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Laminin isoforms in human embryonic stem cells: synthesis, receptor usage and growth support

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Abstract

To reveal the functional intrinsic niche of human embryonic stem cells (hESC) we examined the production of basement membrane (BM) proteins and the presence of their receptors in feeder-free cell culture conditions. In addition, we investigated binding of hESCs to purified human BM proteins and identified the receptors mediating these contacts. Also, we tested whether purified human laminin (Lm) isoforms have a role in hESC self-renewal and growth in short-term cultures. The results show that hESCs synthesize Lm α_1 and Lm α_5 chains together with Lm β_1 and γ_1 chains suggesting the production of Lms-111 and -511 into the culture medium and deposits on cells. hESCs contain functionally important integrin (Int) subunits, Int β_1 , α_3 , α_6 , α_5 , β_5 and α_v , as well as the Lm α_5 receptor, Lutheran (Lu) glycoprotein and its truncated form, basal cell adhesion molecule (B-CAM). In cell adhesion experiments, Int β_1 was crucial for adhesion to most of the purified human BM proteins. Lu/B-CAM mediated adhesion to Lm-511 together with Int $\alpha_3\beta_1$, and was essential for the adhesion of hESCs to embryonic feeder cells. Adhesion to Lm-411 was mediated by Int $\alpha_6\beta_1$. Lm-511 supported hESC growth in defined medium equally well as Matrigel. These results provide consequential information of the biological role of BM in hESCs, warranting further investigation of BM biology of human pluripotent stem cells.

Keywords: human embryonic stem cell • extracellular matrix • basement membrane • laminin • integrin • B-CAM/Lutheran • adhesion • defined cultures

Introduction

Basement membranes (BMs) are thin sheets of specialized extracellular matrix (ECM), underlying all epithelia, and surrounding muscle, endothelial, fat and Schwann cells as well as the whole central nervous system. The function of BMs is to provide structural support, divide tissues into compartments and also to regulate cellular functions. The major structural components of BMs

are sheets of laminins (Lm) and type IV collagens, which are bridged by nidogen into a functional BM structure. Out of the various BM components, Lms have been shown to be the most important regulators of cellular functions [1–3]. Lms are trimeric glycoproteins composed of α , β and γ chain [3]. Among 15 recognized Lms, embryonic Lm-111 ($\alpha_1\beta_1\gamma_1$) and Lm-511 ($\alpha_5\beta_1\gamma_1$) are crucial during early mouse embryogenesis for the survival and development of embryonic epithelial cells [4]. Null mutations in Lm γ_1 or Lm β_1 chains are lethal at E5.5 due to non-functional embryonic BM and Reichert's membrane [5, 6]. Also, mouse embryos lacking Lm α_1 die at E7.5 due to the absence of functional Reichert's membrane [7].

Integrins are heterodimeric cell membrane receptors, consisting of non-covalently linked α and β subunits. They mediate

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cell–cell and cell–ECM interactions by adhering cells to BM proteins. Mouse embryos constitutively express $\text{Int } \alpha_V\beta_3$, $\alpha_6\beta_1$ and $\alpha_5\beta_1$ at two-cell stage, and integrins $\alpha_3\beta_1$, $\alpha_7\beta_1$, $\alpha_6\beta_1$, $\alpha_2\beta_1$ and $\alpha_1\beta_1$ in a regulated manner during further development [4, 8]. Human embryos express $\text{Int } \alpha_V$ subunit throughout the early embryonic development, at least until compaction [9]. Embryonic epithelial cells have been shown to express the dystroglycan glycoprotein complex, which can act as a receptor for Lm-111 [4, 10, 11]. Besides integrins and dystroglycan complex, Lutheran glycoprotein (Lu/B-CAM) acts as a receptor for α_5 -chain containing Lms [12, 13].

Feeder cell-free culturing of human embryonic stem cells (hESC) typically requires an ECM coating, such as Matrigel, and supplementation with basic fibroblast growth factor (bFGF) [14, 15]. A recent study indicated that bFGF might have an indirect effect on hESC cultures *via* hESC-derived fibroblast-like cells [16]. Beside other functions, FGFs modify the ECM production of cells [17]. Stem cells are located in a specific niche composed of soluble factors and the physical interactions between stem cells and the surrounding BM proteins [18, 19]. Because of the origin of hESCs, their *in vivo* niche is difficult to explore and there is little information available on the synthesis of BM proteins by hESCs. In order to better understand the natural ECM of hESCs, we have studied the Lm synthesis by hESCs and the interactions between hESCs and purified human BM proteins.

Materials and methods

hESC culture

The studies presented here were performed on Finnish hESC lines FES 29 and FES 30 [20]. The cells were regularly analysed for the expression of pluripotency markers (tumour recognition antigen [Tra] 1–60 and stage-specific embryonic antigen [SSEA]3) by flow cytometry and found to be positive (85% to 97%). The hESCs were cultured on mouse embryonic fibroblasts (mEF, isolated from day 12.5 ICR fetuses) in KnockOut Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA, USA), supplemented with 20% KnockOut serum replacement, 0.1 mM 2-mercaptoethanol, 1× penicillin-streptomycin-L-glutamine, 1× nonessential amino acids (all from Invitrogen), 1× insulin transferrin selenium (ITS) liquid media supplement (Sigma, St. Louis, MO, USA) and 6 ng/ml bFGF (Sigma). hESCs were passaged by trypsin-like enzyme (TrypLE Select, Invitrogen). Before carrying out the experiments presented here the hESCs were adapted to feeder-free conditions using mEF-conditioned medium supplemented with 12 ng/ml bFGF and Matrigel (BD Biosciences, Bedford, UK) coating as described previously [14]. The feeder-free cultures were passaged every 5 days by collagenase IV (4–5 min., 20 U/ml [Invitrogen]). Lms-111 [21] and -511 [22] were purified by immunoaffinity chromatography from the culture supernatant of Jar cells. Vitronectin was purified as described [23]. Defined cultures were performed using purified human Lm-111 (8 $\mu\text{g/ml}$), Lm-511 (3.5 $\mu\text{g/ml}$) or vitronectin (4 $\mu\text{g/ml}$) as matrix and StemPro (Invitrogen) as culture medium using mechanical passaging.

