Matrix Biology 142 (2025) 21–32

Contents lists available at ScienceDirect

Matrix Biology

journal homepage: www.elsevier.com/locate/matbio

Laminin-511-functionalized fibrin gel enables in-gel proliferation of human induced pluripotent stem cells

Yukimasa Taniguchi, Mamoru Takizawa, Ayaka Hada, Ayano Ishimaru, Kiyotoshi Sekiguchi *

Division of Matrixome Research and Application, Institute for Protein Research, The University of Osaka, 3-2 Yamadaoka, Suita, Osaka 565-0871, Japan

ARTICLE INFO

Keywords:
Laminin
Integrin
Fibrin gel
Pluripotent stem cell
3D culture

ABSTRACT

Fibrin is a biocompatible hydrogel that is widely used as a surgical sealant and as a scaffold for in vitro cell culture. Here, we engineered a heterotrimeric chimera between fibrinogen and laminin-511 by connecting the N-terminal self-polymerization domain of fibrinogen with the C-terminal integrin-binding domain of laminin-511 via their coiled-coil regions. The resulting chimeric protein, designated Chimera-511, binds to fibrinogen in a thrombin-dependent manner and exerts integrin-binding activity in a fibrin(ogen)-bound form. Chimera-511 co-polymerizes with fibrinogen to form a fibrin gel endowed with the potent integrin-binding activity of laminin-511, thereby enabling robust three-dimensional proliferation of human induced pluripotent stem cells while maintaining their pluripotency marker expression and trilineage differentiation potential. This functionalized, biodegradable fibrin gel provides a defined and clinically compatible three-dimensional scaffold for stem cell culture, with potential applications in both basic research and regenerative medicine.

Introduction

Three-dimensional (3D) gel culture systems that mimic complex tissue structures and functions have been developed and are expected to bridge the gap between in vivo microenvironments and conventional two-dimensional (2D) in vitro culture [1,2]. Recently, 3D gel culture systems combined with directed differentiation of human pluripotent stem cells have enabled the generation of organoids comprising diverse cell types [1,2], establishing 3D culture as an essential platform in stem cell biology and regenerative medicine. As organoid technologies continue to advance toward clinical application, the development of clinically compatible 3D scaffolds for transplantation has become increasingly urgent [3].

Various hydrogels have been employed for 3D cell culture systems, including collagen/gelatin, fibrinogen/fibrin, synthetic polymers, and basement membrane (BM) extracts such as Matrigel [4–7]. Among these hydrogels, Matrigel, a mouse tumor-derived extract enriched in BM components, remains the gold standard for many organoid protocols because of its high biological activity. However, the ill-defined composition, batch-to-batch variability, and xenogeneic origin of Matrigel severely limit its clinical applicability [8]. In contrast, fibrin is a biocompatible hydrogel that is widely used in clinical settings as a hemostatic agent and wound dressing [9]. Fibrin is a biopolymer of thrombin-cleaved fibrinogen, one of the major plasma proteins essential for blood coagulation [10]. Fibrin gels have several advantages for application in cell-based regenerative medicine, including their amenability to autologous preparation from patient plasma, minimization of immune rejection, and degradation by fibrinolytic enzymes. However, fibrin itself exhibits limited adhesive affinity and specificity for many cell types, including pluripotent stem cells, with the notable exception of platelets and leukocytes [6,11,12]. Although researchers have attempted to potentiate the cell-adhesive activity of fibrin gels by conjugating laminin-derived synthetic peptides, such as YIGSR [13,14], these peptides cannot recapitulate the high-affinity interactions of laminin with integrins [15,16], highlighting the need for fibrin gels endowed with the authentic integrin-binding activity of laminin.

Laminin, a major cell-adhesive protein in the BM, is a heterotrimeric glycoprotein composed of α, β, and γ chains. In mammals, five α (α1–α5), three β (β1–β3), and three γ (γ1–γ3) chains have been identified, combinations of which give rise to at least 12 isoforms with distinct chain compositions [17]. These laminin isoforms regulate diverse cell behaviors, including survival, proliferation, polarity, and differentiation, through specific interactions with integrins such as α3β1, α6β1, α6β4, and α7β1 [18]. Among them, α6β1 integrin is a high-affinity receptor for laminin-511 (LM-511; composed of the α5, β1, and γ1 chains), and their

* Corresponding author.
E-mail address: sekiguch@protein.osaka-u.ac.jp (K. Sekiguchi).

https://doi.org/10.1016/j.matbio.2025.10.003
Received 23 August 2025; Received in revised form 29 September 2025; Accepted 8 October 2025
Available online 10 October 2025

Abbreviations

BSA	bovine serum albumin
D-PBS	Dulbecco's phosphate-buffered saline
FBS	fetal bovine serum
HBS	Hepes-buffered saline
hiPSC	human induced pluripotent stem cell
HRP	horseradish peroxidase
LM-511E8	the recombinant E8 fragment of laminin-511
mAb	monoclonal antibody
pAb	polyclonal antibody
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBS	Tris-buffered saline

interaction supports efficient expansion of human pluripotent stem cells in an undifferentiated state [19,20]. The integrin-binding site in laminins is mapped to the C-terminal region of laminin heterotrimers, referred to as the E8 region (*Fig. 1**a***). Previously, we reported that the integrin-binding site in LM-511 is composed of two parts: a cluster of basic residues in globular domain 2 of the α5 chain and a Glu residue in the C-terminal tail region of the γ1 chain (*Fig. 1**a***) [21–23]. Importantly, this composite integrin-binding interface is only formed when the α, β, and γ chains are assembled into a heterotrimer through their coiled-coil regions, thus precluding any single-chain-derived peptide from replicating the integrin-binding activity of laminins [24,25].

