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A Synthetic Substrate to Support Early Mesodermal Differentiation of Human Embryonic Stem Cells

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Abstract

Our ability to guide differentiation of human pluripotent stem cells (hPSCs) toward desired lineages efficiently and reproducibly in xeno-free conditions is the key to advancing hPSC technology from the laboratory to clinical use. Here we report an engineered biomimetic substrate functionalized with both peptide ligands for $\alpha 5\beta 1$ and $\alpha 6\beta 1$ integrins to support efficient early mesodermal differentiation of human embryonic stem cells (hESCs) when cultured in a differentiation medium containing BMP4. In contrast, mesodermal differentiation is not induced on substrates functionalized with either ligand alone even though the culture medium is identical. Mesodermal differentiation was characterized by immunofluorescent staining, flow cytometric analysis, and RT-PCR analysis of early mesodermal markers Brachyury, Mixl1, and Wnt3. The early mesodermal progenitors derived on the substrate functionalized with both integrin ligands have the normal developmental potential to further differentiate along the hemato-endothelial and cardiac lineages. Immobilized ligands for $\alpha 5\beta 1$ and $\alpha 6\beta 1$ integrins both are permissive, necessary, and sufficient insoluble ligands in this engineered system to support early mesodermal differentiation of hESCs. This synthetic substrate, in conjunction with defined soluble factors, constructs a well-controlled and xeno-free early mesodermal differentiation niche that offers advantages over the previously reported niche constructed with the Matrigel-coated substrate.

1. Introduction

Cell-based therapy represents an important strategy for treatment of many human diseases such as ischemic diseases, degenerative disorders, and trauma-related tissue loss [1-3]. However, its success has been hampered by limited availability of desired cells. The recent advances in stem cell biology offer an opportunity to address this challenge. In particular, human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs), are able to proliferate extensively and differentiate into all cell types, virtually providing unlimited cell sources [4,5]. The recent success in developing iPSCs from adult cells offers additional advantages in providing immunologically compatible autologous hPSCs and enabling “personalized” therapy in the

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future [6,7]. However, one of the limitations in advancing hPSC technology from the laboratory to clinical use is that most current differentiation methods, such as those with embryoid bodies (EBs), on mouse stromal cells, or on Matrigel-coated surfaces, are not able to guide hPSC commitment toward desired lineages efficiently and reproducibly in xeno-free conditions [8-10].

Using well-defined artificial cell microenvironments that recapitulate the essential characteristics of their *in vivo* counterparts to guide stem cell differentiation may offer opportunities to address this challenge and produce desired cells efficiently and reproducibly in xeno-free conditions. In addition, engineered systems can be systematically and precisely tuned to enable fundamental investigations of stem cell lineage commitment in response to their microenvironments; the obtained understanding can further advance technological development. Rationally engineered biomaterials are important components in artificial cell microenvironments, playing roles ranging from regulating presentation of insoluble and soluble biochemical ligands to controlling microenvironmental elasticity [11-19]. One important family of insoluble ligands is integrin ligands. Integrin ligands bind to and activate integrin receptors on cell surfaces, mediating cell adhesion and a variety of signaling pathways having essential biological consequences [20,21]. It has been revealed that integrin activities modulated by the ligands in extracellular matrices (ECMs) are essential for normal embryonic development [22,23]. Since stem cell differentiation parallels animal development, it is expected that integrin ligands need to be presented properly in artificial cell microenvironments to guide stem cell differentiation *in vitro*. Biomimetic presentation of integrin ligands requires that they are immobilized on materials. Addition of soluble forms of these ligands into cell culture systems could potentially cause detrimental effects because of competitive binding for cell surface receptors [24].