Metabolic labelling and immunoprecipitation of culture medium, matrix and cells

hESCs were cultured in Roswell Park Memorial Institute medium (RPMI) (Sigma) without methionine, serum or serum replacement and supplemented with ITS, non-essential amino acids, L-Glutamine and bFGF (see above) for 30 min. After methionine starvation, 100 μCi of ^{35}S -labelled methionine (Perkin-Elmer, Waltham, MA, USA) was added to the medium and incubated overnight. To identify Lms secreted to the culture medium the medium was first collected and centrifuged. Then the medium was supplemented with normal mouse serum and with 0.5% Triton X-100. The medium was pre-absorbed with GammaBindPlus Sepharose (GBPS) beads (GE Healthcare-Biosciences, Uppsala, Sweden), followed by centrifugation and adsorption with GBPS beads pre-coupled with monoclonal antibodies (MAb) against human Lm α_1 , α_2 , α_3 , α_4 and α_5 (Table 1).

Labelled cells were lysed on ice in radioimmunoprecipitation assay (RIPA) buffer containing 10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate, 5 mM EDTA and protease inhibitor mixture. The cell sample was cleared by centrifugation. For ECM isolation, the cells were extracted with 0.5% deoxycholate in 10 mM Tris-HCl, 150 mM NaCl, 1 mM protease inhibitor, pH 8.0. The remaining material was solubilized in RIPA buffer. The cell samples were supplemented with normal mouse or rat serum, and ECM specimens with normal mouse serum. The samples from cells were absorbed to GBPS beads pre-coupled with MAb against $\text{Int } \alpha_3$, α_5 , α_6 and β_1 subunits (Table 1) and ECM specimens with MAbs to Lm chains as aforementioned.

After overnight immunoprecipitation, bound proteins from cell and matrix samples were first washed three times in RIPA buffer and bound proteins from medium were washed in phosphate buffered saline (PBS) with Triton X-100. Then the proteins were eluted to Laemmli's sample buffer and analysed by SDS-PAGE. After immunoprecipitation the samples from hESC culture medium were exposed to Chondroitinase ABC treatment (0.02U chondroitinase ABC [Sigma] in 50 mM Tris-HCl, pH 8.0, 50 mM sodium acetate) at +37°C for 8 hrs and thereafter treated as other samples. The following cell lines were used as positive controls: Jar [21] for Lm-111 and Lm-511, HPAE [24] or Lm-411, and UT-SCC-43A [25] for Lm-332. The antibody was omitted in negative controls.

Flow cytometry

hESCs were collected by trypsin-EDTA (Invitrogen) treatment (4–5 min.), after which the cells were washed, counted and suspended in FACS staining buffer (5% foetal calf serum [Promocell, Heidelberg, Germany] in PBS). The cells were suspended in 50 μl of primary MAb solution (Table 1) and incubated on ice for 45 min. After three washes, the hESCs were incubated for 30 min. on ice with 2 μl of conjugated secondary antibodies, in the dark, washed and fixed with 0.1% paraformaldehyde in FACS staining buffer. Secondary conjugates were phycoerythrin-coupled anti-rat IgM for SSEA-3 (BD Biosciences, San Jose, CA, USA), fluorescein-isothiocyanate (FITC)-coupled antimouse IgG or FITC-coupled anti-rat IgG (Invitrogen). The primary antibody was omitted in negative controls. Samples were run by FACScalibur (BD Biosciences) using CellQuestPro software (BD Biosciences) and analysed by Flow Jo (Tree Star Inc., Ashland, OR, USA) software.

Immunocytochemistry

For indirect immunofluorescence the cells were cultured on glass cover slips and fixed in methanol at –20°C. After washing in PBS the cells were

Table 1 Antibodies used in the study

MAb clone	Specificity	Reference	Methods
161EB7	Lm α_1 chain	Virtanen <i>et al.</i> [21]	IP
5H2	Lm α_2 chain	Leivo and Engvall [48]	ICC
BM-2	Lm α_3 chain	Rousselle <i>et al.</i> [49]	IP
3H2	Lm α_4 chain	Wondimu <i>et al.</i> [50]	IP
4C7	Lm α_5 chain	Engvall <i>et al.</i> [51]	IP
114DG10	Lm β_1 chain	Virtanen <i>et al.</i> [52]	IP
S5F11	Lm β_2 chain	Wewer <i>et al.</i> [53]	ICC
113BC7	Lm γ_1 chain	Määttä <i>et al.</i> [54]	IP
TS2/7	Int α_1 subunit	Hemler <i>et al.</i> [55]	ICC, FC
CLB-10G11	Int α_2 subunit	Giltay <i>et al.</i> [56]	ICC, FC
J143	Int α_3 subunit	Fradet <i>et al.</i> [57]	ICC, IP, FC
PIB5	Int α_3 subunit	Wayner and Carter [58]	FB
PUJ14	Int α_4 subunit	Hemler <i>et al.</i> [59]	ICC, FC
B1E5	Int α_5 subunit	Werb <i>et al.</i> [60]	ICC, IP, FC
GoH3	Int α_6 subunit	Chemicon, Temecula, CA	ICC, IP, FC, FB
LM 142.69	Int α_v subunit	Cheresh and Spiro [61]	ICC, FC
13	Int β_1 subunit	Yamada <i>et al.</i> [62]	FB
102DF5	Int β_1 subunit	Yläne and Virtanen [63]	ICC, IP, FC
90B10	Int β_2 subunit	Yläne <i>et al.</i> [64]	ICC
AA3	Int β_4 subunit	Tamura <i>et al.</i> [65]	ICC
B5-1A9	Int β_5 subunit	Pasqualini <i>et al.</i> [66]	ICC
BRIC221	Lutheran	Serotec, Oxford, England	ICC
Tra 1-60	Tra 1-60	Chemicon, Temecula, CA	ICC, FC
MC631	SSEA-3	Solter and Knowles [67] Peter Andrews, Sheffield, UK	FC

IP; immunoprecipitation, ICC; immunocytochemistry, FC; flow cytometry, FB; function blocking.

incubated with primary antibodies listed in Table 1. FITC-coupled anti-mouse IgG (Fc-fragment specific [Jackson ImmunoResearch, West Grove, PA, USA]) was used as the secondary antibody, followed by MAb Tra1-60 and finally, after washing, the cells were incubated with goat anti-mouse AlexaFluor 594 IgM (μ chain specific [Invitrogen]). The specimens were embedded in veronal glycerol buffer (1:1) (pH 8.4) and viewed with an AX70 Provis fluorescence microscope (Olympus, Tokyo, Japan). Images were acquired using a computer connected to a cooled digital camera mounted on the microscope.

RT-PCR and quantitative PCR (qPCR) analysis

Total RNA was extracted using NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) without on-column DNase treatment. DNase treatment was done separately as we have noticed that separate DNase treatment is

more complete than on-column treatment. After the DNase treatment, RNA was cleaned by using NucleoSpin RNA Clean-up kit (Macherey-Nagel).

