Human embryonic stem cells : advancing biology and cardiogenesis towards functional applications l

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CHAPTER FIVE

Recombinant vitronectin is a functionally defined substrate

that supports hESC self renewal via αVβ5 integrin

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Abstract

Defined growth conditions are essential for many applications of human embryonic stem cells (hESC). Most defined media are presently used in combination with Matrigel™, a partially defined extracellular matrix (ECM) extract from mouse sarcoma. Here, we defined ECM requirements of hESC by analyzing integrin expression and ECM production and determined integrin function using blocking antibodies. hESC expressed all major ECM proteins and corresponding integrins. We then systematically replaced Matrigel™ by defined media supplements and ECM proteins. Cells attached efficiently to natural human vitronectin, fibronectin and Matrigel™ but poorly to laminin+entactin and collagen IV. Integrin blocking antibodies demonstrated that $\alpha V\beta 5$ integrins mediated adhesion to vitronectin, $\alpha 5\beta 1$ to fibronectin and $\alpha 6\beta 1$ to laminin+entactin. Fibronectin in feeder cell-conditioned medium partially supported growth on all natural matrices but in defined, non-conditioned medium only Matrigel™ or (natural and recombinant) vitronectin were effective. Recombinant vitronectin was the only defined functional alternative to Matrigel™, supporting sustained self-renewal and pluripotency in three independent hESC lines.

Introduction

Human embryonic stem cells (hESC) are self-renewing, pluripotent cells with expected clinical applications in cell therapy, tissue repair and drug development because of their ability to differentiate to all cell types present in the adult body. Since the first derivation of hESC[1], many lines have been generated under a wide variety of culture conditions. Most have included mouse or human feeder cells to inhibit differentiation[2] but also to maintain karyotypic stability[3,4]. In addition, many studies have shown a consistent requirement for FGF2, either secreted by the feeders or added as recombinant protein[5,6]

Attempts to replace feeder cells have shown that hESC can be maintained on Matrigel™ [7], a laminin-111-rich, mouse sarcoma-derived commercial product also containing collagen IV, entactin, heparin sulfate proteoglycan and multiple other components including growth factors, but no vitronectin. More recently defined culture conditions that use a matrix of natural human collagen IV, fibronectin, laminin and vitronectin have been described[8] but whether a specific matrix integrin interaction is essential is unclear. In addition, protein products from natural sources tend to show batch-to-batch variability. In a recent comparison of several commercial human laminin preparations, for example, both compositional and functional differences were found between batches, with highly fragmented proteins, mixtures of isoforms and/or contaminating fibronectin detected in variable amounts[9]. This is an important concern in attempts to understand the role of matrix-integrin interactions in pluripotency and self renewal. Therefore we aimed to establish culture conditions based entirely on recombinant / synthetic substrates.

Integrins are the cell surface receptors that mediate cell-ECM adhesion and signaling[10]. Previously, a number of integrins were detected in hESC by RT-PCR. Among them were α6 and β1 which mediate binding to matrigel™ [7]. Integrins are heterodimeric transmembrane molecules with large extracellular domains and relatively small cytoplasmic domains formed by α and β subunits that can switch between inactive and active conformations. In the inactive state, integrins have a low affinity for ECM proteins. Intracellular signaling can prime the integrins, resulting in conformational changes that expose the ligand-binding site. Ligand binding activates signaling cascades that lead to the assembly of multiprotein focal adhesion signaling complexes at the site of cell adhesion to the ECM. This causes cells to assemble connections between the ECM and the actin cytoskeleton and induces many intracellular signaling pathways. The pathways that are activated depend on the particular ECM ligand-receptor interaction; they may give rise to cytoskeletal changes that result in different migratory behaviour or to changes in growth and/or differentiation[10].

In nearly all cell types, integrin binding activates focal adhesion kinase (FAK)[11]. FAK disseminates integrin signals by forming complexes with signaling proteins that have numerous effects on cell function like proliferation, differentiation and apoptosis[12].

While functional integrin-ECM interaction is clearly crucial for derivation, self renewal and maintenance of pluripotency by hESC, the ECM constituents and their role in signaling have not been systematically investigated. Here we have determined integrin and ECM protein expression profiles in three independently derived hESC lines (HES2, HUES1 and HESC-NL3), with a history of maintenance and culture under widely differing conditions. Functional analyses using integrin blocking antibodies showed that $\alpha V\beta 5$ integrins were responsible for adhesion to vitronectin, $\alpha 5\beta 1$ to fibronectin and $\alpha 6\beta 1$ to laminin+entactin in all three cell lines. Attachment of cells to recombinant vitronectin through $\alpha V\beta 5$ was demonstrated to be sufficient to support proliferation and self-renewal in chemically defined (mTeSR1) medium. Stem cell marker expression and pluripotency *in vitro* was retained over multiple passages without loss of karyotypic stability. Recombinant vitronectin matrix therefore represents a defined functional alternative for Matrigel™ in a chemically defined medium.

Material & Methods

CELL CULTURE AND MATRICES

HUES1 are routinely cultured[13] on CD1 mouse embryonic fibroblast feeders (MEFs) (kind gift of Dr. C. Denning) in KO-DMEM (Invitrogen) containing 10% Plasmanate and 10% Knock Out-serum replacement (KSR) (Invitrogen), HES2[14] on 129SV MEFs as described previously [15] and HESC-NL3 on human foreskin fibroblast feeders in Knockout DMEM/ KSR supplemented with bFGF[16].