Here we report the engineering of a synthetic substrate functionalized with integrin ligands to support efficient early mesodermal differentiation of hESCs in xeno-free conditions. Mesodermal derivatives include highly desirable cell types such as cardiomyocytes, endothelial cells, and hematopoietic cells [5,25]. This study is focused on early mesodermal differentiation of hESCs, which precedes the formation of more mature mesodermal derivatives. The stepwise and complex nature of stem cell differentiation justifies the strategy to dissect the whole differentiation process into separate stages for investigation and optimization. It is expected that efficient and reproducible early mesodermal differentiation in xeno-free conditions is a prerequisite for consistent and high-yield production of clinically applicable mature mesodermal derivatives in the future. Animal studies have revealed that both fibronectin and laminin are present during the early stage of development from inner cell mass cells (from which ESCs are derived) to mesodermal progenitors [26]. The major cell-interacting ligands in fibronectin and laminin are the ligands for $\alpha 5\beta 1$ and $\alpha 6\beta 1$ integrins (L $\alpha 5\beta 1$ and L $\alpha 6\beta 1$), respectively. *In vitro* studies have shown that hESCs interact with fibronectin and laminin through these integrins [27-29]. In this study we have prepared synthetic materials functionalized with L $\alpha 5\beta 1$ and/or L $\alpha 6\beta 1$ and examined whether they represent biomimetic materials supporting early mesodermal differentiation of hESCs in xeno-free conditions.

2. Materials and methods

The commercial sources of reagents and supplies are listed in Table S1 (Supporting Information).

2.1. Synthesis and purification of polypeptides

The genes encoding the polypeptides cysA-L $\alpha 5\beta 1$, cysA-L $\alpha 6\beta 1$, and B were each constructed in the Qiagen pQE9 expression vector (the full sequence of each polypeptide is

shown in Table S2, Supporting Information). The polypeptides were expressed in the *E.coli* strain SG13009 and purified through nickel-nitrilotriacetic acid metal-affinity chromatography [30]. Purified polypeptides were each characterized using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry.

2.2. Immobilization of polypeptides on substrates

The solution of each polypeptide (50 μ M, pH 7.5) was prepared in phosphate buffered saline (PBS) containing 5 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and sterilized via filtration. Gold-coated slides were cleaned in freshly prepared Piranha solution (mixture of H₂SO₄ and H₂O₂ at a ratio of 70:30), rinsed with water thoroughly, sterilized with 70% ethanol, and dried in a sterile environment. Polypeptide solutions containing only cysA-L α 5 β 1, only cysA-L α 6 β 1, and the mixture of cysA-L α 5 β 1 and cysA-L α 6 β 1 (1:1 molar ratio) were each incubated with gold-coated slides at room temperature for 6 hours to prepare polypeptide-functionalized substrates [31,32]. Each modified substrate was rinsed with PBS and placed in a tight-fit well for cell culture. Matrigel-coated substrates were prepared by incubating polystyrene tissue culture plates with Matrigel solution (70 μ g/mL, prepared in DMEM/F12) at 37 °C for 1 hour.

2.3. Characterization of polypeptide density on modified substrates

Each modified substrate was treated with 8 M urea to remove non-covalently attached polypeptides, followed by thorough rinsing with PBS to refold the coiled-coil A in immobilized cysA-L α 5 β 1 and cysA-L α 6 β 1. The substrate was then incubated with large excess of a complementary coiled-coil B (50 μ M) overnight, so that B was co-immobilized through A/B heterodimerization [33,34]. After washing with PBS, the substrate was treated with 8 M urea 3 times to recover the co-immobilized B. For each sample, the B recovered from 72 cm² surface area was concentrated by using a Millipore Microcon centrifugal filter unit (3000 MWCO), followed by buffer exchange to 2 M urea. The polypeptide concentrate was collected; the kit was washed with 2 M urea 3 times and all the washes were combined with the collected concentrate. The weight of the resulting solution was measured to determine the volume (the density of 2 M urea solution was characterized separately). The concentration of B in the solution was characterized using the NanoOrange protein quantification assay on a Cary Eclipse fluorescence spectrophotometer. The revealed volume and concentration were used to determine the total amount of recovered B, which was equal to the total amount of immobilized cysA-L α 5 β 1 and cysA-L α 6 β 1.

2.4. Cell culture

H9 hESCs were maintained as described previously [35,36]. Briefly, undifferentiated hESCs were maintained on mitotically inactivated mouse embryonic fibroblast (MEF) feeder layers in DMEM/F12 supplemented with 15% knockout serum replacer, 1% nonessential amino acids (NEAA), 2 mM L-glutamine, 0.1 mM β -mercaptoethanol (β -ME), 8 ng/mL recombinant human basic fibroblast growth factor (rhbFGF), 100 U/mL penicillin, and 100 μ g/mL streptomycin. Medium was changed daily. Cultures were passaged with 1 mg/mL collagenase type IV at a split ratio of 1:6 every 5 to 7 days.