Total RNA was reverse-transcribed into cDNA by M-MLV Reverse Transcriptase in RT reaction containing Oligo(dT)₁₅ primers, the mix of all four dNTPs, and rRNasin (all from Promega, Madison, WI, USA). The cDNA amount was determined as the synthesized cDNA in a 20 μ l RT reaction containing 1 μ g total RNA. PCR was performed in a total volume of 25 μ l containing 2.5 μ l 10 \times PCR buffer with 15 mM MgCl₂, 0.2 μ l AmpliTaqGold 5U/ μ l (Applied Biosystems, Foster City, CA, USA), 2 μ l 2.5 mM dNTPs mix, 1 μ l of 10 μ M mix of forward and reverse primers, 2 μ l 50% dimethylsulfoxide (DMSO) and 2 μ l RT reaction as a cDNA template. Polymerase was activated at 95°C for 7 min., followed by 36 cycles of 95°C, 20 sec.; 56.5°C, 30 sec.; 72°C, 30 sec., and the final extension at 72°C for 7 min. PCR products were analysed in 2.1% agarose gels. Primers for human Lu, B-CAM, and cyclophilin G were described earlier [26]. The PCR product for human Lu (B-CAM variant 1) is 115 bp, for human B-CAM (variant 2) 109 bp and for human cyclophilin G (CG), 126bp.

Table 2 Examined laminin chains and their synthesis in hESCs

Laminin chain	Method	Medium	Matrix
Lm α_1	IP, ICC	+	+
Lm α_2	ICC	–	–
Lm α_3	IP	–	–
Lm α_4	IP, ICC	–	–
Lm α_5	IP, ICC	+	+
Lm β_1	IP, ICC	+	+
Lm β_2	ICC	–	–
Lm γ_1	IP, ICC	+	+

IP; immunoprecipitation, ICC; immunocytochemistry.

For real-time SYBR (a nucleic acid gel stain) Green qPCR, total RNA was reverse transcribed as described above. Each multiplication reaction, run in duplicate, contained 2 μ l 10 \times PCR buffer (Applied Biosystems), 2 μ l MgCl₂ 25 mM stock, 1.6 μ l dNuTPs mix 2.5 mM each (Promega), 1.6 μ l DMSO 50% stock, 5 μ l mix of F/R primers (both 1.4 μ M in mix), 1 μ l RT reaction as a cDNA template, 0.16 μ l AmpliTaq Gold DNA polymerase 5 U/ μ L (Applied Biosystems), 0.8 μ l SYBR Green (1/500 dilution from stock SYBR Green I nucleic acid gel stain, 10,000 \times concentrate in DMSO [Invitrogen]), and DEPC H₂O ad 20 μ l. The reactions for the qPCR were prepared with a Corbett CAS-1200 liquid handling system and the qPCR was performed using Corbett Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia) as follows: enzyme activation step at 95°C for 7 min. following 40 cycles of 95°C, 20 sec.; 56°C, 20 sec.; 72°C, 20 sec., followed by a melting step. Data were analysed according to the comparative Ct method ($\Delta\Delta$ Ct) (Applied Biosystems, User Bulletin #2). Control samples were hESCs cultured on Matrigel in StemPro medium. The value '1' represents the control in each qPCR result curve.

Primers used for qPCR:

Oct4 (F/R): 5'-TTGGGCTCGAGAAGGATGTG-3' / 5'-TCCTCTCGTTGTG-CATAGTCG-3'

(NM_00271; pos. 856–946; 91bp)

Sox2 (F/R): 5'-GCCCTGCAGTACAACCCAT-3' / 5'-TGCCCTGCTGC-GAGTAGGA-3'

(NM_003106; pos. 1037–1121; 85 bp)

Goosecoid (GSC) (F/R): 5'-GAGAACCTCTCCAGGAGAC-3' / 5'-TTCTTAAACCAGACCTCCAC-3' (NM_173849; pos. 673–776; 104 bp)

Brachyury (Bra) (F/R): 5'-CGCATGATCACCAGCCACTG-3' / 5'-TTTAAGAGCTGTGATCTCCTCG-3' (NM_003181; pos. 1031–1114; 84 bp)

Cyclophilin G (CG) (F/R): 5'-CAATGGCCAACAGGGAAG-3' / 5'-CCAAAAACAACATGATGCCCA-3' (NM_004792; pos. 552–645; 94 bp)

Quantitative cell adhesion experiments

Fibronectin (Fn) was purified from outdated human plasma (Finnish Red Cross Blood Service, Helsinki, Finland) by Gelatin Sepharose 4B affinity chromatography (GE Healthcare-Biosciences), according to Engvall and Ruoslahti [27]. Recombinant human Lm-411 was produced in a mammalian expression system [28]. Lms-111 [21] and -511 [22] were purified

by immunoaffinity chromatography from the culture supernatant of Jar cells. Soluble recombinant Lutheran (Sol-Lu) corresponding to the extracellular domain of Lu/B-CAM was produced as described [12]. Briefly, adhesion experiments were performed using a method based on intracellular acid phosphatase assay, as described [29, 30]. Wells of 96-well plates were first coated with distinct ECM proteins (4 μ g/ml) at RT for 1 hr and then with 3% bovine serum albumin at RT for 1 hr. Alternatively, a monolayer of mEFs was plated 1 day before performing the experiments. When indicated, Sol-Lu (10 μ g/ml) was incubated at RT for 1 hr. Trypsin-EDTA (Invitrogen) treated hESCs were washed by trypsin neutralizing solution (Promocell). 2×10^4 cells per pre-coated well were plated with or without function blocking (FB) MAbs (Table 1) in hESC medium without serum replacement. After 2 hrs incubation in standard culture conditions at +37°C the wells were washed three times and the attached hESCs were exposed to phosphatase substrate (104 phosphatase substrate [Sigma], 6 mg/ml in 50 mM sodium acetate buffer with 0.1% Triton X-100, pH 5.0). The reaction was stopped with 1M NaOH and absorbances were measured at 405 nm. Unspecific binding and background were measured by using either 3% bovine serum albumin treated wells or wells containing only mEFs. The experiments were performed in triplicate. Results were statistically analysed with two-tailed Student's t-test.