In this study, we leveraged the structural similarity between fibrinogen and laminin, as both consist of three-subunit chains that assemble into heterotrimers through their coiled-coil regions, to design a bifunctional chimeric protein. By directly linking the N-terminal self-polymerization domain of fibrinogen to the C-terminal integrin-binding region of LM-511 (*Fig. 1**a***), we created a chimeric protein, designated Chimera-511, that is capable of co-assembling into a fibrin gel in a thrombin-dependent manner with retention of the potent integrin-binding activity of LM-511, thereby enabling undifferentiated growth of human induced pluripotent stem cells (hiPSCs) under 3D in-gel culture conditions. Because fibrin gel is biodegradable and amenable to clinical use, the fibrin gel functionalized with Chimera-511 offers a new type of next-generation 3D culture scaffold for stem cell manipulation in regenerative medicine.

Results

Fabrication of a bifunctional chimeric protein with integrin- and fibrin(ogen)-binding activities

Fibrinogen is a 340-kDa hexameric glycoprotein composed of two Aα, two Bβ, and two γ chains. These chains assemble into an Aα-Bβ-γ heterotrimer through their coiled-coil regions, followed by dimerization into a disulfide-linked hexamer through their N-terminal regions (*Fig. 1**a***) [10,26]. Self-polymerization of fibrinogen into a fibrin gel is initiated by thrombin-catalyzed release of fibrinopeptides A and B from the N-termini of the Aα and Bβ chains (*Fig. 1**a***). The newly exposed N-terminal sequences of the Aα and Bβ chains, called knob-A and knob-B, interact with hole-a and hole-b located within the C-terminal modules of the γ and β chains, respectively (***Supplemental Fig. 1***). The knob-A–hole-a and knob-B–hole-b interactions trigger protofibril formation, a premature form of fibrin gel. The mechanism by which fibrinogen self-assembles into a fibrin gel, together with the similarity in heterotrimeric structures between fibrinogen and laminin, inspired the idea to fabricate a bifunctional chimeric protein by connecting the N-terminal self-polymerization domain of fibrinogen with the C-terminal integrin-binding region of LM-511 through their heterotrimeric coiled-coil regions. Such a chimeric protein is expected to co-assemble into a fibrin gel via the knob–hole interactions in a thrombin-dependent manner, thereby harnessing the potent cell-adhesive activity of LM-511 in the fibrin gel (*Fig. 1**a***).

The Aα, Bβ, and γ chains of fibrinogen and α, β, and γ chains of laminins are aligned oppositely in their coiled-coil regions, namely, clockwise in fibrinogen and counterclockwise in laminins when viewed from the C-termini (*Fig. 1**b***). Accordingly, there are three combination patterns for fibrinogen (Fbg)–laminin (LM) chimeras: Fbg Aα–LM α5, Fbg γ–LM β1, and Fbg Bβ–LM γ1 (combination 1), Fbg γ–LM α5, Fbg Bβ–LM β1, and Fbg Aα–LM γ1 (combination 2), and Fbg Bβ–LM α5, Fbg Aα–LM β1, and Fbg γ–LM γ1 (combination 3), as schematically illustrated in Fig. 1b. When these three combinations were expressed in FreeStyle™ 293-F cells, combination 3 exhibited the highest level of expression as a hexameric chimera (*Fig. 1**c***, ***Supplemental Fig. 2***). Combinations 1 and 2 yielded low levels of chimeric proteins, possibly because of instability of the resulting proteins. The chimeric protein in combination 3, composed of Fbg Bβ–LM α5 (Chimera-α5), Fbg Aα–LM β1 (Chimera-β1), and Fbg γ–LM γ1 (Chimera-γ1), was designated Chimera-511 and used in the following experiments.

Chimera-511 binds to α6β1 integrin and fibrin(ogen)

Chimera-511 is a hexamer composed of two Chimera-α5, two Chimera-β1, and two Chimera-γ1 chains covalently linked to one another by disulfide bonds (*Fig. 2**a***). The functional units for fibrinogen polymerization and integrin binding are located at the N- and C-terminal regions of Chimera-511 (*Fig. 2**a***). Chimera-511 expressed in 293-F cells was purified by Ni-NTA affinity chromatography, followed by molecular sieve chromatography. The authenticity of the purified Chimera-511 was verified by SDS-PAGE under reducing and nonreducing conditions (*Fig. 2**b***).

The integrin-binding activity of Chimera-511 was verified by solid-phase binding assays using recombinant α6β1 integrin, a high-affinity receptor for LM-511 [21,27]. LM-511E8 was used as a control ligand for the integrin. Because LM-511E8 and Chimera-511 contain one and two integrin-binding sites, respectively, Chimera-511 was coated on 96-well microtiter plates at half the concentration of LM-511E8 to normalize the number of integrin-binding sites. α6β1 integrin bound equally well to Chimera-511 and LM-511E8 with similar dissociation constants (*Fig. 2**c***), confirming that the integrin-binding activity of LM-511 was not compromised by the chimerization with fibrinogen at the coiled-coil region.

Chimera-511 contains the N-terminal self-polymerization domain of fibrinogen that yields knob-A and knob-B upon thrombin treatment (***Supplemental Fig. 1***). Solid-phase binding assays showed that Chimera-511 was competent in binding to fibrinogen in a thrombin-dependent manner, while LM-511E8 was not, irrespective of the presence or absence of thrombin (*Fig. 2**d***). Moreover, Chimera-511 captured by fibrinogen in the presence of thrombin was capable of binding to α6β1 integrin, demonstrating that the interaction of Chimera-511 with fibrinogen did not interfere with the binding of Chimera-511 to α6β1 integrin (*Fig. 2**e***). These findings confirm that Chimera-511 is bifunctional and capable of simultaneously interacting with fibrinogen and α6β1 integrin.