To prepare feeder-free hESCs for differentiation experiments, hESCs were passaged to Matrigel-coated polystyrene plates and cultured in the defined mTeSR1 medium. The combined use of the mTeSR1 medium and Matrigel-coated substrates has proven to support feeder-independent maintenance of hESCs [37,38]. Cultures were passaged with 1 mg/mL dispase at a split ratio of 1:6 every 5 to 7 days. The hESCs at the 4th passage under the feeder-free condition had feeders completely depleted, and these cells were used for differentiation experiments.

2.5. Differentiation of hESCs

For early mesodermal differentiation, undifferentiated hESCs with feeders depleted were seeded on substrates functionalized with synthetic polypeptides or coated with Matrigel at a 50% surface coverage, followed by culture in the mTeSR1 medium for 12 hours. The medium was then replaced with a mesodermal differentiation medium prepared by supplementing a serum-free-medium (RPMI 1640 basal medium, 1% insulin-transferrin-selenium A, 0.1 mM NEAA, 2 mM L-glutamine, 0.1 mM β -ME, 100 U/mL penicillin, and 100 μ g/mL streptomycin) with 25 ng/ml rhBMP4 [39]. Medium was changed every 2 days.

Cells cultured in the mesodermal differentiation medium on different substrates for 48 hours were examined for their developmental potential toward more mature mesodermal derivatives. Cells were harvested with 1mg/mL dispase, re-plated on Matrigel-coated substrates, and induced for further differentiation toward hemato-endothelial and cardiac fates, respectively. For hemato-endothelial differentiation, re-plated cells were cultured in the serum-free-medium supplemented with 100 ng/mL rhbFGF for 3 days, followed by culture in a serum-containing medium (RPMI 1640 basal medium, 5% fetal bovine serum, 0.1 mM NEAA, 2 mM L-glutamine, 0.1 mM β -ME, 100 U/mL penicillin, and 100 μ g/mL streptomycin) for 8 days [39]. For cardiac differentiation, re-plated cells were cultured in the serum-free-medium supplemented with 300 ng/mL rmNoggin for 3 days, followed by culture in the serum-containing medium for 9 days [39].

2.6. Cell viability assay

Cell viability was examined using the alamarBlue assay according to the manufacturer's instructions. Cells were incubated with the reagent for 4 hours in a tissue culture incubator. The fluorescent signal of the supernatant was measured with a fluorescence microplate reader (BioTek instrument, Winooski, VT). The cells were washed with PBS gently and new medium was added for continued culture.

2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

The total RNA in cells was collected using TRI Reagent® according to the manufacturer's recommendations. Each RNA sample (1 μ g) was treated with RNase-free DNase, followed by production of cDNA using the Moloney murine leukemia virus (M-MLV) reverse transcriptase system in the presence of RNase inhibitor. PCR was accomplished on a thermal cycler (Fisher) by using the GoTaq® Green Master Mix system. The primer sequences and the conditions for PCR reactions are shown in Table 1 [39,40]. The following temperature cycle was used: hot start for 3 min at 94 °C; denaturation at 94 °C for 30 s; annealing for 30 s; and extension at 72 °C for 60 s. The number of cycles for each set of primers was optimized to ensure the amplicon level was in the linear phase of the reaction. Each PCR product was resolved on a 1.5% agarose gel; the band intensity was analyzed using NIH ImageJ software. The relative levels of gene expression were normalized to the expression of RNA polymerase II (RPII) [40,41].

