STEM CELLS AND DEVELOPMENT
Volume 22, Number 5, 2013

DOI: 10.1089/scd.2012.0365

ORIGINAL RESEARCH REPORT

Lectin from *Erythrina cristagalli* Supports Undifferentiated Growth and Differentiation of Human Pluripotent Stem Cells

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Lectins are carbohydrate-binding proteins, which occur ubiquitously in nature and are abundant in all living organisms from bacteria to mammals. They have several biological functions among which cell adhesion is well known and characterized. Based on the characterization of the glycome of human embryonic stem cells (hESCs), we have investigated the properties of glycan-binding lectins as a novel class of culture support matrices supporting hESC culture. We report that an *Erythrina cristagalli* lectin (agglutinin) (ECA) matrix supported the undifferentiated growth and significantly increased the plating efficiency of both hESC and human induced pluripotent stem cells when used in conjunction with pinacidil, an antihypertensive drug with ROCK inhibition activity. As a matrix, ECA maintained pluripotency, robust proliferation with a normal karyotype, and the ability to differentiate both in vitro and in vivo. Therefore, our findings indicate that lectins are potential candidates for design of culture and differentiation methods, and that ECA is a potent simple defined matrix for human pluripotent stem cells.

Introduction

Human pluripotent stem cells (hPSCs) have great potential in various lines of basic developmental and genetic research, but also in regenerative medicine. However, they are highly sensitive to culture conditions, and there are several technical issues to be resolved before they could be fully exploited in clinics [1–4]. Challenges in expansion of undifferentiated stem cells for clinical applications include the removal of nondefined components in the matrix and the culture medium. Feeder-free culturing of hPSCs typically requires extracellular matrix (ECM) components, which regulate cell behavior via interactions with cell surface receptors and serve as a reservoir of growth factors [5–7]. ECM products typically consist of glycosaminoglycans and proteoglycans together with different types of laminins and collagens [4,8]. Traditionally, Matrigel has been used as an ECM matrix in stem cell cultures [2,9], but as it is of animal origin and contains undefined compounds and introduces remarkable lot-to-lot variation, it is not acceptable for possible clinical application. Purified ECM proteins such as fibronectin [10], vitronectin [11], and various laminins [12–15] can also support hPSC growth. Also, synthetic surfaces [16–18] as well as suspension culture systems [19] have been reported to support hPSC growth. However, none of the methods based on defined matrices are commonly used, and many of them are too expensive for large-scale application. There is still an obvious need for the establishment of fully defined culture and differentiation methods for pluripotent stem cells.

Lectins are carbohydrate-binding proteins, which occur ubiquitously in nature and are abundant in all living organisms from bacteria to mammals. They have several biological functions among which cell adhesion is well known and characterized [20].

In some cases, they have been reported to enhance also cell growth and proliferation. For example, it was reported that the β-galactoside-binding lectin jacalin induces proliferation of T lymphocytes [21], and other β-galactoside-binding lectins from peanut and mushroom stimulate vascular cell proliferation [22]. The reported beneficial effects on cell proliferation have been specific for both the cell type and the lectin, suggesting that specific interaction between lectins and cell surface glycans are essential for the growth supporting effect.

We have previously characterized the expression profile of the *N*-glycans present on the surface of undifferentiated

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human embryonic stem cells (hESCs) [23]. In the current study, we have used this background information to test several lectins, which bind to the epitopes commonly expressed in hPSC in supporting the growth of undifferentiated hPSC.

Best results were obtained with the lectin (agglutinin) from *Erythrina cristagalli*, ECA (also called ESL), which binds to *N*-acetyl lactosamine (type 2 chain) glycoconjugates, a common structure in *N*-glycans of undifferentiated stem cells [23].

Materials and Methods

Cell culture and maintenance

Three hESC lines (FES 29, FES 30, and H9) [24] and 2 human induced pluripotent stem cell (hiPSC) line (FiPS 5-7 [25] and HEL11.4) were included and cultured on Matrigel as previously described [15,24]. HEL11.4 was generated from adult fibroblasts (men, 84 years old) using retrovirus-induced overexpression of 4 genes: *Oct-4*, *Sox2*, *Klf4*, and *c-Myc*. Cells were infected with equal parts of the hES medium and virus-containing supernatant twice at 24-h intervals. Cells were harvested and reseeded on mitotically inactivated treated mouse embryonic fibroblast (mEF) layer 3 days after infection. Twenty-four days post-transduction, ES-like colonies were picked, expanded, and characterized.

Cells were passaged by using 0.1 mg/mL collagenase IV (Invitrogen) for 5 min at +37°C and harvested onto ECA (Sigma-Aldrich) and Matrigel (Becton Dickinson) plates and cultured either in StemPro® or in mEF-conditioned-medium (CM) 9KnockOut™-DMEM supplemented with 20% KnockOut™ serum replacement, 2 mM Glutamax, 0.1 mM β-mercaptoethanol, and 0.1 mM nonessential amino acids, all from Invitrogen and supplemented with 8 ng/mL recombinant human basic fibroblast growth factor (bFGF; Invitrogen]. Pinacidil (Sigma-Aldrich; 100 μM) was added to the culture medium during passaging. In all experiments, Matrigel™ (BD Biosciences) was used as a control matrix. The Matrigel plates were prepared as recommended by the manufacturer.