Furthermore HUES1 cells were cultured feeder-free as a monolayer on Matrix Growth Factor Reduced Matrigel (BD Biosciences) in MEF conditioned medium. Trypsin/EDTA (Invitrogen) was used to release the cells which were then dissociated to a suspension of single cells and small clumps[17,18]. For the preparation of conditioned medium (CM), MEFs (mouse strain CD1) were mitotically inactivated by mitomycin C (MMC) (10 µg/ml, 2.5-3 hours) and seeded at 4,6 x 10^6 cells per 75 cm^2 flask. The next day, MEFs were incubated with 25 ml of hESC medium (Glutamax based DMEM-F12 supplemented with 15% KSR, 100 µM β-mercaptoethanol, 1% non-essential amino acids (all from Invitrogen) and 4ng/ml basic fibroblast growth factor (Peprotech). CM was harvested after 24 hours and supplemented with 4ng/ml bFGF. This procedure was repeated for 7 consecutive days by replacing CM on MEFs with fresh hESC medium.

The ECM protein concentrations for coating culture plates were determined in dose response curves (Figure 5.2A). ECM concentrations used for integrin blocking assays were: 10 ng/µl collagen IV (isolated from human placenta; Chemicon), 40 ng/µl fibronectin (isolated from human plasma; Harbor Bio-products, Norwood, MA), 10 ng/µl vitronectin (isolated from human plasma; Chemicon), 50 ng/µl laminin (laminin-111), 50 ng/µl mLaminin+entactin and Matrigel™ (84 ng/ul) (all isolated from Engelbreth-Holm-Swarm mouse tumor; BD Biosciences). Throughout the manuscript the new nomenclature for laminins is used, i.e. indicating the three chains of each isoform [19].

For feeder-free defined cultures all three lines were cultured in mTeSR1 (Stem Cell technologies) on recombinant vitronectin (5ng/µl) (R&D systems). Cells were grown in colonies as shown in Figure 5.4C-E. Once weekly, the colonies were dissected using glass needles into small pieces of 50-80 µM and transferred manually to new dishes without enzymes[20]. A minority of colonies contained some differentiated cells at the centre; these areas were omitted from transfer. Directed differentiations to meso- endo- and ectoderm were performed as described previously[21].

FACS ANALYSIS

hESC cells were trypsinized, and stained with specific integrin antibodies (supplementary data) and FITC-conjugated secondary antibody. Cells were analyzed on a FACSCalibur or a FACSCanto (BD Biosciences).

IMMUNOFLUORECENT STAINING

Cells on plastic coverslips (Sarstedt Inc, Newton, NC) were fixed with 2% paraformaldehyde and permeabilized with 0.5% Triton X-100. Cells were stained using specific primary antibodies (supplementary data) and fluorophore conjugated secondary antibodies; when applicable cells were co-stained with phalloidin-TRITC (Sigma). Nuclei were stained with TOPRO-3 (Invitrogen) or DAPI. Cells were mounted using Mowiol or using Vectashield hard set with Dapi (Vectalabs). Plastic coverslips were immobilized on glass microscope slides and sandwiched with glass coverslips. Pictures were taken using Laser Confocal Scanning Microscopes TCS-SP2 and TCS-SPE (Leica, Mannheim, Germany).

ADHESION ASSAY

Adhesion assays were essentially performed as described previously[22]. In brief, hESC were trypsinized, counted and incubated for 30 min. at 37°C in the presence or absence of integrin blocking antibodies in IMDM (Invitrogen) containing 0.35% bovine serum albumin (BSA). Purified antibodies were tested at 1 and 10 µg/ml concentrations and hybridoma supernatants at 1/5 and 1/50 dilutions. Dilutions appropriate for blocking were used in all subsequent assays.

They were subsequently seeded into 96-well flat-bottom plates, which were previously coated with various matrix components and blocked with 2% BSA, at 50,000-100,000 cells/well, depending on the substrate. They were left to adhere for 30 min at 37°C. The non-adherent cells were carefully washed away with IMDM/0.35% BSA three times, and finally once with PBS. The adherent cells were fixed with 100% ethanol for 5 min at RT and stained with 0.4% crystal violet in methanol for 5 min at RT. The wells were washed extensively with demi-water. The cells were solubilized by adding 40 µl 1% SDS and cell adhesion was determined by measuring OD_{570}. Results are expressed as relative adhesion normalized to one. Blank controls were without cells but otherwise treated identically to controls

INTEGRIN BLOCKING ASSAYS

HUES1 from Matrigel™ were trypsinized and replated on the indicated matrix in the presence or absence of integrin blocking antibodies. For αVβ5 antibody (Chemicon) a concentration of 2 µg/ml was used, for AIIb2 (anti-β1 integrin) the supernatant was diluted 50 times. The cells were allowed to grow in MEF-CM for 4 days and the medium containing the antibodies was refreshed every day. Coverslips were stained for Tra-1-60, human nuclei and fibronectin or Tra-1-60, phalloidin and OCT4 as described above

CELL EXPANSION ASSAYS

HUES1 from Matrigel™ were trypsinized and replated on the indicated matrix. 4 hours later GoH3 1 µg/ml and / or Alpha5 blocking antibody (Chemicon) 1 µg/ml was added. Cell proliferation was assayed using CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) at 24, 48, 72 and 96h post plating. MEF-CM was refreshed daily. As required, integrin blocking antibodies were added freshly, daily.

KARYOTYPE ANALYSES

hESC cultured on recombinant vitronectin in mTeSR1 were treated with 100 ng/ml colcemid (Karyomax) for 1 hr. hESC colonies were dissociated using TrypLE (Invitrogen). After hypotonic solution treatment (0,6 % citrate buffer), lysed cells were fixed in methanol/glacial acetic acid (5:1). G banding was performed for identification of chromosomes, 30 metaphase spreads were examined; full analysis involving band-by-band comparison between chromosome homologues was performed on 10 spreads, and the presence of gross abnormalities was visually examined in the remaining spreads.