2.8. Immunofluorescence

For each sample, cells were dissociated into individual cells by using 0.05% trypsin-EDTA. The cells were re-plated on a coverslip coated with poly-D-lysine and allowed to adhere at room temperature for 20 min. The cells were fixed with 4% paraformaldehyde for 20 min, washed with PBS 3 times, and permeabilized with 0.2% Triton X-100 at room temperature for 15 min. The cells were incubated with a blocking buffer (PBS containing 5% fetal bovine serum and 5% glycerol) at room temperature for 1 hour, followed by sequential staining with goat anti-human Brachyury antibody at 4 °C overnight and with donkey anti-goat IgG conjugated with Alexa Fluor 488 at room temperature for 1 hour. The sample was

mounted in VECTASHIELD mounting medium with DAPI and examined with a 10× objective on a Zeiss Axio Observer inverted fluorescent microscope.

2.9. Flow cytometry

To analyze Brachyury⁺ cells after early mesoderm differentiation, cells cultured on each substrate were dissociated into a single cell suspension by using 0.05% trypsin-EDTA, followed by fixing with 4% paraformaldehyde at 4 °C for 20 min. The cells were incubated in a blocking buffer, which was prepared by adding 5% fetal bovine serum (FBS) in the staining buffer (Hank's buffered salt solution containing 0.1% saponin, 0.05% sodium azide, and 1% BSA), at 4 °C for 10 min. The cells were stained with phycoerythrin(PE)-conjugated goat anti-human Brachyury antibody at room temperature in dark for 45 min. A control stained with PE-conjugated goat IgG isotype was prepared for each experiment. Flow cytometric analysis was performed on a BD LSR II flow cytometer (BD Biosciences). The data were analyzed using Flowjo software.

To analyze CD34⁺ cells after hemato-endothelial differentiation, cells were dissociated into single cell suspensions by using 0.05% trypsin-EDTA and stained with allophycocyanin(APC)-conjugated mouse anti-human CD34 antibody at 4 °C for 30 min. A control stained with APC-conjugated mouse IgG isotype was prepared for each experiment. Staining, washing, and flow cytometric analysis were all performed in PBS containing 5% FBS and 0.1% sodium azide. Flow cytometric analysis was conducted by using a BD LSR II flow cytometer and Flowjo software.

2.10. Statistical analysis

Each experiment was performed in triplicate. Results are expressed as mean ± standard deviation. Statistics analysis is performed using unpaired Student's *t*-test. The level of significance is specified in figure captions.

3. Results

3.1. Design and preparation of substrates functionalized with integrin ligands

A fibronectin-derived peptide ligand for $\alpha 5 \beta 1$ integrin, PHSRN-(SG)₅-RGDSP (L $\alpha 5 \beta 1$), and a CCN1-derived peptide ligand for $\alpha 6 \beta 1$ integrin, TTSWSQ (L $\alpha 6 \beta 1$), were each fused to a coiled-coil A to yield polypeptides for modifying substrates [42-44]. The polypeptides cysA-L $\alpha 5 \beta 1$ and cysA-L $\alpha 6 \beta 1$ each have an N-terminal cysteine residue to allow polypeptide immobilization on gold-coated slides through the thiol-gold reaction (Fig. 1). Each polypeptide was genetically engineered and biosynthesized, and the identity and purity were confirmed by SDS-PAGE and mass spectrometric characterization (Fig. 2). The coiled-coil A was included as a linker between each immobilized integrin ligand and the substrate to allow quantification of the ligand density: A could heterodimerize with a complementary coiled-coil B and the physically co-immobilized B could be recovered under a denaturing condition for quantification. The ligand density on the substrates used in this study was 2.27 ± 0.21 pmol/cm². The sequences of the domains are shown in Fig. 1; the full sequences of the polypeptides are shown in Table S2 (Supporting Information).

3.2. Viability of hESCs cultured on substrates functionalized with integrin ligands

The substrate functionalized with both L $\alpha 5 \beta 1$ and L $\alpha 6 \beta 1$ and the substrate functionalized with L $\alpha 5 \beta 1$ alone supported cell survival and growth similarly over the whole examined time course of 7 days when hESCs were cultured in the mesodermal differentiation medium containing 25 ng/mL BMP4 (Fig. 3). But cell viability decreased when hESCs were cultured in the same medium on the substrate functionalized with L $\alpha 6 \beta 1$ alone (Fig. 3). These results

suggest that immobilized $\text{La5}\beta 1$ is essential for the survival of hESCs when cultured in this engineered system.