We also produced enhanced green fluorescent protein (EGFP)-tagged Chimera-511 (Chimera-511^{EGFP}) to investigate whether Chimera-511 can assemble into fibrin fibrils. When mixed with a large excess of Alexa Fluor 647-labeled fibrinogen and induced to co-assemble into a fibrin gel, Chimera-511^{EGFP} was detected in a meshwork structure and co-localized with fibrin fibrils (*Fig. 2**f***), confirming that Chimera-511 is capable of co-assembling into a fibrin gel as designed.

Chimera-511-functionalized fibrin gel supports the proliferation of hiPSCs

LM-511 is a major niche component for pluripotent epiblast cells in

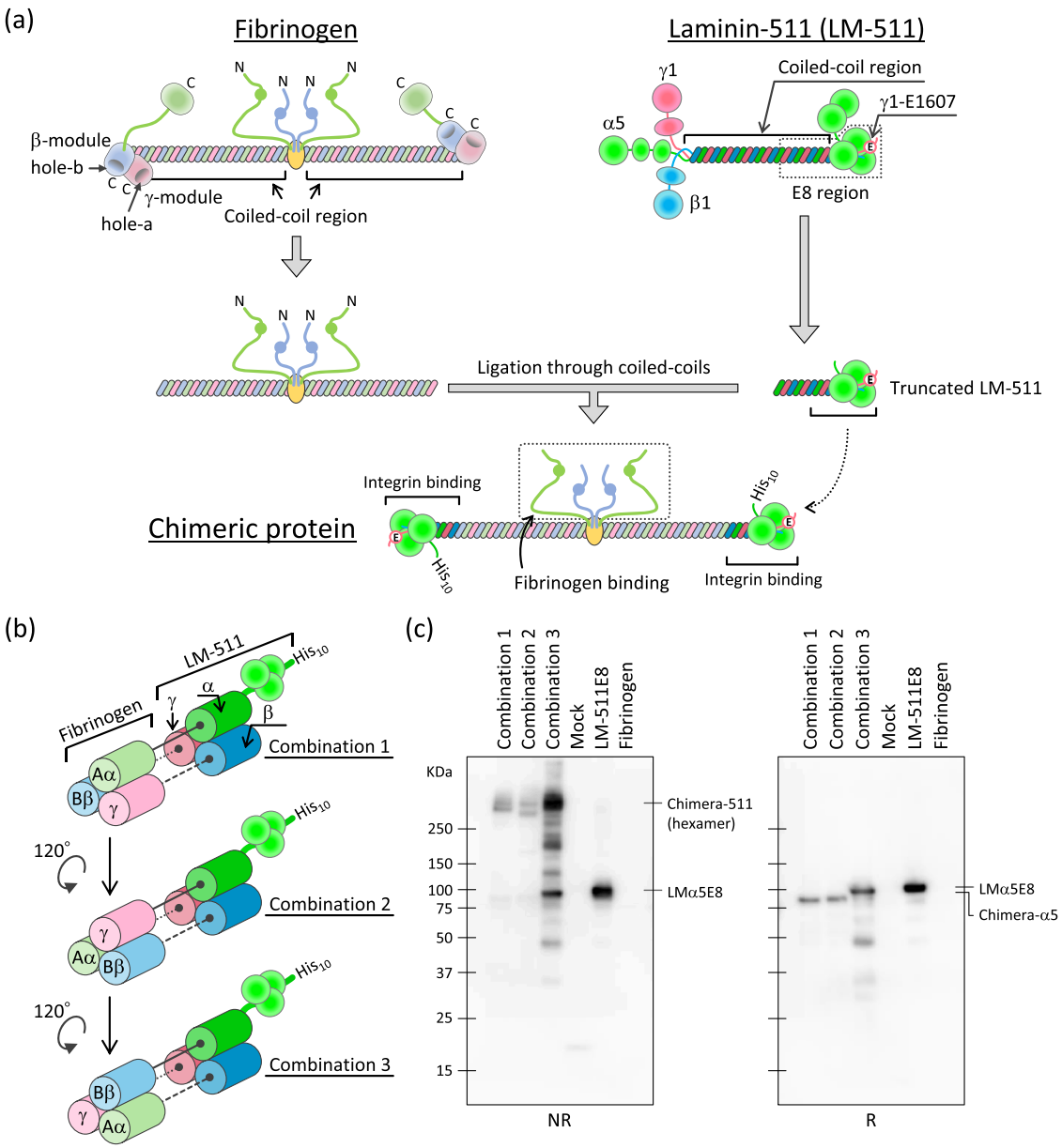


Fig. 1. Generation of a chimeric protein of LM-511 and fibrinogen. (***a***) Schematic diagram showing the construction of a chimeric protein of fibrinogen and LM-511. A truncated fibrinogen lacking the C-terminal coiled-coil part and β- and γ-modules is fused to the integrin-binding region of LM-511. A glutamic residue (*E*) in the LM γ1 chain that is essential for integrin binding is indicated by a *hot pink circle*. (***b***) Three different combinations of the Aα (*green*), Bβ (*bluish purple*), and γ (*pink*) chains of fibrinogen (Fbg) and the α5 (*lime*), β1 (*deep sky blue*), and γ1 (*hot pink*) chains of LM-511. combination 1: Fbg Aα–LM α5, Fbg γ–LM β1, and Fbg Bβ–LM γ1; combination 2: Fbg γ–LM α5, Fbg Bβ–LM β1, and Fbg Aα–LM γ1; combination 3: Fbg Bβ–LM α5, Fbg Aα–LM β1, and Fbg γ–LM γ1. (***c***) Chimeric proteins secreted into the conditioned media using the three combinations were analyzed by SDS-PAGE, followed by immunoblotting with an anti-PentaHis mAb. Purified LM-511E8 and fibrinogen were used as the positive and negative control antigens, respectively. The results with an anti-fibrinogen pAb are shown in *Supplemental Fig. 2*.