Results

EXTRACELLULAR MATRIX EXPRESSION IN HESC CULTURES

Understanding the composition of ECM components in hESC cultures on feeder layers may provide clues on which substrates favor sustained undifferentiated proliferation of hESC. MEFs are most commonly used and produce a complex matrix of many structural ECM proteins including laminins, various collagens and fibronectin[23]. Detail is lacking on the exact composition of feeder derived matrix components making it difficult to predict which components are essential versus those that are redundant. In addition the exact interaction between hESC and feeder-derived ECM has not been investigated in detail. Therefore we first immuno-stained ECM proteins in three hESC lines that have all been derived and cultured under completely different culture conditions. HUES1[13], is cultured on MEFs in plasmanate/KO-SR containing medium and passaged by trypsinization. HES2[14] is cultured on MEFs in FCS-containing medium and passaged mechanically using dispase. HESC-NL3 was derived in our lab on human foreskin feeders (HF), and is cultured in serum replacement medium and passaged mechanically without the use of enzymes.

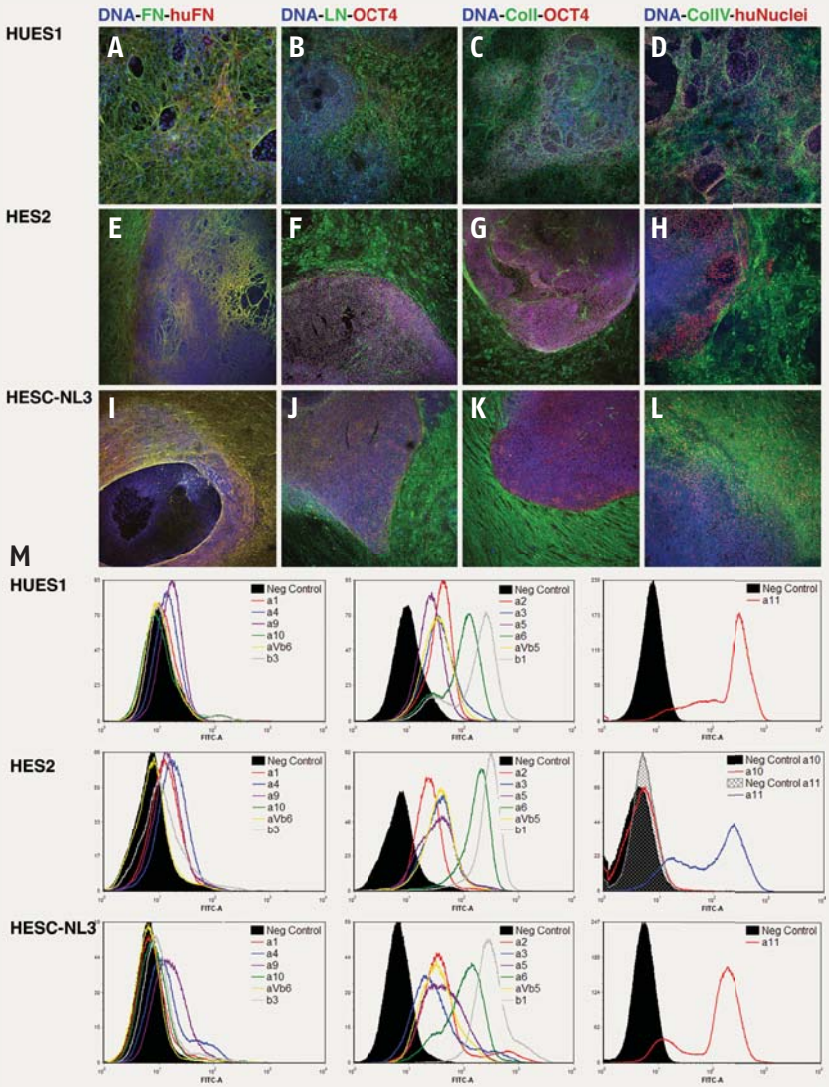

Figure 5.1

Extracellular matrix and integrin protein expression in hESC cultures

HUES1, HES2 and HESC-NL3 stained for the ECM components fibronectin, laminin-α1, collagenI and collagenIV. (A,E,I) polyclonal anti-fibronectin antibody recognizing both mouse and human fibronectin is shown in green, human fibronectin (mAb) is shown in red. (B,F,J) polyclonal anti laminin-α1 recognizing both mouse and human is shown in green, with OCT4 shown in red. (C,G,K) polyclonal anti-collagenI recognizing both mouse and human is shown in green, with OCT4 shown in red. (D,H,I) CollagenIV is shown in green, with human nuclei in red. In all pictures nuclei are counterstained in blue with TO-PRO (M) FACS analysis for the majority of integrin chains on the three hESC lines. All integrins tested were expressed at essentially the same level on the three lines.

These diverse hESC lines were selected to address the question whether culture conditions themselves caused hESC to adapt with respect to their molecular mechanisms for cell–cell or cell substrate interactions.

As shown in Figure 5.1A, E and I large quantities of fibronectin were present in all three cultures under native conditions. There is abundant staining of fibronectin in the feeder areas, which is not recognized by the antibody specific for human fibronectin, and therefore appears to be of mouse origin. (Figure 5.1E and not shown). However, human fibronectin was found at the edges and at the center of the hESC colonies (Figure 5.1E and not shown). These are the areas that usually contain mostly differentiated hESC. It is most likely, therefore, that fibronectin is mainly secreted and assembled into bundles by the feeders and differentiated hESC, although we cannot exclude secretion by undifferentiated hESC. Importantly the human specific fibronectin antibody stained the human fibroblast feeders (HFs) exclusively (Figure 5.1I) but not the MEFs (Figure 5.1A,E). Both Laminin and collagen I were also found predominantly in the feeder area, again suggesting mouse origin (Figure 5.1B,F,J and 5.1C,G,K). However there was some staining between hESC. Collagen IV was found exclusively in the feeder area, again suggesting mouse origin (Figure 5.1D,H,J). Immunolabeling with anti-vitronectin antibodies from multiple sources resulted in no obvious staining. Thus a number of major ECM components were present in the native cultures of three different hESC lines, primarily associated with the feeders or differentiated cells around the hESC, but independent of the type of feeder used and method of passage or culture medium.