Coating of plates with ECA

ECA lectin (Sigma-Aldrich) solution [1 mg/mL in phosphate-buffered saline (PBS)] was let to passively adsorb onto surface of the cell culture plates (5 μg/cm^2) (Nunc, Corning Life Sciences) o/n at +4°C, followed by washing twice with PBS. The coated plates were stored at +4°C and used within 4 weeks.

Flow cytometry analysis of surface antigens

Single-cell suspensions were generated by incubation with TrypLE (Gibco) for 5 min at +37°C. Cells were stained with specific cell surface antibodies (SSEA-1, SSEA-3, Tra 1-60, H type 1, and CXCR4) and fluorescein-conjugated secondary antibody before analysis by flow cytometry (FACS Calibur; BD Biosciences). Antibodies are listed in Table 1.

Immunohistochemistry of the cells on ECA

Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) if needed. The antibodies used are listed in Table 1. The cells were probed with secondary antibodies for 30 min in the dark at room temperature. Cells were mounted using a Vectashield mounting medium with 4,6-diamidino-2-phenylindole (Vector Laboratories).

Table 1. Antibodies Used for Analysis

Primary antibody	*Manufacturer/catalog number*
SSEA-3	Millipore/MAB 4303
Tra 1-60	Millipore/MAB4360
H-type1	Abcam/Ab3355
SSEA-1	Millipore/MAB4301
CXCR4	BD/555974
Oct-4	Santa Cruz Biotechnology/sc9081
Nanog	Cell Signalling/4903
Sox2	Cell Signalling/3579
Anti-human alpha-1-fetoprotein	DAKO/A0008
FOXA2	Santa Cruz Biotechnology/sc-9187
Anti-human/mouse serum albumin	R&D Systems

Validation of ECA binding specificity

Cells were passaged and plated as described earlier. The 2 compounds expected to act as specific binding inhibitors for ECA were lactose monohydrate (Sigma-Aldrich; 100 mM) and lacto-*N*-neotetraose (Kyowa Hakko Kogyo; 100 mM). Saccharose (Sigma-Aldrich; 100 mM) was used as a control. The inhibitors were added to the culture medium at the time of passaging, and the attached cells were counted after 20 h.

In vitro differentiation

Hepatic differentiation was done on FES 29, H9, and HEL11.4 cells, which had been cultured either on ECA or Matrigel for at least 10 passages. The differentiation protocol is described in Table 2.

Teratoma formation

Cells were harvested with collagenase IV from ECA and Matrigel plates, and ca. 100,000 cells from each matrix were injected into nude mouse testis. After 7–8 weeks, tumors were dissected, fixed with 4% paraformaldehyde, and hematoxylin and eosin-stained sections histologically examined. The animal experiments were approved by the experimental animal welfare committee of the District Government of Southern Finland.

RNA isolation and quantitative polymerase chain reaction

Total RNA was isolated using NucleoSpin® RNA II (Macheray-Nagel GmbH & Co. KG) according to the manufacturer's instruction. Complementary DNA was synthesized from 50 μg of total RNA using iScript™ cDNA Synthesis Kit (Biohit) according to manufacturer's instruction.

Real-time SYBR Green quantitative polymerase chain reaction (qPCR) analyses were performed with Corbett Rotor-Gene 6000 (Corbett Life Science) using the following conditions: 95°C 7 min and 40 cycles of 95°C, 20 s; 56°C, 20 s;

Table 2. Protocol for Hepatic Differentiation

Stage 1		*Stage 2*	*Stage 3*
1 day	*4 days*	*5 days*	*10 days*
The first day of DE induction	DE differentiation	Differentiation to hepatic progenitors	Hepatocyte maturation
RPMI1640 + Glutama ×	RPMI1640 + Glutama ×	KO-DMEM	Leibovitz's L-15 (Invitrogen)
B27 2% (v/v)	B27 2% (v/v)	KO-SR 20% (v/v)	FBS 8.2% (v/v)
Wnt3a 75 ng/mL	Act A 100 ng/mL	NEAA 1% (v/v)	Tryptose phosphate broth 8.3% (v/v)
Act A 100 ng/mL	NaB 0.5 mM	Glutamine 1 mM	Hydrocortisone-21-hemisuccinate 10 μM
NaB 1 mM		β – MeOH 0.1 mM	Insulin 1 μM
		DMSO 1% (v/v)	Glutamine 2 mM
			HGF 10 ng/mL
			Onc M 20 ng/mL

DE, definitive endoderm; Act A, activin A; NaB, natrium butyrate; KO-DMEM, KnockOut™-DMEM; KO-SR, KnockOut™ serum replacement; NEAA, nonessential amino acids; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; HGF, hepatocyte growth factor; Onc M, oncostatin M.

72°C, 20 s. The data were analyzed according to the comparative Ct method (Applied Biosystems; User Bulletin No. 2). Cyclophilin gene expression was an internal reference for normalization. All samples and controls were analyzed in duplicates. Primers used for qPCR are shown in Table 3.

PCR arrays

FES 29 cells were cultured for 9 passages on ECA or Matrigel in CM. Total RNA was isolated from 3 separate plates using RNeasy Mini kit (Qiagen), and complementary DNA was synthesized from 1 μg of total RNA using RT^2 First-Strand Kit and RT^2 qPCR Master Mixes (SABiosciences) according to manufacturer's instruction. The RT^2 qPCR primer assays (SABiosciences) were used to study the gene expression profile of genes related to the identification, growth, and differentiation of stem cells (array PAHS-081).