the BM [28]. LM-511E8 supports efficient expansion of human pluripotent stem cells when coated on culture vessels [19,20], prompting us to examine whether human pluripotent stem cells can be expanded within the Chimera-511-functionalized fibrin gel. We seeded hiPSCs into fibrin gels containing increasing concentrations of Chimera-511 and cultured the cells for 7 days. hiPSCs were also seeded into a fibrin gel containing LM-511E8 as a control. While hiPSCs showed no proliferation within the control fibrin gel containing LM-511E8, they proliferated in the Chimera-511-functionalized fibrin gels in a dose-dependent manner, reaching a plateau at Chimera-511 concentrations above 50 nM and exhibiting > 25-fold amplification under 3D culture conditions (*Fig. 3a and 3b*, ***Supplemental Fig. 3 and 4*; see *Experimental procedures***). No expansion of hiPSCs was observed in fibrin gels containing <5 nM Chimera-511. hiPSCs grown in Chimera-511-functionalized fibrin gels formed sphere-like structures and persistently expressed the pluripotency markers OCT3/4 and NANOG (*Fig. 3**c***). The resulting spheres were surrounded by endogenously secreted laminin detected with a polyclonal antibody recognizing the intact laminin γ1 chain but not Chimera-γ1 (*Fig. 3**d***). Consistent with these findings, α6β1 integrin in hiPSCs was co-localized with the cell-derived laminin deposited at the periphery of the hiPSC spheres.

Pluripotent stem cells express α6β1 integrin as the major integrin species [19,29]. The interaction of α6β1 integrin with LM-511 triggers the PI3K-Akt signaling cascade and rescues hiPSCs from apoptosis [19,30]. To investigate whether the adhesive interaction of hiPSCs with Chimera-511 via α6β1 integrin is required for cell proliferation in spheres, we generated a mutant Chimera-511 in which Glu1607 in the C-terminal tail region of laminin γ1, the Glu residue essential for the