3.3. Early mesodermal differentiation of hESCs on substrates functionalized with integrin ligands

Immunofluorescence analysis revealed that Brachyury (a pan-mesodermal marker) was expressed in cells cultured for 48 hours in the presence of BMP4 on the substrate functionalized with both $\text{La5}\beta 1$ and $\text{La6}\beta 1$ (Fig. 4a). Brachyury expression on this substrate was similar to that on the Matrigel-coated substrate (positive control) [39]. But Brachyury was undetectable in cells cultured on substrates functionalized with either $\text{La5}\beta 1$ or $\text{La6}\beta 1$ alone even though the differentiation medium was identical, suggesting that both immobilized $\text{La5}\beta 1$ and $\text{La6}\beta 1$ are required in this engineered system to support early mesodermal differentiation. This conclusion was further confirmed by flow cytometric analysis, which revealed a significant fraction of Brachyury⁺ cells after hESCs were cultured for 48 hours in the mesodermal differentiation medium on the substrate functionalized with both $\text{La5}\beta 1$ and $\text{La6}\beta 1$, but not on substrates functionalized with either ligand alone (Fig. 4b).

To further evaluate how these synthetic substrates affect early mesodermal differentiation, expression of Brachyury, Mixl-1, and Wnt3 (three early mesodermal markers) was examined over a time course of 7 days using RT-PCR. In normal development, these three markers are transiently expressed during early mesodermal differentiation [45-47]. Their transient expression has also been observed in hESCs cultured on Matrigel-coated substrates in a mesodermal differentiation medium [39]. Our experiments showed that when hESCs were cultured on the substrate functionalized with both $\text{La5}\beta 1$ and $\text{La6}\beta 1$, each of these three genes was transiently expressed with a peak observed at approximately 2–4 days (Fig. 5). In contrast, over the entire examined time course, expression of all these genes was significantly lower when hESCs were cultured on the substrate functionalized with $\text{La5}\beta 1$ alone. Expression of these genes was undetectable when hESCs were cultured on the substrate functionalized with $\text{La6}\beta 1$ alone. It is concluded that immobilized $\text{La5}\beta 1$ and $\text{La6}\beta 1$ are sufficient and necessary insoluble ligands in this engineered system to support early mesodermal differentiation of hESCs.

3.4. Hemato-endothelial and cardiac differentiation of early mesodermal progenitors

The developmental potential of the early mesodermal progenitors derived on the synthetic substrate functionalized with both $\text{La5}\beta 1$ and $\text{La6}\beta 1$ toward downstream mesodermal derivatives was examined. Human ESCs cultured on different substrates in the presence of BMP4 for 48 hours were re-plated on Matrigel-coated surfaces and cultured in identical conditions favorable for hemato-endothelial differentiation (in the serum-free medium supplemented with bFGF for 3 days and the serum-containing medium for 8 days sequentially) [39]. RT-PCR analysis revealed that cells undergoing early mesodermal differentiation on the substrate functionalized with both $\text{La5}\beta 1$ and $\text{La6}\beta 1$ or the Matrigel-coated surface (positive control) during the first 48 hours were able to further differentiate into hemato-endothelial progenitor cells expressing a prominent level of CD34 (Fig. 6) [35,36]. In contrast, gene expression of CD34 was approximately 10 times lower if cells were cultured on substrates functionalized with either $\text{La5}\beta 1$ or $\text{La6}\beta 1$ alone during the initial 48 hours. The developmental potential of the early mesodermal progenitors derived on the substrate functionalized with both $\text{La5}\beta 1$ and $\text{La6}\beta 1$ toward hemato-endothelial progenitors was further confirmed by flow cytometric analysis. The fraction of CD34⁺ cells in the sample resulted from further differentiation of the early mesodermal progenitors derived on this substrate was approximately 3 times higher than that in the samples initially cultured on substrates functionalized with either ligand alone (Fig. 7).