Karyotype analysis

Karyotype was detected by G-banding technique in the cytogenetics laboratory of the Yhtyneet Medix Laboratories, Inc. Twenty metaphases were examined from each sample.

Clonogenicity assay

Cells were dissociated with TrypLE for 5 min and passed through an 80-μm cell strainer (Becton Dickinson). Dissociated single cells from either ECA or Matrigel were seeded onto both ECA and Matrigel (35 cells/cm^2) and cultured in an mEF-CM supplemented with 8 ng/mL bFGF. Pinacidil (100 μM) was used during passaging. To evaluate clonogenic capacity, cells were alkaline phosphatase stained, and colony numbers were counted 10 days after plating.

Cell viability analysis

Cells were plated and cultured on ECA and Matrigel 6 days. Cell viability was analyzed in the beginning, on day 3 and on day 6 using Trypan Blue staining of dissociated cells. The results represent 8 separate experiments, each performed in duplicate. Cell viability was tested also on plate without dissociation using the Live/Dead Viability/Cytotoxity Kit (Invitrogen) according to manufacturer's instructions.

Determination of cell growth rate

FES 29 and HEL11.4 cells were passaged by collagenase IV to small clumps from ECA and Matrigel and plated on 12-well plates, ~6,000 cells/well on both matrices. Cells were counted at 2 time points, day 3 and 6.

Live cell imaging was used as an alternative method. For this purpose, FES 29 and HEL11.4 cells were dissociated by collagenase IV to small aggregates of 10–20 cells from ECA and Matrigel and plated on 12-well plates, ~1,000 cells/well on both matrices. Cells were let to adhere in the cell culture incubator for 24 h, and the plates were then transferred into Cell IQ culture platform (CM-Technologies). All wells were imaged every second hour for 5 days. The images were analyzed using a Cell IQ Analyzer.

Table 3. Primers Used in Quantitative Polymearse Chain Reaction Analysis

Primer	*5′-Sequence*	*3′-Sequence*
Oct4	TTGGGCTCGAGAAGGATGTG	TCCTCTCGTTGTGCATAGTCG
Sox2	GCCCTGCAGTACAACTCCAT	TGCCCTGCTGCGAGTAGGA
Nanog	CTCAGCCTCCAGCAGATGC	TAGATTTCATTCTCTGGTTCTGG
Brachuyru	GCATGATCACCAGCCACTG	TTAAGAGCTGTGATCTCCTC
Goosecoid	GAGAACCTCTTCCAGGAGAC	TTCTTAAACCAGACCTCCAC
Cyclophilin	CAATGGCCAACAGAGGGAAG	CCAAAAACAACATGATGCCA
FOXA2	AAGACCTACAGGCGCAGCT	CGTTGAAGGAGAGCGAGTG
CER1	CATTGGGAGACCTGCAGGAC	CCCAAAGCAAAGGTTGTTCTG