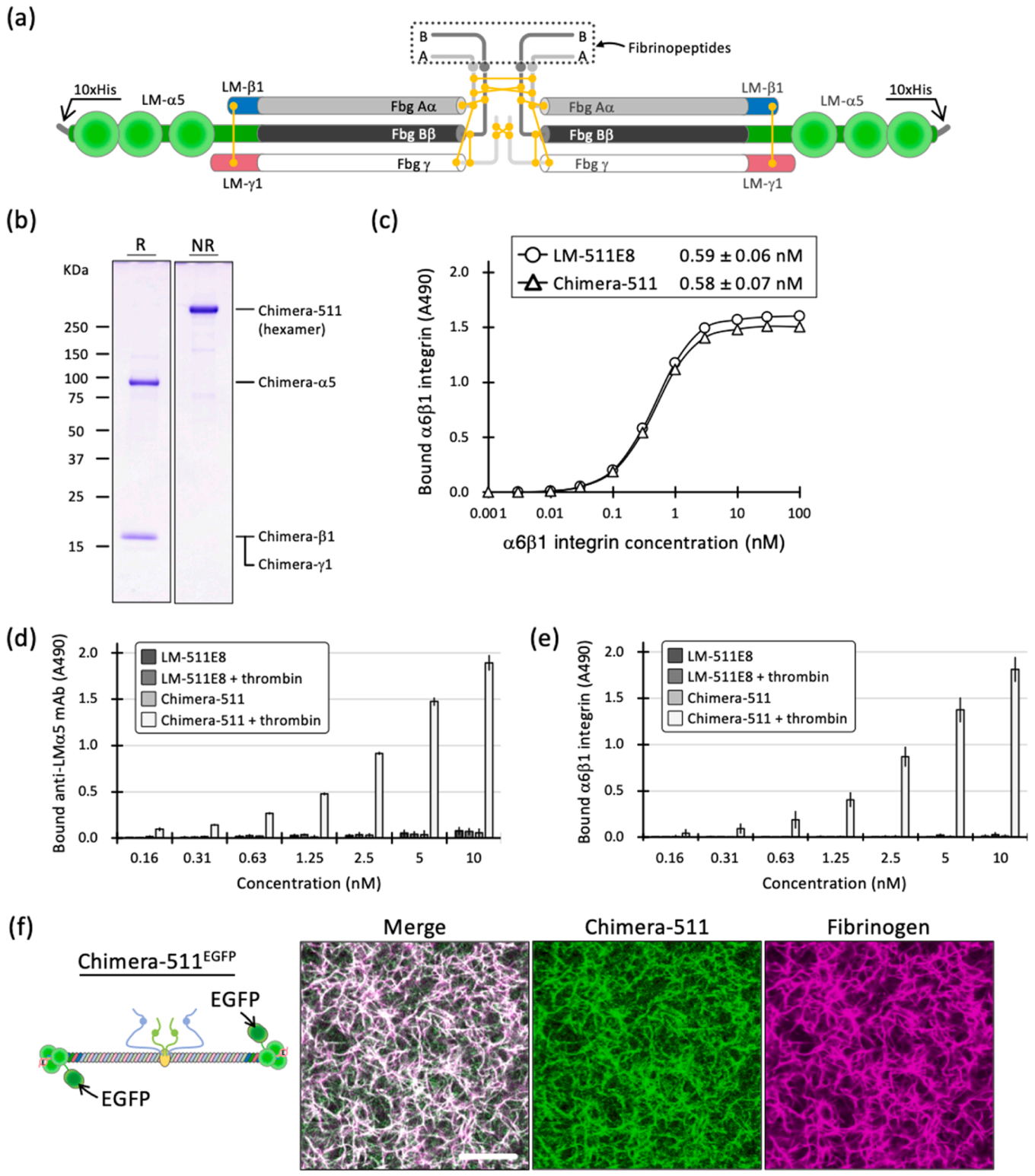


Fig. 2. Binding activities of Chimera-511 to α6β1 integrin and fibrinogen. (***a***) Schematic diagram of Chimera-511 in combination 3. Disulfide bonds are indicated by *orange lines*. (***b***) Purified Chimera-511 was verified by SDS-PAGE using 5 %–20 % gradient polyacrylamide gels under reducing (*left*) and nonreducing (*right*) conditions. The positions of the molecular weight markers are shown in the *left margin*. (***c***) Titration curves of LM-511E8 and Chimera-511 binding to α6β1 integrin. Increasing concentrations of α6β1 integrin were allowed to bind to a ninety-six-well microtiter plate coated with LM-511E8 (*open circles*) or Chimera-511 (*open triangles*) in the presence of 1 mM $MnCl_2$. Bound integrin was quantified as described in *Experimental procedures*. Nonspecific binding, defined as binding in the presence of 10 mM EDTA, was subtracted as background. Apparent dissociation constants (*inset*) were determined by Scatchard plot analysis. (***d*** and ***e***) Binding activities of LM-511E8 and Chimera-511 to fibrinogen. Ninety-six-well microtiter plates precoated with 100 nM fibrinogen were allowed to interact with increasing concentrations of LM-511E8 or Chimera-511 with or without thrombin treatment. Bound LM-511E8 or Chimera-511 was detected with an anti-human LM α5 mAb (***d***) or α6β1 integrin (***e***). The amounts of mAb or α6β1 integrin were quantified as described in *Experimental procedures*. (***f***) Confocal images showing the colocalization of Chimera-511^{EGFP} (*green*) and Alexa Fluor™ 647-labeled fibrin gel (*magenta*). The scheme on the left shows Chimera-511^{EGFP}. *Scale bar*, 25 μm.

integrin-binding activity of LM-511 [21], was substituted by Gln. The mutant Chimera-511, designated Chimera-511(EQ), was inactive in binding to α6β1 integrin (***Supplemental Fig. 5b***). The fibrin gels containing Chimera-511(EQ) did not support the proliferation of hiPSCs, even when Chimera-511(EQ) was added at 500 nM (*Fig. 3**e**, Supplemental Fig. 6*), confirming that the interaction of Chimera-511 with α6β1 integrin is required for the proliferation of hiPSCs in the composite fibrin gel.

We also generated another mutant Chimera-511, designated Chimera-511(RS), in which both Arg20 in knob-A and Arg18 in knob-B in fibrinogen were substituted by Ser (***Supplemental Fig. 5a***). These substitutions abrogate the self-polymerization of fibrinogen [31]. Solid-phase binding assays confirmed that Chimera-511(RS) was inactive in binding to fibrinogen (***Supplemental Fig. 5b***). When hiPSCs were seeded in fibrin gels containing Chimera-511(RS), no significant expansion of the cells was observed, even when Chimera-511(RS) was added at 500 nM (*Fig. 3**e, Supplemental Fig. 6***). The results with these two mutant chimeric proteins underscore the importance of the bifunctionality of Chimera-511, i.e., binding to α6β1 integrin through the C-terminal region derived from LM-511E8 and co-assembly into a fibrin gel through the N-terminal self-polymerization domain of fibrinogen, for the growth of hiPSCs in the Chimera-511-functionalized fibrin gel.

hiPSCs can be serially passaged in the Chimera-511-functionalized fibrin gel

Next, we assessed the potential of the Chimera-511-functionalized fibrin gel to support serial passaging of hiPSCs under 3D culture conditions. After dissociation of 2D-cultured hiPSCs (see ***Experimental procedures***), the cells were seeded into the Chimera-511-functionalized fibrin gel and cultured for 7 days. The cultured cells were harvested by digestion of the composite fibrin gel with Accumax™, and passaged into fresh composite fibrin gel. We defined this 3D-to-3D subculture as passage 0 (3D-P0) and repeated the serial passaging five times using the composite fibrin gel (*Fig. 4**a***). The cell numbers robustly increased for at least five serial passages in the composite gel (*Fig. 4**b***). Moreover, immunohistochemical and flow cytometry analyses demonstrated that almost all of the dissociated 3D-P5 hiPSCs were positive for the pluripotency markers SSEA-4, BC2LCN, OCT3/4, SOX2, and NANOG, and negative for SSEA-1 (*Fig. 4**c and 4d, Supplemental Fig. 7***). When cultured in medium lacking FGF-2, 3D-P5 hiPSCs were spontaneously induced to differentiate into three germ layers, as evidenced by positive immunoreactivity for β3-tubulin (ectoderm), α-smooth muscle actin (mesoderm), and SOX17 (endoderm) (*Fig. 4**e, Supplemental Fig. 8***). These findings indicate that hiPSCs maintain their pluripotency and trilineage differentiation potential after five consecutive passages in the Chimera-511-functionalized fibrin gel.