The potential of the early mesodermal progenitors to develop toward the cardiac cells was also examined. Human ESCs cultured on different substrates in the presence of BMP4 for 48 hours were re-plated on Matrigel-coated substrates and cultured in identical conditions favorable for cardiac differentiation (in the serum-free medium supplemented with Noggin for 3 days and the serum-containing medium for 9 days sequentially) [39]. RT-PCR analysis revealed that cells undergoing early mesodermal differentiation on the substrate functionalized with both $\text{La5}\beta 1$ and $\text{La6}\beta 1$ or the Matrigel-coated surface (positive control) during the first 48 hours were able to further differentiate into cells expressing Gata4 and Mef2, two cardiac-restricted transcription factors (Fig. 8) [39,48]. In contrast, expression of these two genes was undetectable in cells that were cultured on substrates functionalized with either ligand alone during the initial 48 hours. These results confirmed that the substrate functionalized with both $\text{La5}\beta 1$ and $\text{La6}\beta 1$ indeed supports early mesodermal differentiation of hESCs into progenitors having the normal developmental potential toward more mature mesodermal derivatives.

4. Discussion

This study shows that the synthetic substrate functionalized with both integrin ligands $\text{La5}\beta 1$ and $\text{La6}\beta 1$ is able to support early mesodermal differentiation of hESCs when cultured in a differentiation medium containing BMP-4, while substrates functionalized with either $\text{La5}\beta 1$ or $\text{La6}\beta 1$ alone do not. Immunofluorescent staining, flow cytometric analysis, and RT-PCR analysis all suggest that immobilized $\text{La5}\beta 1$ and $\text{La6}\beta 1$ are sufficient insoluble ligands that, together with defined soluble biological factors in the medium, construct a well-controlled synthetic niche that guides efficient early mesodermal differentiation of hESCs (Fig. 4,5). These results also suggest that immobilized $\text{La5}\beta 1$ and $\text{La6}\beta 1$ are both required in this engineered system. The early mesodermal progenitors derived on the substrate functionalized with both $\text{La5}\beta 1$ and $\text{La6}\beta 1$ are able to further differentiate along the hemato-endothelial and cardiac lineages given appropriate conditions (Fig. 6–8), suggesting that this synthetic substrate indeed supports normal early mesodermal differentiation of hESCs.

This study has revealed that insoluble ligands are essential for creating synthetic niches to guide early mesodermal differentiation of hESCs *in vitro*. Our results suggest that the role of the immobilized integrin ligands used in this study is more than just allowing cell adhesion and survival: similar cell viability and growth were observed on the substrate functionalized with both $\text{La5}\beta 1$ and $\text{La6}\beta 1$ and the substrate functionalized with $\text{La5}\beta 1$ alone (Fig. 3); but early mesodermal differentiation only occurred on the substrate functionalized with both ligands (Fig. 4–8). Previous studies demonstrating differentiation of hESCs toward early mesodermal progenitors and cardiomyocytes (a mesodermal derivative) on Matrigel-coated substrates focused on optimization of soluble factors in culture media [8,39]. Whether the insoluble ligands in immobilized Matrigel have roles other than supporting cell adhesion and survival in those differentiation processes was not investigated and remains unclear. Since naturally derived Matrigel has complex components and little flexibility for adjustment of material properties [49], such investigation might be difficult. The well-controlled synthetic system reported here allows us to demonstrate the importance of insoluble ligands in niches guiding early mesodermal differentiation.

Our results show that immobilized $\text{La6}\beta 1$ is not only permissive, but also necessary for early mesodermal differentiation of hESCs in this engineered system (Fig. 4–8). The conclusion that $\text{La6}\beta 1$ is permissive is consistent with the results in previous studies showing that hESCs can differentiate toward early mesodermal progenitors and cardiomyocytes on substrates coated with Matrigel, which contains laminin carrying a ligand for $\alpha 6\beta 1$ integrin [8,39]. These studies suggest that although Matrigel-coated