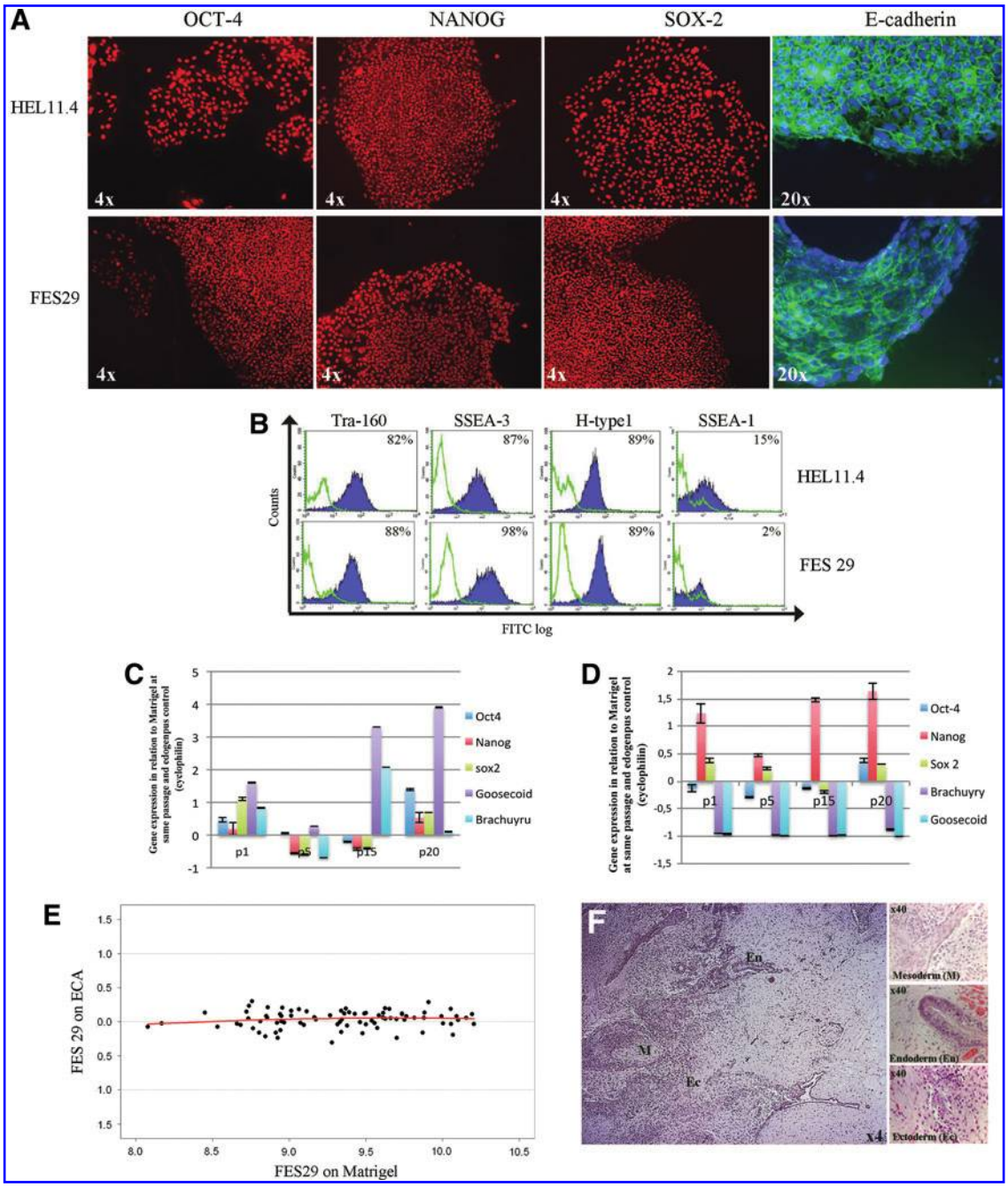

FIG. 1. **(A)** Immunohistochemistry of human embryonic stem cell (hESC; FES 29) and human induced pluripotent stem cell (hiPSC; HEL11.4) lines cultured on *Erythrina cristagalli* agglutinin (ECA) for 20 passages. **(B)** Fluorescence-activated cell-sorting (FACS) analysis of pluripotency-associated cell surface markers after 20 passages on ECA. **(C, D)** Relative expression level of pluripotency-associated genes (*Oct4*, *Nanog*, and *Sox2*) and early differentiation-associated genes (Brachyuru, Goosecoid) by quantitative polymerase chain reaction (qPCR) at passages 1–20. The data were normalized against the level in cells cultured on Matrigel for the same time. **(C)** hESC (FES 29); **(D)** hiPSC (HEL11.4). Error bars indicate standard error of the mean (SEM). **(E)** PCR array analysis of pluripotency and early differentiation gene expression of cells growing either on Matrigel or on ECA at passage 9. Gene profiles were compared between FES 29 on Matrigel and on ECA. *Y*-axis is the intensity ratio, and *X*-axis is the average intensity for a given gene measured on 2 similar high throughput qPCR arrays. All differences were <2-fold. **(F)** Hematoxylin and eosin staining of a teratoma derived from FES 29 cultured on ECA 9 passages. Derivatives of all germ layers can be detected. Color images available online at www.liebertpub.com/scd

Statistics

Statistical significance between 2 groups was tested using Students *t* test. One-way analysis of variance (ANOVA) was used for comparison of multiple groups, followed by Tukey's test.

Results

Testing of lectins

Based on our previous analysis of the *N*-glycans expressed on the surface of hESCs [23], we selected specific lectins as candidates for the cell culture matrix. ECA (binding specificity in type 2 *N*-acetyl-lactosamine structures), *Maackia amurensis* agglutinin (MAA, specific for α-2,3-linked sialic acid), *Wisteria floribunda* agglutinin (binding preferentially to *N*-acetylgalactosamine in α- or β-linkage), and *Phytolacca americana* agglutinin (with *N*-acetylglucosamine specificity, binding also to polylactosamine structures) were tested for their ability to act as a growth supporting matrix for ESC lines FES 29 and FES 30 in mEF-CM. In cell culture conditions, stem cells attached onto ECA and MAA, but continuous growth, was acquired only on an ECA matrix (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/scd).

Basic characteristics of hPSCs cultured on ECA

Long-term culturing on ECA-coated plates was evaluated with hPSC lines (FES 29 and HEL11.4), and the results were compared to the same cell lines cultured on Matrigel. For most of the experiments, the cells were cultured in the mEF-CM and treated with pinacidil during passaging. Without pinacidil the cells did not attach as effectively, and they also partly changed morphology forming a lot of feeder-like cells. In long-term cultures, the analysis of these cell lines by immunocytochemical stainings (Oct4, Nanog, Sox2, and E cadherin) and flow cytometry (Tra 1-60, SSEA-3, H-type1, and SSEA-1) demonstrated a profile characteristic for undifferentiated hESCs (Fig. 1A, B). The expression levels of major pluripotency-associated genes remained essentially