Live-cell imaging for the determination of cell growth rate

hESCs were passaged mechanically from Matrigel to Lm-111, Lm-511, Vitronectin, Matrigel, or Gelatin- (Sigma) coated 48-wells in StemPro medium and let to adhere in a cell culture incubator for 24 hrs. Then, the culture medium was changed and the culture plate was transferred to a Cell-IQ Cell Culturing Platform (ChipManTechnologies, Tampere, Finland). Selected positions of the wells were imaged every hour for 24 hrs. Then, the images were analysed for colony size and presence of dead cells, using protocols developed by ChipManTechnologies. Results are presented as relative changes in cell surface area in three replicate culture wells.

Results

hESCs synthesize and deposit laminins-111 and -511

First we studied which Lm chains are synthesized by undifferentiated hESCs. By using metabolic labelling and MAbs specific for human Lm chains, we found that hESCs produce a limited set of Lm subunits, regardless of the culture conditions. Of the analysed Lm α chains (α_1 – α_5), only Lm α_1 and α_5 could be precipitated from the culture medium (Fig. 1a and b; Table 2). Also, Lm β_1 and γ_1 chains were precipitated from the medium. To ascertain the functionality of the Lm chains, we also isolated the ECM produced by hESCs. These experiments showed that the hESCs deposited Lm α_1 , α_5 , β_1 and γ_1 chains into the matrix (Fig. 1a and b; Table 2). By contrast, neither Lm α_3 nor Lm α_4 chains were detected in the hESC culture medium supernatant (Fig. 1c and d; Table 2) or in the hESC-ECM material (not shown).

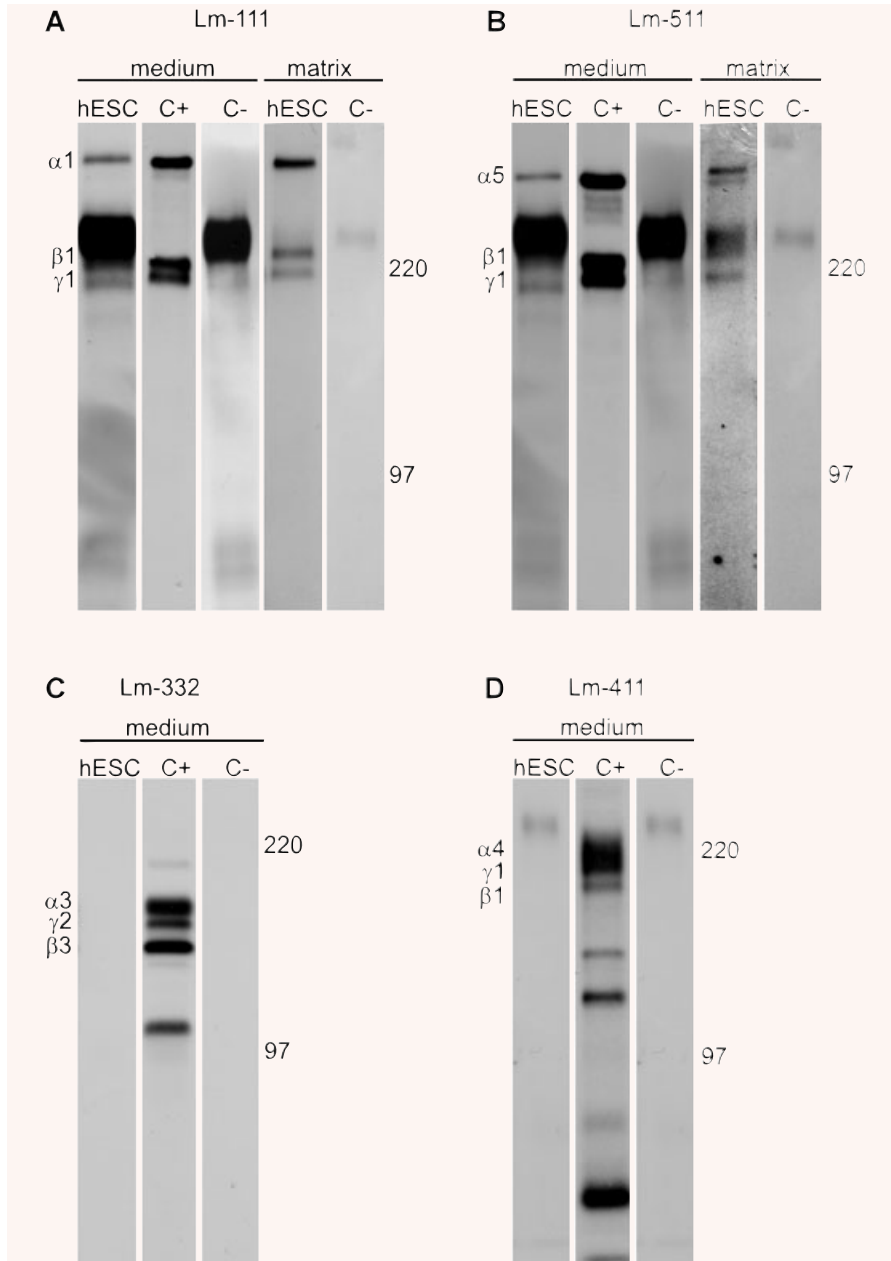


Fig. 1 Production of laminin (Lm) chains by hESCs. hESCs were metabolically labelled with radioactive methionine followed by the immunoprecipitation of Lm chains. (a) and (b) hESCs synthesized and secreted chains of Lm-111 ($\alpha_1\beta_1\gamma_1$) and Lm-511 ($\alpha_5\beta_1\gamma_1$) into culture medium and matrix. (c) and (d) hESCs did not produce Lm-332 or Lm-411. C+; positive control, C-; negative control without antibody.