INTEGRIN EXPRESSION IN HESC

Undifferentiated hESC need signaling factors from feeders for undifferentiated growth. This signaling may be mediated by growth factors but also ECM integrin interactions may contribute to signaling. Four major ECM components were present in hESC cultures, most of them secreted by the feeder cells, as described above. However, it is not known which ECM receptors on hESC mediate adhesion to the matrix present in the cultures. An important class of ECM receptors is the integrin family of proteins. In order to further investigate ECM–hESC interaction at the molecular level we determined cell surface expression of the majority of the α- and β-integrin chains by FACS analysis (Figure 5.1M). The three hESC lines tested exhibited comparable integrin expression profiles. Integrin chains α1, α4, α10, β3 and integrins α9β1 and αVβ6 were not detected or at very low levels (Figure 5.1M, left panels). Integrin chains α2, α3, α5, α6, α11, β1 and integrin αVβ5 were expressed (Figure 5.1M, middle and right panels). These integrin chains can form the combinations α2β1 (binding collagen, laminin), α3β1 (binding nidogen, laminin, collagen I, fibronectin), α5β1 (binding fibronectin), α6β1 (binding laminin-111, 211 and 411), α11β1 (binding collagen), αVβ5 (binding vitronectin, fibronectin) and αVβ1 (binding fibronectin). Thus hESC have cell surface receptors for all major ECM components, independent of their method of derivation or culture (Figure 5.1M). Even though α4 and α9β1, and sometimes α1 or β3, were detected at low levels by FACS analysis, immunofluorescent staining of these integrins showed only background levels (not shown) and are therefore unlikely to play a major functional role in adhesion. By contrast, integrin

chains α2, α3, α5, α6, α11, β1, and integrin αVβ5 all showed distinct plasma membrane localization in the three hESC lines (Figure S1). Furthermore independent mass-spectroscopy analyses of membrane proteins from a fourth hESC line, HUES7, confirmed the presence of α2, α3, α5, α6, αv, and β1 integrin peptides[24] (Table S1). The peptides identified were blasted against reference databases, confirming their human origin. In summary, it is evident that the collagen receptor α2β1 and α11β1, the fibronectin receptor α5β1, the vitronectin receptor αvβ5 and the laminin receptor α6β1 are expressed on all three hESC lines.

Next we characterized the ability of hESC to attach to various ECM coated substrates. Since we observed no major differences in integrin expression profiles among the three tested lines, HUES1 was chosen for a detailed dose-response curve of their adhesion to laminin-111, laminin-111+entactin, Matrigel™, collagen IV, fibronectin and vitronectin (Figure 5.2A). Monolayer HUES1 cultures using Matrigel™-coated substrates in KSR-supplemented MEF conditioned medium (CM) have already been shown to be beneficial for cell manipulation. These conditions support undifferentiated proliferation of various hESC lines and to maintain their karyotype stably for at least 10 passages[17]. Since this system is based on trypsin passage it was clearly beneficial for attachment assays. Attachment was very efficient on vitronectin and Matrigel™ but poorer on fibronectin and collagen IV. Attachment to pure laminin-111 was extremely weak. Since laminin-111 and Matrigel™ are both isolated from the EHS tumor this suggests that the rigorous chemical purification of the commercial laminin product destroys its functionality. Indeed the less pure laminin+entactin combination from the same source rescued the binding to some extent (Figure 5.2A). Therefore throughout the manuscript laminin+entactin will be used when referring to laminin-111.

FUNCTIONAL VALIDATION OF DETECTED INTEGRINS

To investigate the functionality of these integrins in HUES1 further, we performed short term adhesion assays but now in the presence of specific integrin blocking antibodies. hESC were first incubated with the antibodies, then allowed to adhere mouse laminin+entactin, fibronectin, vitronectin or collagen IV (Figure 5.2B).

Both anti-α6 and anti-β1 antibodies significantly reduced adhesion of hESC to laminin+entactin, indicating that α6β1 was indeed functional in binding its major ligand laminin-111 in hESC (Figure 5.2B). Blocking the integrin chains that make up the fibronectin receptor α5β1, as expected, significantly impaired adhesion to fibronectin, while blocking the vitronectin receptor αVβ5 reduced binding to vitronectin (Figure 5.2B). The functionality of α2β1 and α11β1 could not be tested, as there where no specific blocking antibodies available. Taken together α6β1 was shown to mediate adhesion of hESC to laminin-111, α5β1 to fibronectin and αVβ5 to vitronectin.