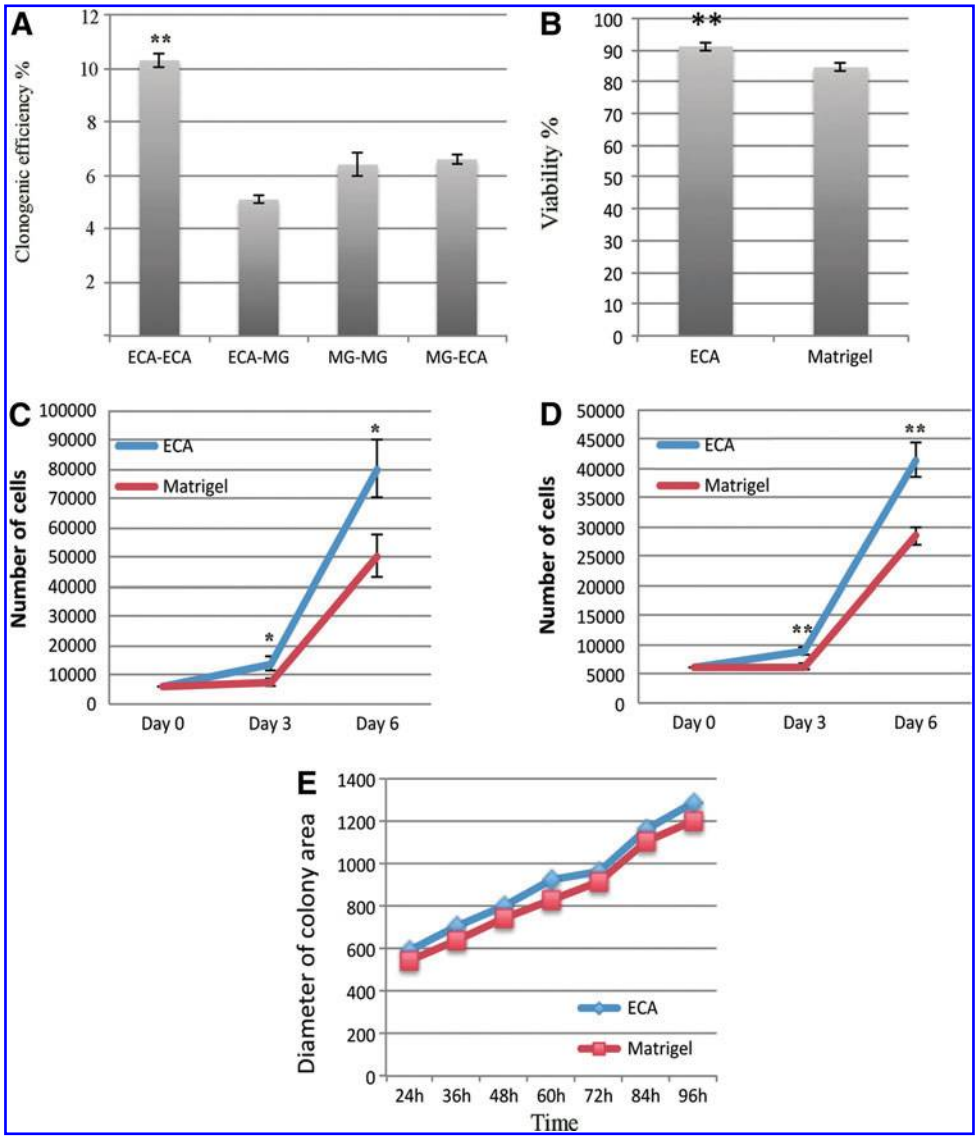

FIG. 2. (A) Clonogenicity assay on dispersed single cells (35 cells/cm^2). Cloning efficiency was calculated as the number of clones per the total number of plated cells when transferring the cells between ECA and Matrigel (MG) substrates ($P = 0.003$, one-way analysis of variance). Error bars indicate SEM. **(B)** Cell viability during passaging. Cells were counted by trypan blue exclusion ($N = 8$). On ECA the average cell viability rate was 92%, whereas on Matrigel it was 84% ($P < 0.01$). Data represent the mean (±SEM) of 8 separate experiments. **(C, D)** Analysis of cell growth rate. Data represent the mean of 12 wells of hESC [FES 29, **(C)**] or hiPSC [HEL11.4, **(D)**]. $^*P < 0.05$; $^{**}P < 0.01$. **(E)** Colony area (2 wells/matrix) for FES 29 cell line. Cells were passaged in small clumps and cultured in live cell-imaging system (Cell IQ; CM-technologies). Areas of the colonies were analyzed in a Cell IQ Analyzer program, and results are shown as average size. *Y*-axis is the diameter of colony area counted as pixels. Color images available online at www.liebertpub.com/scd

similar throughout 20 passages on both matrices. Minor upregulation of primitive streak/early differentiation markers Brachyuru and Goosecoid occurred at later passages of FES 29 cells on ECA (Fig. 1C). With the iPSC line HEL11.4, the pluripotency genes tended to remain higher and the differentiation genes lower on ECA throughout the culture period (Fig. 1D). In general, FES 29 showed a constant gene expression pattern independent of the matrix as tested by the PAHS-081 qPCR array, including 84 genes controlling growth and differentiation of stem cells (Fig. 1E). The ECA-cultured cells also retained their full in vivo differentiation capacity as indicated by highly complex teratoma containing all 3 germ layer derivatives (Fig. 1F). Both cell lines were karyotypically normal after 18 passages on ECA (not shown).

We next tested the ability of ECA to support the growth of undifferentiated hPSC in a defined cell culture medium StemPro™. Cells were first adapted to StemPro for 1 passage using 1:1 mix of StemPro and CM media, and then only StemPro was used. The results indicated that also defined media supported self-renewal, and cells maintained stem cell markers and normal karyotype detected after 9 passages on ECA in StemPro (data not shown).