Undifferentiated hESCs contain integrin receptors for laminins

Flow cytometry, immunocytochemistry and immunoprecipitation analysis were carried out to find out which integrin subunits were present on hESCs (Fig. 2a and b; Table 3). Immunocytochemistry analysis was performed together with the pluripotency marker Tra1-60 to confirm the quality of the cells. Int β_1 subunit was highly expressed, as 99% of the hESCs contained the antigen with high intensity (Fig. 2a) together with the pluripotency marker

Tra1-60 (Fig. 2b). Lm binding integrin subunits Int α_3 (95%) and α_6 (98%), were also highly present on hESCs (Fig. 2a and b), while α_1 and α_2 subunits, specifically binding collagen, could not be found (Fig. 2a). Int α_V (Fig. 2a and b) and β_5 (Fig. 2b) subunits were strongly present in prominent point adhesion sites of hESCs (as suggested by lack of talin and vinculin, not shown), but not elsewhere in the cells. Also, Int α_5 (90%) was present in most of the hESCs (Fig. 2a and b) while immunoreactivity for Int α_2 , α_4 , β_3 or β_4 subunits could not be found (not shown). Immunoprecipitation by MAbs against human integrin subunits confirmed that the hESCs

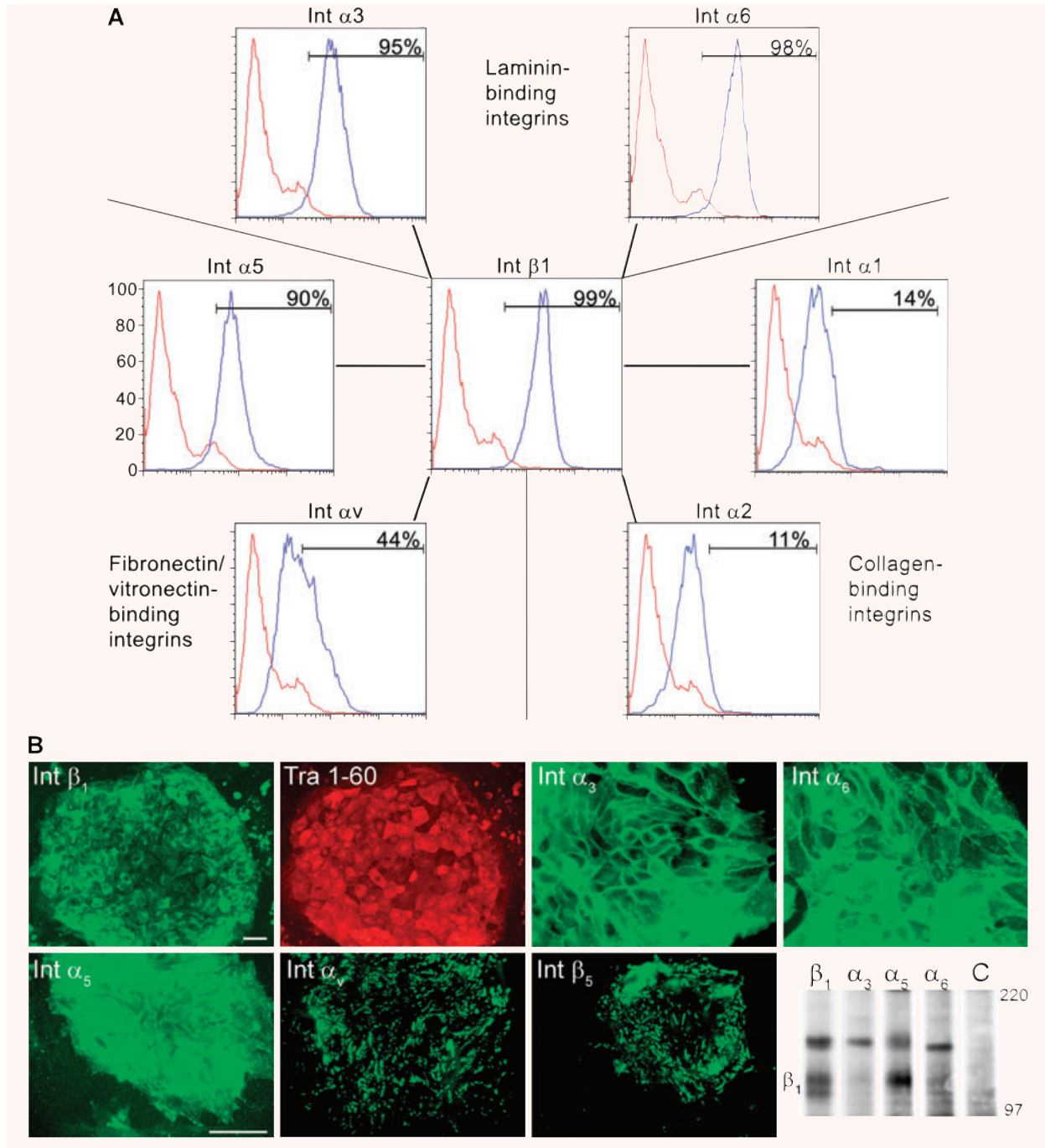


Fig. 2 hESCs contain laminin (Lm-), fibronectin- and vitronectin-binding integrins. (a) Flow cytometry analysis showed that hESCs contained Int β_1 , α_3 , α_6 , α_5 and $\alpha\nu$ subunits. Int β_1 subunit can bind different Lms by pairing with Int α_3 and α_6 subunits, fibronectin with Int α_5 subunit, and vitronectin with Int $\alpha\nu$ subunit. Functions of fibronectin and vitronectin receptors may overlap. hESCs did not contain collagen binding Integrins α_1 and α_2 . (b) hESCs were double-stained by monoclonal antibodies against integrin subunits and Tra1-60 antigen. Tra1-60-positive hESCs showed immunoreactivity for Int β_1 , α_3 , α_6 and α_5 subunits in a cell surface-confined manner (Tra1-60 shown only for Int β_1). Int $\alpha\nu$ and β_5 subunits were seen as a pattern resembling point adhesions. Scale bar, 50 μm . Immunoprecipitation confirmed production of Int β_1 , α_3 , α_5 and α_6 subunits.

Table 3 Examined integrin subunits and their presence in hESCs

Integrin subunit	Method	Localization
Int α_1	ICC, FC	-
Int α_2	ICC, FC	-
Int α_3	ICC, FC, IP	Cell surface
Int α_4	ICC, FC	-
Int α_5	ICC, FC, IP	Cell surface
Int α_6	ICC, FC, IP	Cell surface
Int α_v	ICC, FC	Point adhesions
Int β_1	ICC, FC, IP	Cell surface
Int β_3	ICC	-
Int β_4	ICC	-
Int β_5	ICC	Point adhesions

IP; immunoprecipitation, ICC; immunocytochemistry, FC; flow cytometry.

contained Int β_1 , α_3 , α_5 and α_6 subunits produced by themselves (Fig. 2b). The results were highly similar in two different hESC lines in all tested culture conditions. Pluripotency markers Tra1–60 and SSEA3 were expressed (from 80% to 95%) by both hESC lines throughout the study (not shown).