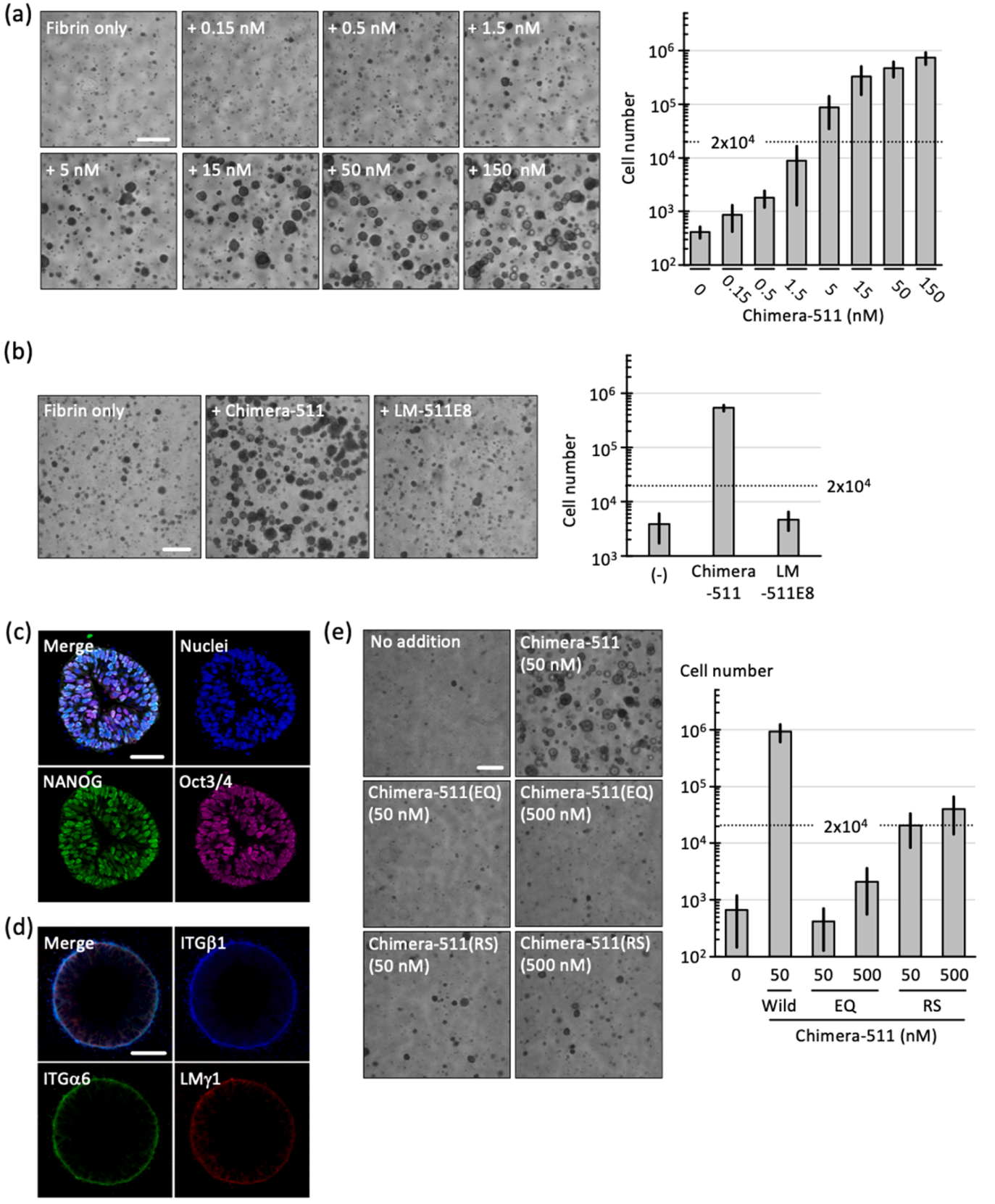
Discussion

Despite the biological importance of laminin family proteins in the extracellular matrix niches for many types of stem cells, it has been challenging to functionalize fibrin gels with the potent integrin-binding activity of laminin. In this study, we succeeded in fabricating a chimeric protein between fibrinogen and LM-511 by directly connecting the N-terminal self-polymerization domain of fibrinogen to the C-terminal integrin-binding domain of LM-511 through their heterotrimeric coiled-coil domains. The resulting chimeric protein, Chimera-511, was confirmed to be bifunctional, binding to fibrinogen in a thrombin-dependent manner and being fully competent in binding to α6β1 integrin in a fibrin(ogen)-bound form. Chimera-511 co-assembled into a fibrin meshwork, and the resulting composite fibrin gel supported the proliferation of hiPSCs without compromising their pluripotency. The hiPSCs maintained their undifferentiated state and pluripotency after at least five passages in Chimera-511-functionalized fibrin gels. To the best of our knowledge, the fibrin gel functionalized with Chimera-511 is the first 3D cell culture scaffold to exhibit the full integrin-binding activity of laminin other than mouse tumor-derived BM extracts (i.e., Matrigel).