Clonogenicity and cell growth

The ability of the ECA matrix to support clonogenicity of hPSC cells was studied by plating dispersed cells first adapted to ECA or Matrigel for at least 2 passages on either of the 2 matrices at the density of 35 cells/cm^2. In the presence of pinacidil, the colony-forming efficacy was clearly highest (10.3%,) when ECA-adapted cells were plated on ECA, as compared with all other conditions where the efficacy was ~6% ($P<0.05$, one-way ANOVA, Tukey's post hoc test) (Fig. 2A). Pinacidil was found to be essential for the development and survival of the single-cell-derived clones in these experiments.

Long-term cell imaging was used to study colony area and cell growth. Colonies were imaged every second hour during 4 days after plating to record colony areas and the number of cells in the colonies. In accordance with the clonogenicity assay, the initial number of colonies was higher on ECA than on Matrigel. An explanation to this was provided by cell viability analysis, which showed higher viability for cells grown on ECA than on Matrigel (90.1% vs. 82.9%, $P<0.01$, Fig. 2B). The cells were counted 3 and 6 days after plating. The number of cells was significantly higher on ECA than on Matrigel at both time points (Fig. 2C, D). No difference in speed of cell division was detected, and the size of the colonies growing on ECA and Matrigel was similar (Fig. 2E). These results show that culture on ECA generates more cells based on better attachment and survival after dissociation and plating.

Validation of binding specificity

To assess the specificity of the cell–lectin interaction in supporting stem cell attachment to the growth surface, we performed inhibition experiments with specific disaccharide inhibitors and control disaccharides. Lactose (composed of galactose β1,4-linked to glucose) inhibited cell attachment effectively at 100 mM concentration, while the same concentration of saccharose (fructose α1,1-linked to glucose) had no inhibitory activity. Further, lacto-*N*-neotetraose oligosaccharide, which contains the β1,4-linked galactose epitope, was as effective as lactose ($P<0.001$) (Fig. 3). The inhibition experiments thus demonstrated that initial cell attachment to the ECA matrix was dependent on specific interaction of the surface-bound lectin with stem cell glycan ligands. The experiments were performed either in the presence (Fig. 3A) of absence (Fig. 3B) of pinacidil. Even if the effect of the inhibitors was similar in both conditions, the total number of attached cells was 4-fold higher with pinacidil.

Hepatocyte differentiation

The cells were differentiated into hepatocyte-like cells (HLCs) on ECA side by side with Matrigel® as a control. For hepatocyte differentiation, we used a 3-step protocol, modified from the one established by Hay et al. [26]. During the course of differentiation, the expression of the pluripotency marker gene *OCT4* was efficiently downregulated while the endoderm marker FOXA2 and anterior definitive endoderm (DE) marker Cerberus (CER1) were strongly upregulated (Fig. 3A). The cells on both coatings changed into morphologically typical DE cells and stained positive for FOXA2

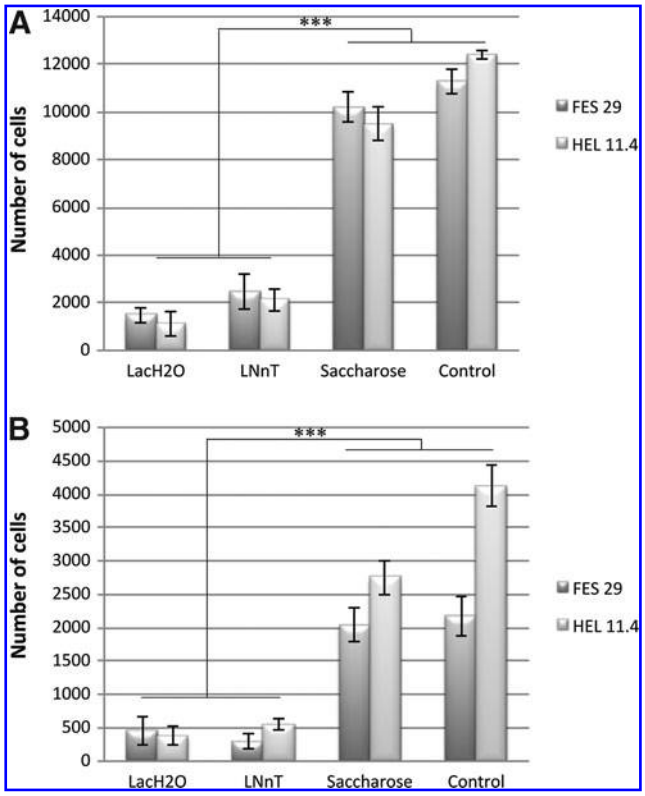

FIG. 3. Validation of the binding specificity of hESC and hiPSC to ECA using specific competitive inhibitors either in the presence **(A)** or in the absence **(B)** of Pinacidil. The attached cells were counted 20 h after plating. Data represent the mean (±SEM) of 3 experiments of both cell lines, FES 29 and HEL11.4. The total number of attached cells was 2-fold to 4-fold higher when Pinacidil was used ($P<0.001$). The disaccharides lactose monohydrate ($LacH_2O$) and lacto-*N*-neoteraose (LnNT) inhibited significantly cell attachment onto ECA (***$P<0.001$). The control disaccharide, saccharose, had no inhibitory activity.