Undifferentiated hESCs contain Lutheran glycoprotein

MAb against human Lu/B-CAM uniformly stained undifferentiated hESC colonies with high intensity, together with Tra1–60 (Fig. 3a). Lu and B-CAM arise from a single mRNA *via* alternative splicing and differ in the length of their cytoplasmic tail. Since the antibody against Lu/B-CAM recognizes both variants of the proteins, we analysed the expression of these splice variants in hESCs by RT-PCR. Both forms of the gene, B-CAM variant 1 (Lu) and B-CAM variant 2 (B-CAM), were expressed in hESCs (Fig. 3b).

hESCs adhere to laminin-511 through lutheran/B-CAM and Int $\alpha_3\beta_1$

Next, we performed functional adhesion experiments to quantify hESC adhesion to distinct ECM and BM proteins in conditions devoid of serum and serum replacement. The experiments were performed with hESCs cultured in feeder-free conditions. While these simplified conditions do not represent true culture conditions, our aim was to decipher interactions between the hESCs and the BM proteins at the receptor level. Based on findings performed by flow cytometry, immunoprecipitation and immunocytochemistry, we selected function blocking MAbs for certain highly

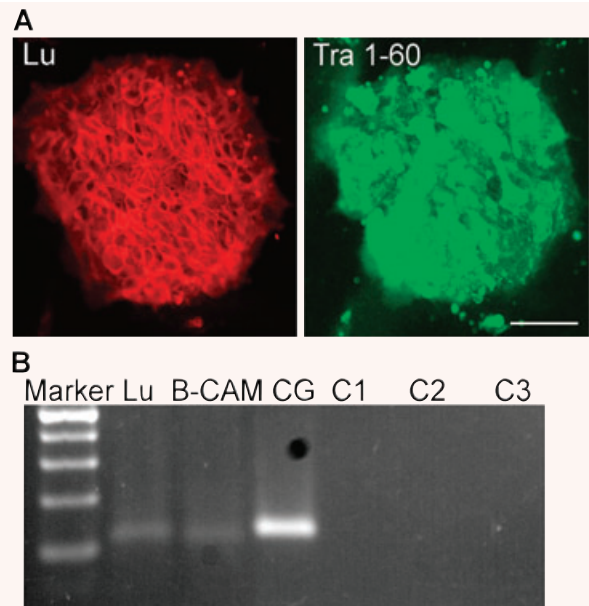
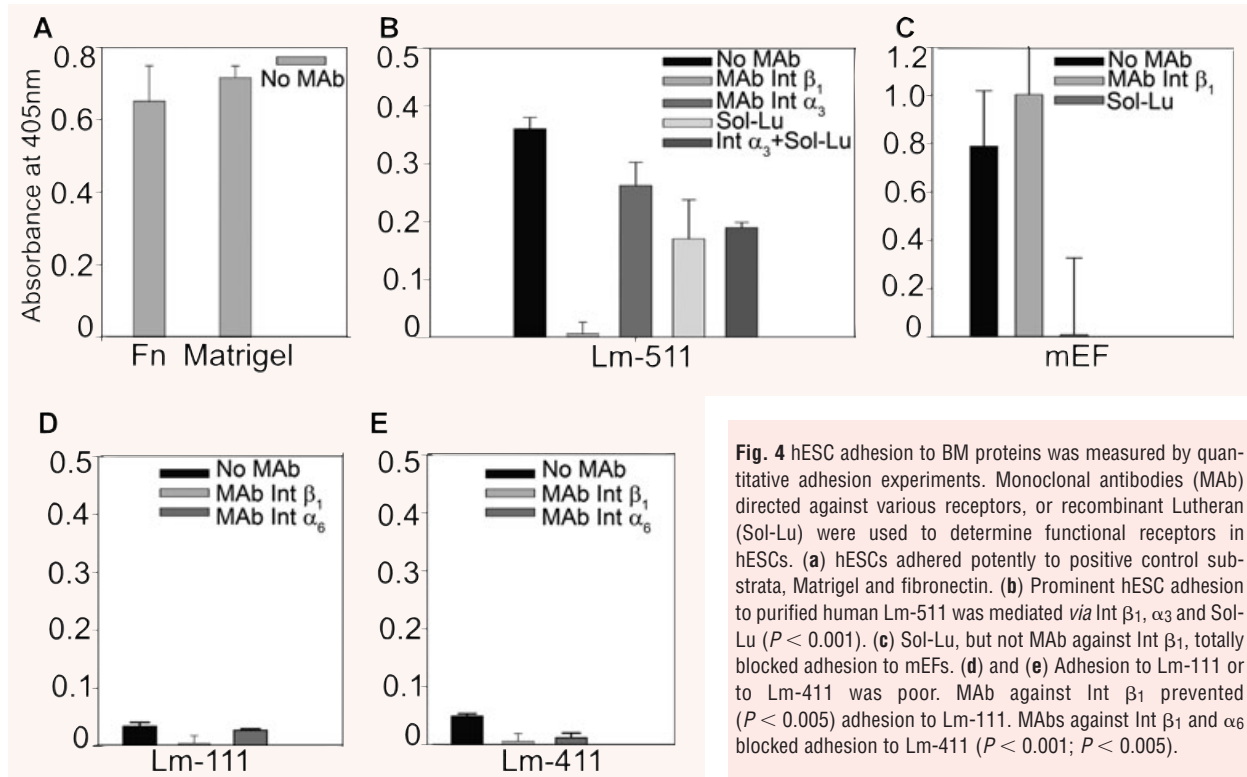


Fig. 3 Undifferentiated hESCs contain a known Lm α_5 receptor, Lutheran/B-CAM. (a) Antibody against Lutheran/B-CAM intensively stained Tra1–60-positive hESCs. Scale bar, 50 μm . (b) Both mRNA variants of the same gene, 115 bp for Lutheran and 109 bp for B-CAM, were expressed by hESCs. Negative controls; C1 for Lutheran, C2 for B-CAM, C3 for Cyclophilin G.

expressed integrin receptor subunits to find out their functional properties in the adhesion of hESCs. The cells adhered well to Matrigel and fibronectin, both of which are known to function as potent cell adhesion substrata (Fig. 4a). Lu/B-CAM receptor mediates adhesion to Lm α_5 chain in many epithelial and endothelial cells [31]. Hence, we quantified adhesion of hESCs to Lm-511 in presence of either function-blocking MAb against Int β_1 subunit alone, or function-blocking MAb against Int α_3 subunit alone. In addition, the adhesion was measured in presence of recombinant, soluble Lutheran (Sol-Lu) alone, or Sol-Lu together with function-blocking MAb against Int α_3 subunit. hESCs adhered strongly to purified human Lm-511 (Fig. 4b). MAbs against Int $\alpha_3\beta_1$ and Sol-Lu inhibited hESC adhesion, thus suggesting that Lutheran mediates their adhesion to Lm-511 in cooperation with Int $\alpha_3\beta_1$ ($P < 0.001$) (Fig. 4b). Then, we quantified the adhesion of hESCs to mEFs either in presence of MAb against Int β_1 or Sol-Lu. Surprisingly, the adhesion to mEFs was not blocked by MAb against Int β_1 , but with sol-Lu (Fig. 4c).