THE VITRONECTIN RECEPTOR αVβ5 RESCUES THE β1 INTEGRIN BLOCK

Since most functional integrin combinations rely on the β1 chain, we examined whether this chain was a specific signaling intermediate crucial for stem cell proliferation by adding

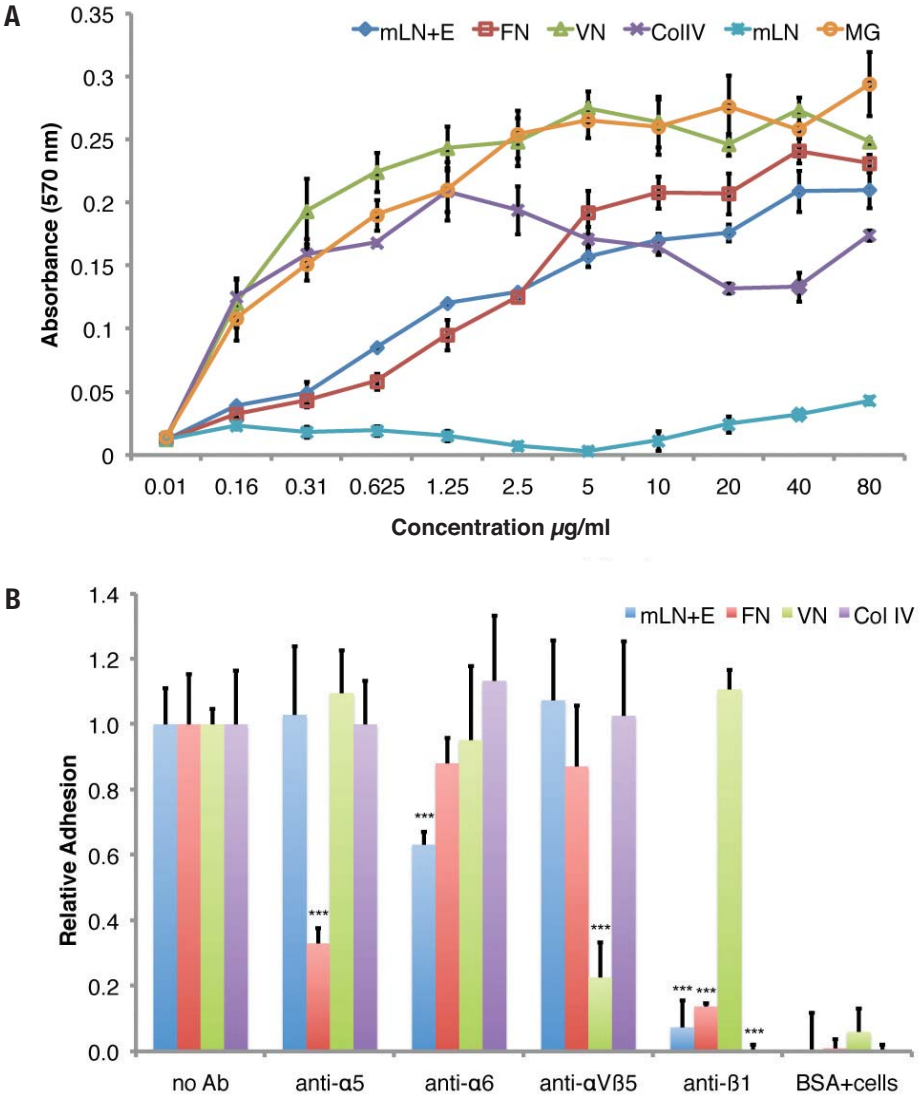

Figure 5.2

hESC Integrin function in initial substrate adhesion

(A) HUES1 cell attachment dose response binding curve to various natural matrices, (B) Functional validation of integrin binding capacity on various matrices using integrin specific blocking antibodies. *** p≤0.01. Data from at least three independent experiments were collected and displayed as average +/- SEM.