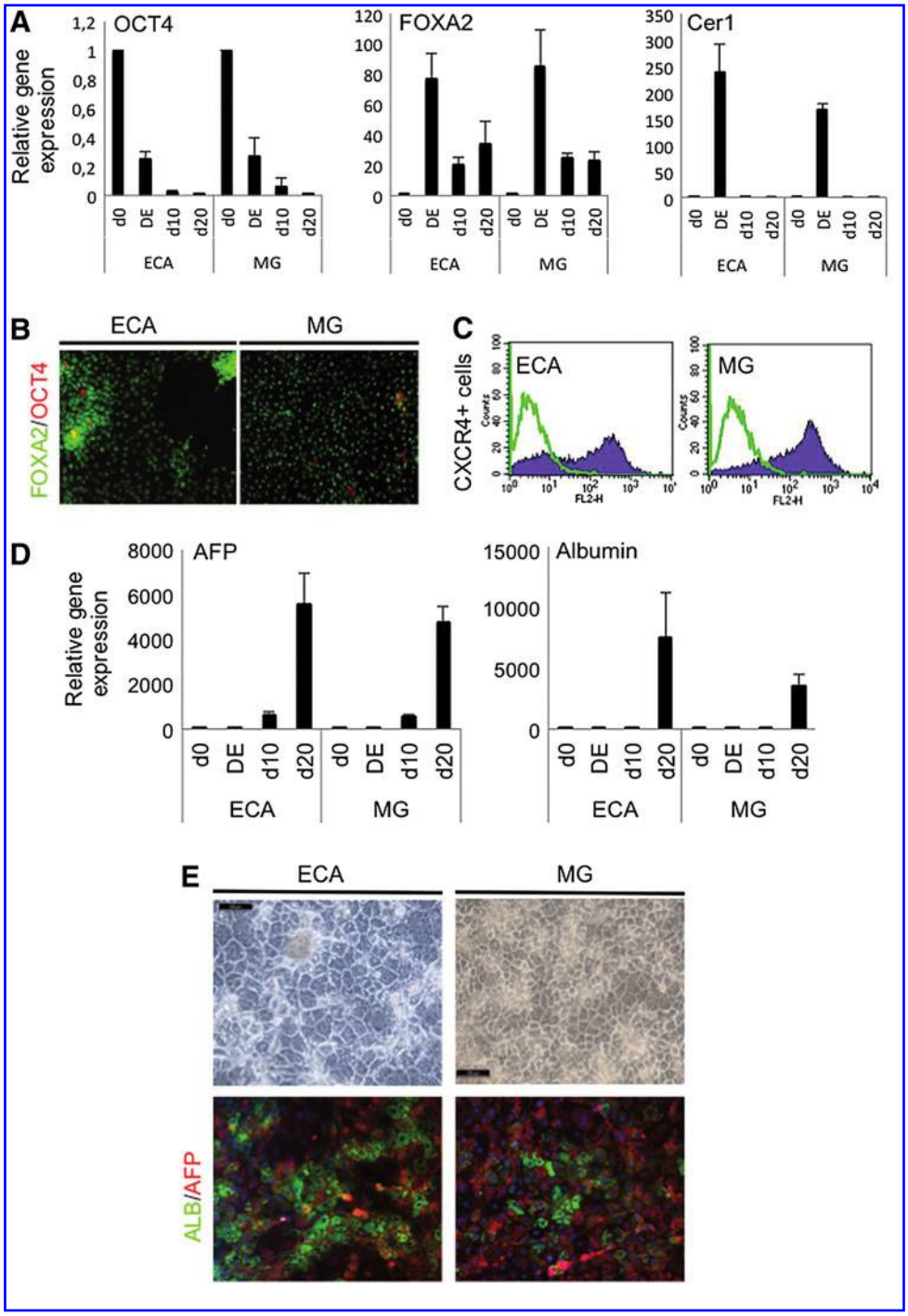

FIG. 4. Hepatic differentiation of HEL11.4 cells cultured on either ECA or Matrigel (MG). **(A)** qPCR analysis for pluripotency and endoderm marker gene expression. Pluripotency gene *OCT4*, anterior definitive endoderm (DE) marker FOXA2, and CER1. Error bars indicate SEM. **(B)** Immunocytochemical characteristics of the DE cells differentiated on ECA and MG. FOXA2 (*green*) and OCT4 (*red*). **(C)** FACS analysis of DE cells expressing the endoderm marker CXCR4 differentiated on ECA and on MG. **(D)** qPCR results for hepatic markers α-fetoprotein and Albumin gene expression. **(E)** Cell morphology and immunocytochemical characteristics of hepatocyte-like cell differentiated on ECA or on MG. Scale bar 100 μm. Color images available online at www.liebertpub.com/scd

(Fig. 3B). DE induction yielded on average 68%±5% cells positive for CXCR4 on ECA, while using Matrigel, the average was 78%±17% (Fig. 3C). However, cells detached easier from ECA than from Matrigel during the DE stage. The DE cells were further differentiated into hepatocyte progenitors with 5 days of dimethyl sulfoxide treatment. The cells formed hepatic endoderm with α-fetoprotein-positive progenitors on both matrices (data not shown). When the cells were matured into HLCs with hepacyte growth factor and Oncostatin M treatment (d10–d20), the more mature hepatocyte marker albumin became strongly expressed as shown by qPCR and immunocytochemistry (Fig. 4D, E). Taken together, hPSCs were successfully differentiated into HLCs on ECA-matrix, and no significant difference was detected when compared to cells differentiating on Matrigel.