Adhesion to purified human Lm-111 was relatively weak and it was completely blocked by function-blocking MAb against Int β_1 subunit ($P < 0.005$) (Fig. 4d). Function-blocking MAb against Int α_6 tended to reduce the adhesion to Lm-111, suggesting that Int $\alpha_6\beta_1$ may be involved in the adhesion (Fig. 4d). However, this effect was not statistically significant. Figure 4e shows minor



hESC adhesion to Lm-411, which was completely blocked by function-blocking MAb against either Int β_1 ($P < 0.001$) or Int α_6 ($P < 0.005$), suggesting that adhesion is mediated *via* Int $\alpha_6\beta_1$. The adhesion of hESCs to purified human fibronectin and Matrigel was also significantly reduced by the Int β_1 antibody (results not shown). The results were similar when the hESCs were cultured on mEFs.

BM proteins differ in their ability to maintain hESC self-renewal

We measured hESC proliferation on Lm-111, Lm-511 or vitronectin in defined culture conditions. Cell growth was measured by changes in the hESC colony surface area in function of time with simultaneous monitoring of cellular morphology. The proliferation rates detected on hESCs growing on Matrigel or purified human Lm-511 were highly comparable (Fig. 5a). Also vitronectin could support the growth, whereas proliferation was weak on purified human Lm-111 and no proliferation was detected on gelatin (Fig. 5a). We then cultured hESCs for 5 passages on either Lm-111, Lm-511, or on heparin-binding form of vitronectin (all purified from human sources) in StemPro culture medium. Typical undifferentiated morphology persisted on Lm-511 (Fig. 5b, II and V) and on vitronectin (Fig. 5b, III and VI). However, distinct

differentiation events occurred on Lm-111 (arrows in Fig. 5b, I and IV). In order to further verify the self-renewal capacity of the hESCs under the defined culture conditions we quantified gene expression levels of pluripotency marker genes Sox2 and Oct4, and early differentiation marker genes, Brachyury (Bra) and Goosecoid (GSC). Matrigel with StemPro was used as the reference culture condition, representing the value '1' in each qPCR panel (Fig. 5c). The expression levels of Sox2 and Oct4 remained relatively stable in all conditions (Fig. 5c). However, the levels of Bra and GSC decreased during culture on Lm-511 or vitronectin. In contrast, hESCs tended to differentiate when cultured on Lm-111, as shown by increased expression of both Bra and GSC (Fig. 5c).

Discussion

Stem cell niches composed of neighbouring cells, ECM proteins, and soluble factors have been recognized crucial for regulating tissue specific stem cell self-renewal and differentiation [32–34] and it has been proposed that ECM has an important regulatory role also for the hESC niche [35, 36]. In this study, we have analysed the production of Lm chains and the ECM and BM receptors by hESCs and further investigated the functional role of these receptors in adhesion to purified human BM proteins. We show that Lm isoforms differ in their ability to support hESC