substrates have been used to maintain undifferentiated hESCs [37,38], Matrigel does not prevent mesodermal differentiation of hESCs. It has also been revealed that the interaction between $\alpha 6 \beta 1$ integrin on hESC surfaces and the laminin-nidogen complex is required for EB formation and subsequent differentiation toward all three germ layers [50]. Studies with mouse ESCs have also shown that laminin is necessary and sufficient to mediate epiblast differentiation, which precedes the development of precursors of three germ layers [51]. Treatment of laminin- $\gamma 1$ -null mouse EBs with laminin rescues mesodermal differentiation [52]. However, there has been discrepancy in the literature regarding favorable ECM molecules in cell microenvironments to support mesodermal differentiation. One study comparing differentiation of wild-type and laminin- $\gamma 1$ -null mouse EBs concluded that laminin inhibits mesodermal differentiation [53]. It is unclear what caused such discrepancy, probably relevant to poorly defined cell microenvironments in these studies. The study reported here is based on a well-controlled synthetic system, which allows us to conclude that the ligand for $\alpha 6 \beta 1$ integrin does not appear to inhibit early mesodermal differentiation of hESCs and it is even necessary in certain engineered mesodermal differentiation niches.

The efficiency of early mesodermal differentiation of hESCs on the synthetic substrate functionalized with both L $\alpha 5 \beta 1$ and L $\alpha 6 \beta 1$ is comparable to that on the Matrigel-coated substrate (Fig. 4–8). Immobilized L $\alpha 5 \beta 1$ is required in this engineered system, but fibronectin, from which L $\alpha 5 \beta 1$ is derived, is not contained in Matrigel. However, the two major components in Matrigel, laminin and type IV collagen, both contain the RGD peptide [49,54,55]. This suggests that although a ligand having both the RGD peptide and the synergy peptide PHSRN was used in this study to mimic the ligand in fibronectin that specifically binds to $\alpha 5 \beta 1$ integrin [44], it is possible that the RGD peptide alone is sufficient. Another possibility is that redundant mechanisms support early mesodermal differentiation and other components in Matrigel elicit similar biological responses as the fibronectin-derived L $\alpha 5 \beta 1$.

The substrate functionalized with both L $\alpha 5 \beta 1$ and L $\alpha 6 \beta 1$ is biomimetic, because the interactions between cells and these ligands mimic those between cells and fibronectin and laminin, two ECM proteins found during *in vivo* differentiation from inner cell mass cells toward mesodermal progenitors [26]. This well-defined synthetic substrate can replace the substrate coated with animal-derived Matrigel to support early mesodermal differentiation of hESCs, providing several advantages because it is xeno-free and has no batch-to-batch variations [39]. Cells produced in such synthetic microenvironments are more desirable for future clinical use. In addition, such synthetic materials allow their properties to be systematically tuned to dissect the role of each component, which is difficult to access with naturally derived materials having complex components.

Although we used the thiol-gold reaction to immobilize ligands on gold-coated slides to demonstrate the design of the biomimetic substrate supporting early mesodermal differentiation in this study, the molecular design can be readily extended to other types of materials (such as hydrogels) by using different chemical approaches for surface immobilization. Examples include the photo-initiated thiol-ene reaction and other orthogonal chemical approaches enabled by unique functional groups introduced through non-natural amino acid analogs [56,57].

5. Conclusions

The synthetic substrate functionalized with both peptide ligands for $\alpha 5 \beta 1$ and $\alpha 6 \beta 1$ integrins supports efficient early mesodermal differentiation of hESCs when cultured in a differentiation medium containing BMP4, while substrates functionalized with either ligand alone do not. The early mesodermal progenitors derived on the substrate functionalized with

both ligands have the normal developmental potential to further differentiate along the hemato-endothelial and cardiac lineages given appropriate conditions. When combined with defined soluble factors, immobilized $\alpha 5\beta 1$ and $\alpha 6\beta 1$ are sufficient insoluble ligands to construct a well-controlled cell microenvironment to guide early mesodermal differentiation of hESCs in xeno-free conditions. Both immobilized $\alpha 5\beta 1$ and $\alpha 6\beta 1$ are not only permissive but also necessary in this engineered differentiation niche.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Molecular design of the substrates functionalized with L α 5 β 1 and/or L α 6 β 1. Substrates were prepared by immobilizing polypeptides cysA-L α 5 β 1 and/or cysA-L α 6 β 1 on gold-coated slides.

Fig. 2. Characterization of purified polypeptides. (a) SDS-PAGE of cysA-L α 5 β 1 and cysA-L α 6 β 1. (b,c) Mass spectrometric characterization of cysA-L α 5 β 1 and cysA-L α 6 β 1, respectively.

