESC lines, while α1 was expressed at levels 10-fold less than those of α5. The other α chains were hardly detected. Among three β subunits, β1 and β2 were expressed in all hESCs examined at almost equal levels, although β3 was not detectable except in the KhES-2 cells that expressed a low level of the β3 transcripts. Among three γ subunits, γ1 was found to be the major γ chain expressed in all three hESCs (Fig. 4B). These results indicate that hESCs predominantly produce laminin-511/-521, which have a high affinity to Integrin α6β1.

Discussion

Laminins are major adhesive proteins in the basement membrane and play a key role in a wide range of cellular adhesion, which is specified by the combination of the type of integrins expressed on the cell. We showed that three hESC lines commonly expressed abundant integrin α6β1. Integrin α6β1 is known to be a laminin-binding type which binds to laminin-111, -332, and -511/-521. Therefore, those laminin isoforms corresponding to integrin α6β1 were thought to be good candidates for the culture substrate of hESCs. Indeed, when hESCs were seeded on the repertory of laminin isoforms, they adhered and proliferated well on rhLM-332, and also rhLM-511, -111. Since the plating efficiency of the hESCs against rhLMs increased according to the ligand-binding specificities of the integrins to laminins, these findings may indicate that integrin is a key factor for adhesion of hESCs onto substrates, and the rhLM-332, rhLM-511, and rhLM-111 are good adhesive materials due to the high expression of integrin α6β1 on hESCs. Xu et al. also reported that the H1 cell expressed abundant integrin α6β1 [2]; these rhLMs are, therefore, assumed to function as substrates to support the undifferentiated growth of this hESC line. The hESCs also expressed the RGD-binding type

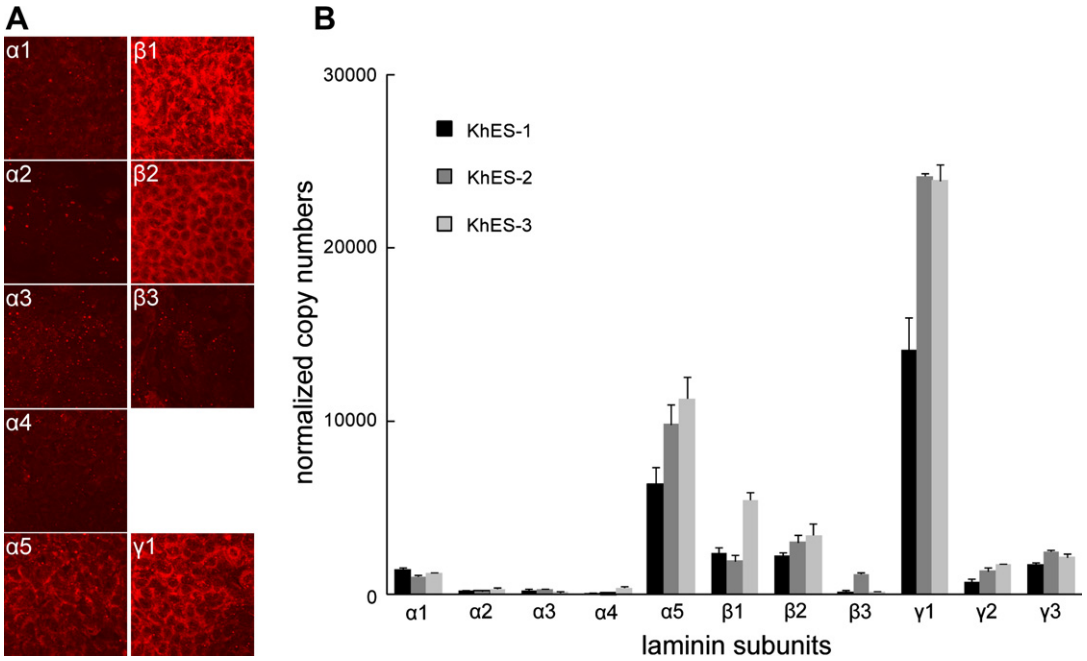

Fig. 4. The laminin isoforms produced by hESCs. (A) Immunostaining for the human laminin subunits. The photos were taken at the same exposure time. (B) A qPCR analysis of the laminin subunits expressed on hESCs. Total RNA of three hESC lines on MEF feeder was used for the analysis. The copy numbers were normalized by using plasmids which have cDNA of each laminin subunit as templates.

integrin αVβ1 and its ligand such as fibronectin can be an alternative substrate for hESC culture. However, hESCs are subject to lose the undifferentiated state during long period culture on fibronectin as compared with laminin [2]. Therefore, the ligands for integrin α6β1 abundantly expressed on hESCs are considered as the most useful substrates.

The hESCs abundantly produced laminin-511/-521, while laminin-111/121 were barely expressed. It has been reported that the basement membrane underlying the ICM contains laminin-511 as the major laminin isoform, while a small amount of laminin-111 is also present in the embryonic basement membrane [21]. It is also reported that laminin-511/-521 and laminin-111 are the only laminins expressed in the early mouse embryos [10,21]. Therefore, it is supposed that the higher affinity of hESCs to these laminins can be attributed to the fact that hESCs are derived from ICM.

Although laminin-332 was not expressed in the hESCs, the hESCs adhered and proliferated well on rhLM-332, preserving their pluripotency for several passages. These findings suggest that hESCs can be cultured on any laminin isoform corresponding to integrin α6β1 due to its high avidity, regardless of being a laminin isoform which the hESCs produce or not. Similarly, rhLM-521, which was not tested in this report, would also function as well as rhLM-511 because it possesses the same ligand-binding specificity [20]. The adhesion to the matrix is crucially important for anchorage-dependent cells like hESCs to grow. However, proliferation of hESCs is not supported simply by the adhesion to the surrounding matrix but by various factors in culture medium such as FGF-2. The interaction between laminin and integrin might be necessary for prevention of aggregation of hESCs to form an EB-like structure, since hESCs undergo spontaneous differentiation in an EB-like structure under poorly adhesive conditions.

In summary, we herein showed that hESCs express integrin α6β1 and can be maintained on rhLM-111, -332, and -511 in the undifferentiated state. Because the potential of hESCs for medical application can be fully explored only when appropriate qualified materials that enable the safe and reproducible cultivation of them become available, the rhLMs appear to be promising components of the chemically defined substrate that facilitates the sustained cultivation of hESCs in regenerative medicine and tissue engineering.

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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.bbrc.2008.07.111.

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