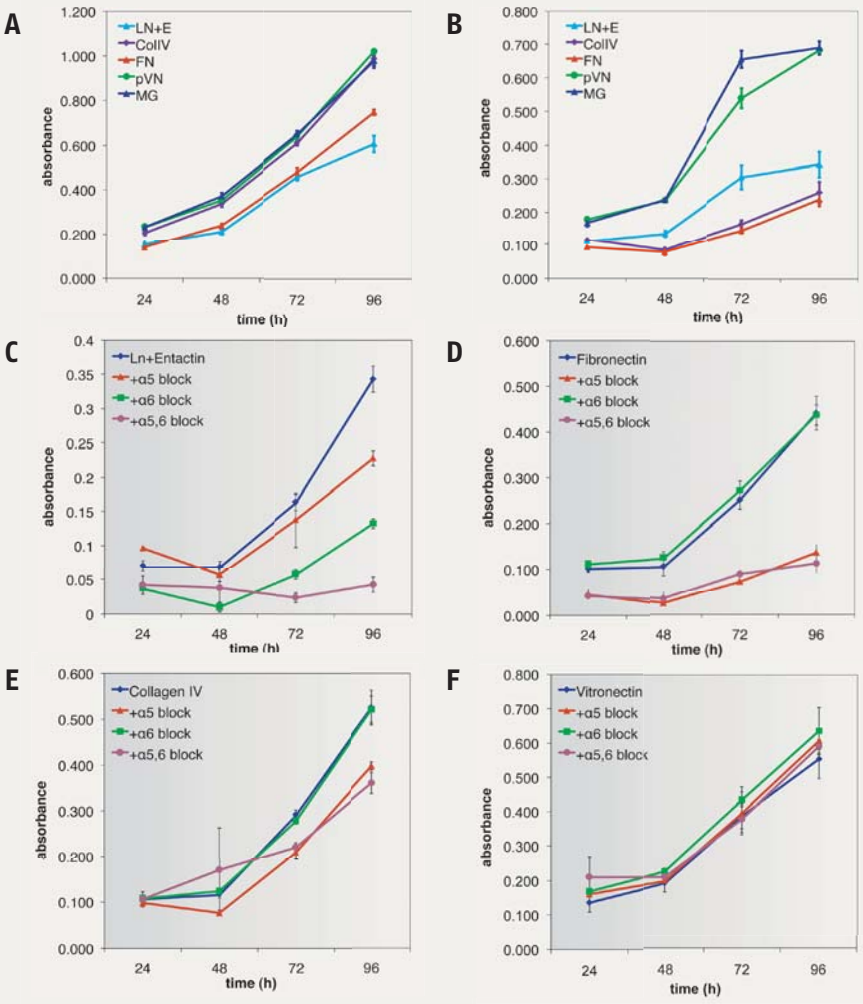

Figure 5.3

hESC expansion in various media is matrix dependent

HUES1 cells were trypsinized and replated in triplo in 96-well plates on various matrices. Cell proliferation was measured quantitatively at different time-points (A) HUES1 cell expansion on various substrates in MEF-CM, (B) HUES1 cell expansion on various substrates in mTeSR1, chemically defined medium. (C-F) HUES1 cell expansion assays in the presence or absence of integrin α5 and α6 blocking antibodies which were added 4-hours post-plating to circumvent differential attachment on (C), Ln+entactin (D), fibronectin (E) collagen IV and (F) vitronectin. Experiments were repeated at least three times, one representative experiment is shown here. Each timepoint represents the mean absorbance of a triplicate, +/- SEM.

blocking β1 antibodies to HUES1 cultured on Matrigel™. This completely inhibited attachment/proliferation, while blocking the vitronectin receptor αVβ5 had as expected no effect (Figure S2). The combination of Matrigel™ with vitronectin but not fibronectin however, rescued the β1 block, whereas the combination of Matrigel™ and fibronectin had no effect (Figure S2). The rescue of vitronectin was mediated by the αVβ5 integrin since a combination of β1 and αVβ5 blocking antibodies blocked the attachment and growth completely (Figure S2). These results indicated that the attachment and growth of hESC not necessarily require binding via the β1 chain but that ligation of αVβ5 to its ligand vitronectin could act as a functional substitute to mediate adhesion and support undifferentiated growth.

MATRIGEL™ AND VITRONECTIN SUPPORT ROBUST HESC PROLIFERATION IN DEFINED MEDIA

To test the functionality of hESC expressed integrins further, we examined the ability of purified ECM preparations to support proliferation of HUES1 in MEF conditioned media and mTeSR1. As expected, cells cultured in both media proliferated well when seeded on Matrigel™ coated plates, as reported previously[7,25]. However on all other matrices tested there was a clear difference between mTESR and MEF conditioned medium. Cells cultured in mTeSR1 supported hESC growth exclusively on purified vitronectin but not on fibronectin, laminin and collagen IV while MEF conditioned medium supported hESC growth on any tested substrate (Figure 5.3A,B). To test these effects further, cells cultured in MEF-CM on various substrates were stained for OCT4 expression (Figure 5.3) and cultured for extended periods on the same ECM coated substrates. All cells retained OCT4 expression although we observed higher culture expansion rates of hESC on Matrigel™ and vitronectin (passage 7), than on laminin, fibronectin and collagen IV (passage 5) after one month of continuous culture. A possible explanation for the observed differences between MEF-CM and mTeSR1 would be the presence of additional factors present in KSR or secreted by the feeders that mediate binding of hESC to their substrate. FIBRONECTIN and LAMININ were selected as candidate proteins mediating the observed difference in culture expansion rates between the two media. This was based on extrapolation of the data in Figure 5.1 and a published secretome analyses of human fetal, human neonatal and mouse embryonic feeder fibroblast respectively[26]. We therefore carried out culture expansion assays in MEF conditioned media in the presence or absence of α6β1 (laminin receptor) and α5β1 (fibronectin receptor) blocking antibodies on laminin+entactin, fibronectin, collagen IV and vitronectin (Figure 5.3C-F). Surprisingly, α5β1 reduced culture expansion on collagenIV and laminin+entactin, indicating that fibronectin secreted by the feeders is a major bioactive molecule in MEF conditioned medium. As expected, blocking α6β1 reduced culture expansion on laminin+entactin without a significant effect on the other matrices tested and blocking α5β1 reduced culture expansion on fibronectin as expected. Interestingly, culture expansion on vitronectin was not impaired by blocking fibronectin signaling. This confirmed the suitability of vitronectin as replacement for Matrigel™.

hESC culture on a completely defined substrate

The aim of this study was to find defined synthetic ECM substrates for sustained self renewal of hESC without loss of pluripotency or changes in karyotype. We identified purified vitronectin as a candidate ECM protein for this purpose. Since human recombinant vitronectin is commercially available, we next tested whether this would be as effective as purified human vitronectin from plasma, preferably in combination with defined medium rather than medium conditioned by MEFs or HFs. HUES1 grown on plasma vitronectin expanded at the same rate as cells grown on recombinant vitronectin (Figure 5.4A) and used integrin $\alpha V\beta 5$ for cell attachment (Figure 5.4B). Next we tested the ability of recombinant vitronectin to support long term self-renewal in defined (mTeSR1) medium. For this we used all three lines used at the outset (HUES1, HES2 and HESC-NL3) to examine the general applicability of this culture method. Each of the lines proliferated robustly in a 'cut and paste' culture in continuous culture for up to 12 weeks, the maximum presently tested. Under these culture conditions hESC had a similar growth rate to conventional cultures grown on feeders cells and were passaged once a week. After minimally 5 weeks of culture (5 passages) on recombinant vitronectin in mTeSR1 medium cells were analyzed in detail for karyotypic abnormalities and pluripotency in culture.