To date, several methods have been developed to functionalize fibrin gels with cell-adhesive peptides/recombinant proteins [13,14,32]. Synthetic cell-adhesive peptides, including YIGSR- and RGD-containing peptides, have been conjugated to fibrin gels using either chemical crosslinkers [14] or the transglutaminase Factor XIIIa [13]. Here, we developed a new method to functionalize fibrin gels with the potent integrin-binding activity of LM-511 by fabricating the gels using Chimera-511, which co-assembles into fibrin fibrils through knob–hole interactions, the same mechanism by which fibrinogen polymerizes into fibrin protofibrils. The hexameric Chimera-511 is equipped with two knob-A and knob-B pairs, which can simultaneously engage a pair of fibrin(ogen) protomers through two-by-two interactions between knob-A/B and hole-a/b, achieving high-affinity co-assembly of Chimera-511 into fibrin fibrils (***Supplemental Fig. 1***). Previously, Barker and colleagues reported fibrin matrices functionalized with the integrin-binding fragment of fibronectin, to which either the knob-A or knob-B sequence had been recombinantly attached [32]. However, the resulting fibronectin-derived fragments containing a knob sequence were not stably retained in the fibrin gels, possibly because of insufficient affinity arising from the monovalent knob–hole interaction [32]. In contrast, Chimera-511 can co-assemble into fibrin gels through two-by-two knob–hole interactions, ensuring its longer retention within the fibrin gels. It should be noted that Chimera-511 lacks hole-a and hole-b as the binding pockets for knob-A and knob-B, respectively, and acts as a terminator of fibrin protofibril elongation, potentially reducing the mechanical strength of the composite fibrin gels. However, the amount of Chimera-511 required to functionalize the fibrin gels was $<1/100$ of the amount of fibrinogen based on the molar ratio, i.e., 50 nM Chimera-511 versus 7350 nM (2.5 mg/mL) fibrinogen, and such adverse effects should not have a significant effect on the mechanical properties of the fibrin gels.

Our results demonstrate that anchorage of Chimera-511 to fibrin fibrils is important for supporting robust proliferation of iPSCs within the composite fibrin gels. No proliferation of hiPSCs was observed within the fibrin gel lacking Chimera-511 or the fibrin gel supplemented with LM-511E8 that does not interact with fibrin(ogen). Furthermore, the mutant Chimera-511 with mutations in knob-A and knob-B that abrogate the knob–hole interactions supported only marginal proliferation of hiPSCs in the fibrin gel, underscoring the importance of the anchorage of Chimera-511 to the fibrin fibrils through the knob–hole interactions. Previously, Polisetti and colleagues reported that LM-511E8 enhanced the adhesion, migration, and proliferation of human limbal epithelial stem/progenitor cells in a fibrin gel when simply added to the gel, indicating that LM-511E8 does not need to be anchored to the fibrin gel to potentiate the cell-adhesive activity of the fibrin gel [33]. This apparent discrepancy may be partly because of the difference in stiffness of the fibrin gels used — Polisetti and colleagues used a hard gel composed of 10 mg/mL fibrinogen, while we used a relatively soft gel made of 2.5 mg/mL fibrinogen. Furthermore, they seeded limbal stem/progenitor cells on the fibrin gel, while we seeded hiPSCs within the gel. It should also be noted that they observed cell proliferation on the control fibril gel without supplementation of cell-adhesive proteins, whereas we observed no apparent proliferation of hiPSCs within the fibrin gel lacking Chimera-511. There is accumulating evidence that cells sense the stiffness of the extracellular environment via integrins and modulate their fate and behaviors [34]. Mechanosensing by integrins triggers signal cascades and controls cellular behaviors, including survival, proliferation, and differentiation, through actin skeletal reorganization [34]. Under in-gel culture conditions, it is conceivable that LM-511E8 needs to be anchored to the fibrin gel to trigger the mechanotransduction of the signaling cascades and mimic the extracellular environment in vivo.

In summary, we have developed a new 3D culture scaffold for stem



(caption on next page)

Fig. 3. Spheroid culture of hiPSCs in Chimera-511-functionalized fibrin gel. (***a***) Phase-contrast images of hiPSC spheroids cultured for 7 days in fibrin gels with increasing concentrations (0.15–150 nM) of Chimera-511. *Scale bar*, 500 μm. The fibrin gels with cultured cells were digested with Accumax, and the cell numbers were counted (*right panel*). The dotted line in the *right panel* indicates the number of seeded cells on day 0. The bars in the *right panel* show the mean ± standard deviation ($N = 3$). (***b***) Phase-contrast images of hiPSC spheroids cultured for 7 days in fibrin gels with no addition (*left*), Chimera-511 (*middle*), and LM-511E8 (*right*). After 7 days of culture, the gels were digested with Accumax, and the cell numbers were counted (*right panel*). The dotted line indicates the number of seeded cells on day 0. *Scale bar*, 500 μm. The bars in the *right panel* show the mean ± standard deviation ($N = 3$). (***c***) Immunofluorescence staining of cryosections of hiPSC spheroids grown in fibrin gels functionalized with Chimera-511. Fibrin gels containing hiPSC spheroids were fixed with 4 % paraformaldehyde, snap-frozen, and sectioned at 7-μm thickness as described in *Experimental procedures*. Cryosections were immunostained with antibodies against NANOG (*green*) and OCT3/4 (*magenta*). Nuclei were stained with Hoechst 33342 (*blue*). *Scale bar*, 50 μm. (***d***) Whole-mount immunofluorescence staining of hiPSC spheroids after 7 days of culture. After fixation with 4 % paraformaldehyde and blocking, the fibrin gels were immunostained with antibodies against integrin β1 (*blue*), integrin α6 (*green*), and LM γ1 (*red*). *Scale bar*, 50 μm. (***e***) hiPSC spheroids were grown in fibrin gels functionalized with Chimera-511 or its mutant proteins Chimera-511(EQ) and Chimera-511(RS). The *left panels* show phase-contrast images of hiPSCs grown in fibrin gels containing 50 nM or 500 nM mutant proteins for 7 days. No addition indicates the control fibrin gel without the mutant proteins. The *right panel* shows the numbers of hiPSCs grown in the fibrin gels containing the mutant proteins. The dotted line indicates the number of seeded cells on day 0. *Scale bar*, 500 μm. The bars in the *right panel* show the mean ± standard deviation ($N = 3$).