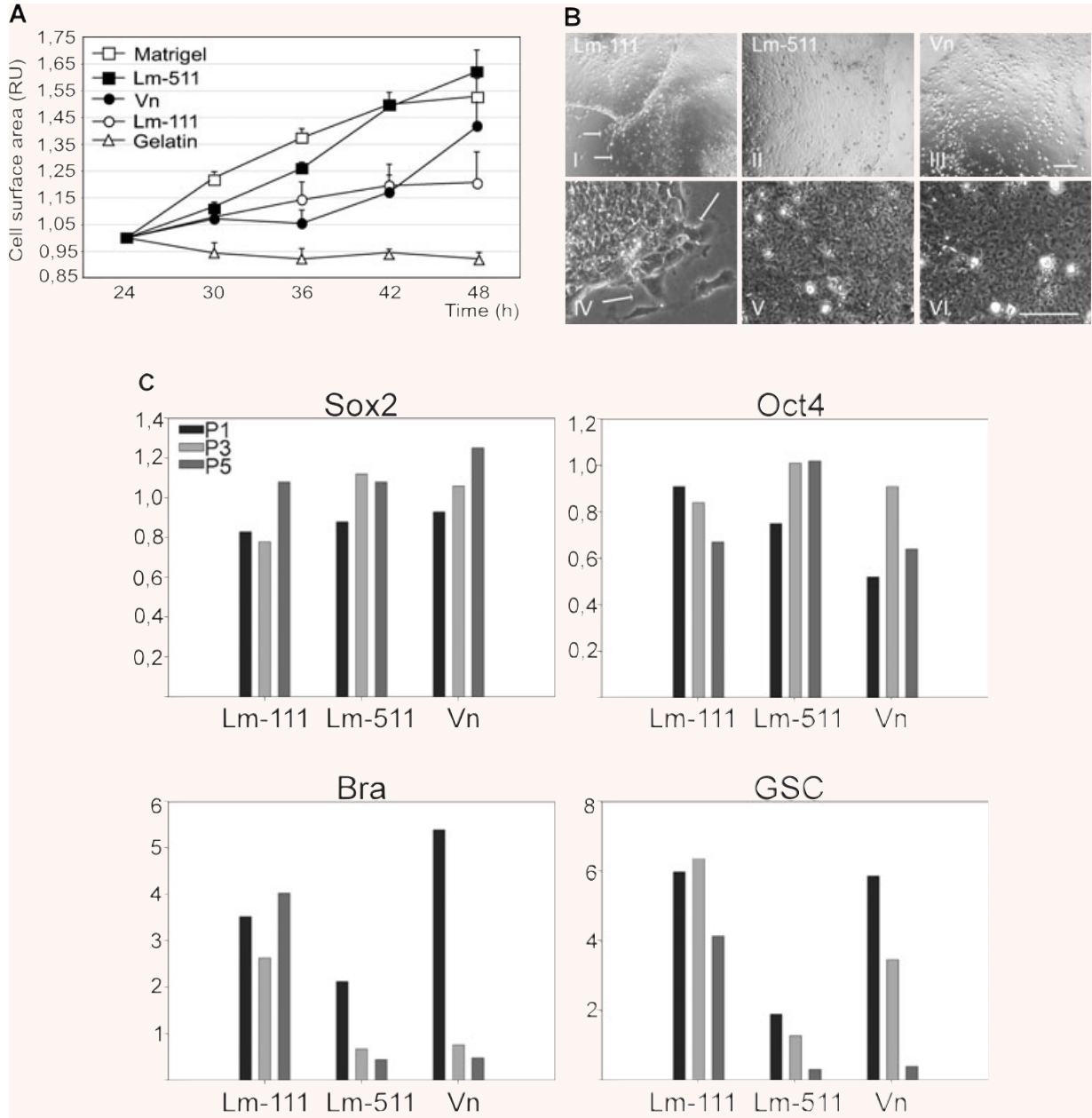


Fig. 5 Individual laminin isoforms have distinct effects on the hESC self-renewal. **(a)** Proliferation was measured in a continuous live-cell imaging system. The area of hESC colonies in relative units is shown in function of time (hours). Results are presented as the mean \pm S.E.M. for three culture wells. The growth rate was highest on Matrigel and on purified human Lm-511. In contrast, proliferation on Lm-111 was low. **(b)** hESCs cultured on purified human Lm-111 (I and IV), Lm-511 (II and V) or Vn (III and VI) in defined medium, StemPro, at passage 5. hESCs tended to differentiate on human Lm-111 (I, IV; arrows) whereas typical characteristics of undifferentiated cells were present on Lm-511 and on Vn. Scale bars, 100 μ m. **(c)** qPCR analysis of hESCs cultured on BM proteins in StemPro, at passage 1, 3 and 5. The relative expression levels of the pluripotency marker genes, Sox2 and Oct4 sustained, while the expression of the differentiation markers GSC and Bra decreased during culture on Lm-511 or Vn. In contrast, differentiation occurred when the hESCs were cultured on Lm-111, as shown by the presence of GSC and Bra and a decreasing level of Oct4. Data are presented as the mean expression level relative to cells grown on Matrigel, determined in duplicate.

proliferation and/or maintain their undifferentiated state, even in short-term culture.

Our results show that hESCs produce Lm α_1 , α_5 , β_1 and γ_1 chains and that those chains are also deposited as Lms-111 and -511 into hESC-produced ECM. According to our flow cytometry, immunocytochemistry and immunoprecipitation analyses the undifferentiated hESCs selectively contain Lm binding integrin β_1 , α_3 and α_6 subunits. In contrast, α_1 , α_2 and α_4 integrin subunits involved in collagen and/or Lm binding could not be detected in hESCs (not shown). Functional adhesion experiments confirmed that hESCs do adhere both to Lm-111 and Lm-511 utilizing their specific integrin receptors. In short-term culture experiments especially Lm-511 seemed to support both self-renewal and proliferation of hESCs. The early differentiation markers, Brachyury and Goosecoid, were declined during cultures on Lm-511 further strengthening its role as a biologically relevant BM component for undifferentiated hESCs. Supporting this, it has recently been shown that Lm-511 supports the self-renewal of mouse ES cells [37]. In contrast, Lm-111 as such did not support growth or self-renewal of the undifferentiated hESCs, as the same differentiation markers were constantly present when the hESCs were cultured on Lm-111. Whether Lm-111 has a role alone or in combination with other BM components in the maintenance or early differentiation of hESCs remains to be elucidated.

In previous studies, hESCs have been shown to have characteristics typical for epithelial cells [38–40]. Specifically, cells covering hESC colonies have a polarized structure and contain epithelial proteins, *e.g.* E-cadherin. Of the studied BM proteins, Lm-111 and Lm-511 are, besides embryonic, also typical epithelial Lms. The same Lm chains have been shown to be essential for the formation of the embryonic BM and survival of the mouse embryo [6, 7].

It has also been suggested for mice that if the embryonic BM is not correctly formed, ICM cells are not able to polarize towards embryonic epiblastic cells, normally occurring prior to gastrulation [41, 42]. Thereby, it is reasonable that hESCs, as epithelial epiblast-like cells, produce and deposit embryonic Lm chains. Taken together, we suggest that Lm-111, and especially Lm-511, are functionally important Lms for hESCs.

Majority of the hESCs showed intensive staining for Int α_5 , α_V and β_5 subunits that in conjunction with Int β_1 are known to form a set of functional fibronectin and vitronectin receptors. The functionality of these integrins was also confirmed by adhesion and short term culture assays. Our results are in good agreement with previous studies, showing that both fibronectin and vitronectin can support hESCs in long-term cultures [15, 43]. There are only few studies about the expression of integrins or Lms in early human embryos, the closest *in vivo* equivalents to hESC. In blastocyst/implantation stage of human embryos, a mainly trophecto-

dermal expression of Int β_1 , β_5 and α_6 subunit proteins has been detected [44]. The human blastocyst also produces the Int α_V subunit [9]. There is no evidence of the role of integrins in the commitment or maintenance of the ICM or trophectodermal cells and no data are available about the epiblastic/hypoblastic differentiation in the human embryo. An analysis of integrin expression of hESCs has recently appeared [43]. The only major difference between the results of Braam *et al.* and our study is that we did not detect immunoreactivity for Int α_2 .

Low Int α_2 level has also been reported based on gene expression data [45]. The integrin β_1 subunit is known to have a central role in various mammalian cell types and it was also found to be involved in interactions between hESCs and all the explored BM/ECM components in this study. The use of MAb blocking Int β_1 function prevented the adhesion to BM proteins nearly totally. Blocking of Int α_6 subunit function with MAb totally prevented hESC adhesion to Lm-411. The result is in line with the results that Int $\alpha_6\beta_1$ functions as a receptor for Lm-111 in mouse embryonic and human epithelial cells [4, 21] as well as for Lm-411 in human endothelial cells [24].

Interestingly, hESCs showed a high content of Lutheran/B-CAM (Lu/B-CAM) glycoprotein on their surface. Lu/B-CAM is a transmembrane receptor of the immunoglobulin superfamily and it is known to mediate cell adhesion to Lms-511 and -521 independently or in concert with integrins [31, 46]. Our results show that independently of culture conditions hESC adhesion to Lm-511 is mediated *via* cooperation of Lu/B-CAM and Int $\alpha_3\beta_1$. Importantly, Sol-Lu also blocked hESC adhesion to mEFs in adhesion experiments. This is consistent with our unpublished observation that mEFs produce large amounts of Lm-511, the target of Lu/B-CAM. Little is known about the role of Lu/B-CAM in early development. However, mice lacking Lutheran are viable, without severe defects [47]. The exact role of Lu/B-CAM in hESC biology and culture adaptation remains to be examined.

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