Fig.3. Viability of hESCs cultured on substrates functionalized with different integrin ligands. Cells were cultured in the mesodermal differentiation medium containing 25 ng/mL BMP4; cell viability was determined by the fluorescence-based alamarBlue assay.

Fig.4.

Expression of the mesodermal marker Brachyury in hESCs cultured for 48 hours on substrates functionalized with different bioactive molecules. (a) Immunofluorescent staining of Brachyury. Cells were cultured in the mesodermal differentiation medium containing 25 ng/mL BMP4 except for the negative control, in which cells were maintained undifferentiated on a Matrigel-coated substrate in the mTeSR1 medium. The scale bars are 200 μ m. (b) Flow cytometric histogram profiles of Brachyury expression. Cells were cultured in the mesodermal differentiation medium containing 25 ng/mL BMP4. Samples stained with the antibody are demonstrated by filled plots and those stained with isotype control by lines.

Fig. 5.

Time-course expression profiles of three early mesodermal markers Brachyury, Mixl1, and Wnt3 in hESCs cultured on different substrates and in the mesodermal differentiation medium containing 25 ng/mL BMP4. All these three markers were not detectable on the substrate functionalized with Lα6β1 alone. The expression of each gene is normalized to RP II expression. The relative expression of Brachyury, Mixl1, and Wnt3 detected in undifferentiated hESCs was 0.015, 0, and 0, respectively. N/D: not detected. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$: significantly different from that on the Lα5β1-modified substrate at the same time point.

Fig.6.

Relative gene expression of CD34 after hemato-endothelial lineage differentiation of mesodermal progenitors. Human ESCs were cultured on different substrates and in the mesodermal differentiation medium for 48 hours, followed by being re-plated in identical conditions favorable for hemato-endothelial differentiation and cultured for 11 days. The gene expression is normalized to RP II expression. The relative gene expression of CD34 detected in undifferentiated hESCs was 0.01. *** $P < 0.001$.

Fig.7. Flow cytometric analysis of CD34⁺ cells after hemato-endothelial lineage differentiation of mesodermal progenitors. Human ESCs were cultured in different conditions for 48 hours, followed by being re-plated in identical conditions favorable for hemato-endothelial differentiation and cultured for 11 days. ****P* < 0.001.

Substrate used during the first 48 hours of culture

- 1: functionalized with La5 β 1 and La6 β 1;
- 2: functionalized with La5 β 1;
- 3: functionalized with La6 β 1;
- 4: coated with Matrigel.

Fig.8.

Relative gene expression of Gata4 and Mef2 after cardiac differentiation of mesodermal progenitors. Human ESCs were cultured on different substrates in the mesodermal differentiation medium for 48 hours, followed by being re-plated in identical conditions favorable for cardiac differentiation and cultured for 12 days. The gene expression is normalized to RP II expression. Gene expression of Gata4 and Mef2 was not detected in undifferentiated hESCs. $**P < 0.01$.

Table 1

Primers and conditions for RT-PCR.

		Oligo sequence (5'→3')	Amplicon size (bp)	Annealing temperature (°C)	Cycle number
Brachyury	Forward	GCGGGAAGAGCCTGCAGTA	300	55	32
	Reverse	TTCCCCGTTACGTACTTC			
MixII	Forward	GGTACCCCGACATCCACTT	120	55	32
	Reverse	TGGAAGGATTTCCCACTCTG			
Wnt3	Forward	GGCCATGAACAAGCACAA	368	58	32
	Reverse	TGCCGTGGGAGGTGACATT			
CD34	Forward	CCTAAGTGACATCAAGGCAGAA	475	55	32
	Reverse	GCAAGGAGCAGGGAGCATA			
Gata4	Forward	GACGGTCACTATCTGTGCAAC	563	55	32
	Reverse	AGACATCGCACTGACTGAGAAC			
Mef2	Forward	AGATTACGAGGATTATGGATGAACGT	201	55	31
	Reverse	ACCTGCACCTGGAGGTCGATGTG			
RPII	Forward	ACTGGAGACAGCAAG GTCGT	222	55	32
	Reverse	CAGGTGGATGTTGAAGAGCA			