Discussion

In this study, we have demonstrated the ability of ECA–lectin to support hESC and hiPSC cell self-renewal in mEF-CM and in commercial defined media. We also show that when passaged with pinacidil, the expansion of the cells is more efficient than on Matrigel without any impairment of quality. As ECA is a small-sized single protein that can be

easily produced recombinantly [27], and therefore it is a suitable matrix for a good manufacturing practice use.

It has been reported in many studies that culture adaptation is an evident phenomenon in hPSC cultures. Adapted stem cells proliferate better and quickly become a dominant population in the culture [28–30]. Extreme adaptation may lead to impaired differentiation capacity of the cells and enrichment of chromosomal abnormalities [31]. The cells cultured on ECA maintained a normal karyotype and pluripotent differentiation capacity. An important feature of the current ECA-based culture method is use of Pinacidil (or ROCK inhibitor) at the time of passaging. Pinacidil is a small molecule that has been widely used for years as a vasodilating drug. It mimics efficiently the effect of ROCK inhibitors in supporting the attachment and viability of dispersed stem cells [32–34]. Even after, more than 15 passages on ECA, the benefit from use of Pinacidil remained unchanged. This suggests that our culture method does not select for an adapted subpopulation that over time survives on the single-compound matrix. The teratoma formation and hepatocyte differentiation experiments suggest that long-term culture on ECA does not alter the differentiation capacity of the cells. However, further studies of various differentiation pathways are needed to confirm this.

Lectins, the carbohydrate-binding proteins, are identified in a great number of animal cells. The carbohydrate structures to which ECA binds, terminal *N*-acetyl lactosamine (Galβ1-4GlcNAc) and 2′-fucosyl-*N*-acetyl lactosamine (Fucα1-2Galβ1-4GlcNAc) are frequently found in cell glycoproteins and glycolipids of mammalian origin, also in undifferentiated hESC and their early differentiation derivatives [23,35,36]. Also galectins, the mammalian counterparts of ECA have been implicated in many biological phenomena, including cell–cell and cell–ECM adhesion [37].

Human galectin-1 has binding specificity related to ECA and in preliminary experiments, we were able to support both hESC attachment and undifferentiated proliferation by using recombinant galectin-1 (unpublished results). In general, the adhesion activity of lectins correlate with the presence of its receptors on the cell surface [38]. However, the affinity between a lectin and its receptor may vary a great deal due to small changes in the carbohydrate structure of the receptor and it is difficult to identify the key interactions mediating a cell's binding to ECA.

Various specific glycan structures are known to be expressed in stem cells in developmentally regulated fashion [23,39]. However, little is known about their potential biological functions, but a plausible mechanism is interaction with cellular lectins. Adhesion molecules, including fibronectin and CD44, contain functionally important lectin domains mediating interaction with the ECM. The use of lectins as cell culture matrixes is not self-evident. For example, lectins binding to mammalian cell surface can be mitogenic [21,22], and the β-galactoside-binding lectin ricin is highly toxic. This was also demonstrated by our initial functional experiments; only few of the tested lectins that bound to hESCs supported their initial attachment, and only ECA was able to support continuous cell culture. The capacity of ECA to act as a support for undifferentiated growth is likely to depend on high-affinity cell-type specific adhesion, which is not associated with any toxic or differentiation-inducing effects. There is also evidence for the activation of specific growth-stimulating signaling pathways after binding of mouse ESCs to a β-galactoside-binding lectin, galectin-1 [40]. However, we did not observe direct mitogenic effects for ECA.

The primary role of a cell culture matrix is to stimulate the cell-dependent attachment response. In addition, the matrix provides signals for growth and differentiation of the cells. However, these functions may be less important in a complete complex culture medium. Adhesive epitopes, which often are glycans, and their density on the substrate as well as combination with other ligands influence the cell adhesion. Previous reports have indicated that at least laminins 511 and 111 as well as vitronectin and fibronectin are effective for the attachment and growth of undifferentiated hPSC [11,14,15,41]. However, these large and complex proteins are difficult and expensive to produce. Using currently commercially available preparations, the cost of plate coating is approximately similar with ECA and Matrigel (1:20). When comparing costs and benefits of ECA and Matrigel, the cell expansion is clearly more effective on ECA, saving both money and time. In our hands, 10,000 hESCs plated on ECA produce over 2 million cells during 8–10 days while on Matrigel this takes 12–14 days. For many purposes, clonal expansion is essential, and this is significantly better on ECA than on Matrigel.

Overall, our results suggest that lectins may serve as a natural platform for culture and differentiation of stem cells. Lectins may also provide a simple and cost-effective platform for studies of additional signals in self-renewal and differentiation. Based on the efficient expansion of karyotypically and functionally intact cells, recombinant ECA is a useful GMP-applicable alternative as a defined coating substrate for hPSC culture.

Acknowledgments

The authors are grateful to Eila Korhonen, Heli Mononen, Jaan Palgi, and Jarkko Ustinov for help with cell culture and analysis. This study was supported by the Finnish Funding Agency for Technology and Innovation (TEKES), the ES-TOOLS project of the EU 6th FP, and the LIV-ES project of the 7th FP. Further support was received from the Academy of Finland and the Sigrid Jusélius Foundation.

Author Disclosure Statement

T. S., J.N., and J.S. are shareholders of Glykos Finland Ltd.

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Received for publication June 29, 2012
Accepted after revision October 27, 2012
Prepublished on Liebert Instant Online XXXX XX, XXXX