Under these defined conditions cells grew in compact colonies (Figure 5.4B-D) without obvious morphological differentiation. Karyotypic analyses of HESC-NL3 and cultured on recombinant vitronectin showed a normal 46xy karyotype in all cells analyzed (Figure 5.4E). Maintenance of stemness was demonstrated by staining for the stem cell markers E-CADHERIN, GCTM2, OCT4, SOX2, SSEA-3 and SSEA-4 (Figure 5.4F-K). To examine their differentiation potential, hESC cultures on recombinant vitronectin in mTeSR1 were treated with growth factor supplemented N2/B27 medium as described previously [21]. Cell types derived from each of the three germ layers were present for each of the line. Endoderm differentiation was confirmed using antibodies against alpha-fetoprotein (AFP), ectoderm using antibodies against betaIII-tublin, a neuronal protein and mesoderm differentiation was confirmed using antibodies recognizing alpha-actinin, a cardiac protein (Figure 5.4L-N). These results confirmed the potency of our fully defined hESC culture system.

Discussion

For both clinical applications as well as addressing fundamental questions regarding hESC biology and the molecular nature of `stemness'. it is clearly beneficial to culture with fully defined culture substrates and media based on recombinant (or clinical grade) proteins[27]. We demonstrated here that hESC express integrin receptors for laminin, fibronectin, collagen and vitronectin and that all of these receptors are functional in mediating adhesion. MEFs and HFs secrete large quantities of at least laminin, collagen I, collagen IV and fibronectin. Although all cell lines attached and proliferated well on the major ECM proteins if grown in MEF-CM. However if the fully defined mTeSR1 medium was used, only vitronectin supported

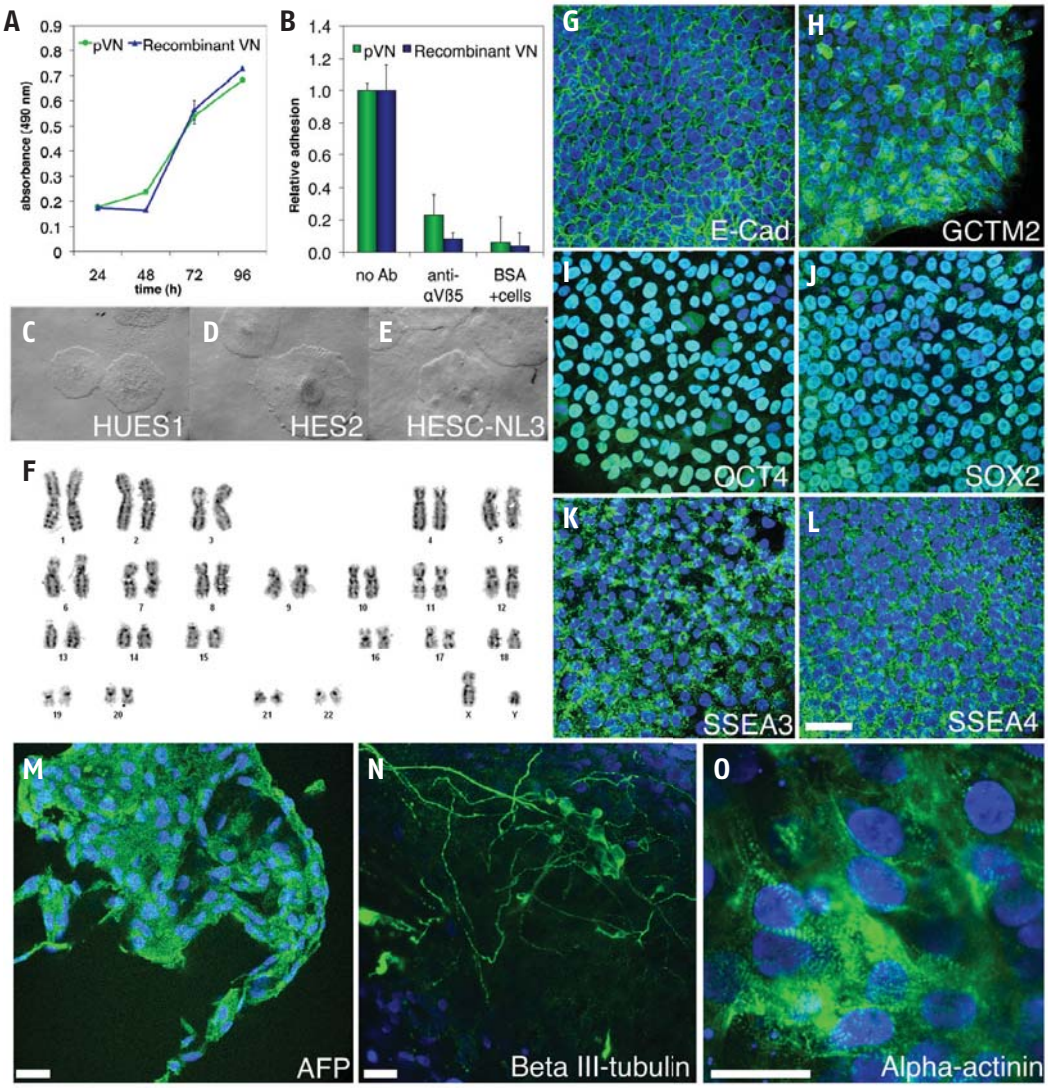

Figure 5.4

recombinant vitronectin supports hESC in mTeSR1

(A) HUES1 cell expansion assay on plasma Vitronectin (pVN) and recombinant vitronectin. (B) HUES1 cell attachment in the presence and absence of integrin αvβ5 blocking antibodies on both plasma vitronectin and recombinant vitronectin (C–E) representative brightfield pictures of HUES1, HES2 and HESC-NL3 cultured for 8 passages on recombinant vitronectin in mTeSR1 (F) Karyogram of HESC-NL3 cultured for 7 passages on recombinant vitronectin in mTeSR1 showing a normal diploid 46,xy karyotype (G–L) Immunostaining for stem cell markers and DNA for HESC-NL3 (overlay). (G) E-cadherin, (H) GCTM2, (I) OCT4, (J) SOX2, (K) SSEA3 (L) SSEA4. (M–O) Immunostaining for HESC-NL3 differentiation markers representing the three germ layers (M) AFP, endoderm (N) beta III tubulin, ectoderm and (O) α-actinin, mesoderm. Scale bars, 50 μm.

hESC growth as well as Matrigel™. We identified recombinant vitronectin as a suitable and functional alternative for Matrigel™. Three independently derived cell lines (HUES1, HES2 and HESC-NL3) were cultured long term on recombinant vitronectin in mTeSR1 medium and all maintained their characteristic compact morphology, expression of stem cell markers, normal karyotype and differentiation potential, demonstrating the general applicability of these conditions.

Most of the >400 hESC lines derived to date have been traditionally cultured on a feeder layer of MEFs to maintain self renewal and pluripotency. It was of interest to note in our study that MEF conditioned medium reduced the stringent ECM requirements for hESC i.e. fibronectin, collagen IV and laminin could replace Matrigel™ as well as vitronectin. We showed that feeder cells produce a highly complex matrix consistent of several ECM molecules and that secreted fibronectin is likely responsible for this loss of matrix stringency. However, it is of interest that FGF2 signaling, the primary signaling pathway for hESC self-renewal, is highly dependent upon the ECM. FGF family members bind to the extracellular matrix (ECM) becoming concentrated in specific regions where they are protected from proteolytic degradation[28]. FGF receptor activation is dependent upon heparan sulphate side chains on either ECM or cell surface proteoglycans[29]. Many of the growth factor signaling pathways are shared with integrin pathways[30]. ECM modulated signals may convergence with FGF signaling and be involved in the maintenance of "stemness".

Indeed, it has been suggested that integrin signaling in mouse ES cells converges with growth factor signaling to maintain stemness. Recently is has been shown that cells cultured on type I and IV collagen or poly-d-lysine remained undifferentiated while laminin or fibronectin induced differentiation[31]. Unfortunately vitronectin was not investigated in this study. However, as many studies have shown, results obtained in mouse ES cannot always easily be translated to hESC, particularly with respect to cell surface proteins and signal transduction. This is likely related to their different state of differentiation[32,33]. Nevertheless it might be interesting to study the relationship between integrin signaling in differentiation and self-renewal of hESC in more depth in the future.

Almost all of our knowledge related to hESC is based on studies carried out under the influence of feeder cells and/or serum. This might explain several inconsistencies in the literature, for example; the debated role of Activin A in hESC self-renewal[6,21]. Over recent years, a number of papers have described defined media for hESC but the majority made use of Matrigel™ [6,21,25,34-37] which is still incompletely defined. Apart from laminin, entactin, collagen and heparin sulfate proteoglycan Matrigel™ contains several growth factors, even in its growth factor reduced variant. Among these is TGF-β which might contribute to hESC self-renewal[38]. Therefore, under Matrigel™ culture conditions it is difficult to define growth factor requirements for undifferentiated growth or directed differentiation. Furthermore, Matrigel is derived from a tumour of mouse origin, a potential xenorisk, since it contains the non human sialic acid Neu5Gc, an immunogenic molecule that might be transferable to hESC[39].

Based on the work of Xu et al (ref 7) in which $\alpha 6\beta 1$ integrin was detected on the cell surface of hESC and feeder-free culture of hESC on Matrigel™ in MEF-CM was described, it has been generally assumed that laminin is the most important ECM protein for hESC in culture. Indeed hESC culture on purified laminin has been reported[7,40]. Both studies were however performed in MEF-CM. It is likely that these results were influenced by ECM proteins secreted by MEFs. Since feeders secrete fibronectin[26] and blocking the fibronectin receptor $\alpha 5\beta 1$ reduced hESC expansion on laminin and collagen IV it is likely that fibronectin is the major bioactive protein in MEF-CM in this respect. In fact, it has been reported that purified fibronectin supports hESC growth even in semi-defined hESC medium[41]. Here we observed even better cell growth and attachment on purified vitronectin. Furthermore, on vitronectin we observed no delay in cell expansion by blocking fibronectin signaling indicating that ligation of hESC through $\alpha v\beta 5$ is sufficient for hESC growth.

The recombinant vitronectin, used was produced in the mouse myeloma cell line, NS0 and therefore could potentially contain Neu5Gc. However, biotechnological innovations may and should overcome this problem. Previously, yeast was successfully genetically engineered to produce humanized glycosylated proteins[42]. Another possibility could be to use human protein expression platforms, such as the PER.C6 cell line[43]

Concurrent with the present study, two papers reported defined media for hESC culture. Gerecht et al. (2007) described a defined hydrogel based on hyaluronic acid. For routine use however, this approach has certain drawbacks: cells are difficult to access for subsequent analysis and use and encapsulation of cells depends on UV photoactivation. This might cause point mutations in the DNA. Furthermore the authors did not demonstrate serial passage, prolonged culture and karyotypic stability in this system[44]. Derda et al. used SAM surface arrays to detect peptides derived from laminin suitable for hESC-self-renewal[45]. The biological relevance of that study might be limited by the fact that surface arrays were coated with serum which contains high levels of fibronectin and the use of MEF-CM. As shown in our study fibronectin from MEF-CM mediated binding on several substrates that were not supportive in defined media. This illustrates the importance of media and substrate development progressing in parallel.

In conclusion; we defined a culture system for hESC based entirely on human/recombinant proteins that can be used for long-term self-renewal and selected differentiation of hESC. This will be essential for both basic research on hESC pluripotency and clinical applications.

SUPPLEMENTAL DATA

Supplemental Data contains three Supplemental Figures and two supplemental tables which can be found on-line at http://www3.interscience.wiley.com/journal/121587696/suppinfo

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