cells by conferring the potent integrin-binding activity of LM-511 on a fibrin gel by fabricating a chimera between fibrinogen and LM-511. Fibrin gels functionalized with Chimera-511 support the in-gel growth of hiPSCs in spheroids with retention of their pluripotency and trilineage differentiation potential. Unlike mouse tumor-derived BM gels, fibrin gels are free from xenogeneic contaminants and have been used as surgical glues and wound dressings. Importantly, Chimera-511, composed of human fibrinogen and LM-511, can be readily produced in cGMP-compatible CHO cells, as is the case with LM511E8. In conventional 2D culture, LM511E8 has been shown to be superior to mouse tumor-derived BM gels (e.g., Matrigel) in promoting hiPSC adhesion and proliferation [19]. Although further work is needed to extend our 3D culture platform to organoid systems, fibrin gels functionalized with recombinant Chimera-511 offer promising 3D scaffolds for manipulation of stem cells within a BM-like environment for tissue engineering and regenerative medicine, owing to their proven biosafety, biocompatibility, and biodegradability.

Experimental procedures

Antibodies and reagents

The horseradish peroxidase (HRP)-conjugated monoclonal antibody (mAb) against the penta-His sequence was purchased from Qiagen (Hilden, Germany; #34,460). The rabbit polyclonal antibody (pAb) against human fibrinogen was from GeneTex (Irvine, CA, USA; #GTX79760). The mouse mAb 4C7 against human LM α5 chain (#MAB1924) and rabbit pAb against human SOX2 (#AB5603) were from Merck (Darmstadt, Germany). The recombinant rabbit mAb against human NANOG was from Cell Signaling Technology (Danvers, MA, USA; #4903). The mouse mAbs against Oct-3/4 (#sc-5279) and α-smooth muscle actin (#sc-32251) were from Santa Cruz Biotechnology (Dallas, TX, USA). The mouse mAb against tubulin β3 was from BioLegend (San Diego, CA, USA; #MMS-435P). Rat anti-human integrin α6 mAb (#555734), normal rat IgG2a (#553927), Alexa Fluor 488-conjugated mouse anti-human Oct3/4 mAb (#560253), Alexa Fluor 488-conjugated mouse anti-human Nanog mAb (#560791), FITC-conjugated mouse anti-SSEA-1 mAb (#560127), FITC-conjugated mouse anti-SSEA-4 mAb (#560126), FITC-conjugated mouse IgG3 (#555578), FITC-conjugated mouse IgM (#555583), and Alexa Fluor 488-conjugated mouse IgG1 (#557782) were from BD Biosciences (Franklin Lakes, NJ, USA). The rabbit pAb against human LM γ1 was from Atlas Antibodies (Stockholm, Sweden; #HPA001908). The goat pAbs against mouse immunoglobulin G (IgG) with Alexa Fluor™ 405 (#A-31553), against mouse IgG with Alexa Fluor™ 488 (#A-11001), against rat IgG with Alexa Fluor™ 488 (#A-11006), against rabbit IgG with Alexa Fluor™ 488 (#A-11008), against rabbit IgG with Alexa Fluor™ 546 (#A-11035), donkey pAb against mouse IgG with Alexa Fluor™ 647 (#A-31571), normal rabbit IgG (#10500C), Hoechst 33,342 (#H3570), human fibrinogen with Alexa Fluor™ 647 (#F35200), and HRP-conjugated streptavidin (#21126) were from Thermo Fisher Scientific (Waltham, MA, USA). Normal mouse IgG1 (#M075–3), mouse IgG2a (#M076–3), IgG2b (#M077–3), rabbit IgG (#PM035), and goat IgG (#PM094) were from MBL (Tokyo, Japan). The goat pAb against human SOX17 was from R&D Systems (Minneapolis, MN, USA; #AF1924). The rabbit pAb against the ACID/BASE coiled-coil region was produced as described previously [27]. The pAb was biotinylated using EZ-Link™ Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific; #21335) in accordance with the manufacturer's instructions. The rabbit pAb against LG1–3 of the human LM α5 chain was prepared as described previously [23]. The mAb TS2/16 against human integrin β1 was purified on a Protein G Sepharose 4 Fast Flow column (Cytiva, Marlborough, MA, USA; #17–0618–05) from the conditioned media of hybridoma cells obtained from the American Type Culture Collection. The HRP-conjugated goat pAb against rabbit IgG (#711–035–152), donkey anti-mouse IgG (#715–035–150), and Alexa Fluor™ 488-conjugated donkey anti-goat pAb (#705–545–147) were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Dulbecco's modified Eagle's medium (DMEM; #08456–65), D-PBS(-) (#14249), skim milk (#31149–75), Y-27,632 (#18188–04), tranexamic acid (#02134–84), and Fluoro-KEEPER Antifade Reagent (#12745–74) were from Nacalai Tesque (Kyoto, Japan). Hydrogen peroxide (#081–04215), 37 % formaldehyde solution (#064–00406), and rBC2LCN-FITC (#180–02991) were from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). HRP-conjugated streptavidin was from Proteintech (Rosemont, IL, USA; #SA00001–0). All restriction enzymes used in the study were obtained from New England Biolabs (Ipswich, MA, USA). Human plasma fibrinogen was purchased from Enzyme Research Laboratories (South Bend, IN, USA; #FIB 3). After thawing in a water bath at 37°C, the fibrinogen solution was passed through a 0.44-μm pore-size filter, followed by a 0.22-μm pore-size filter. The protein concentration of the filtered fibrinogen solution was determined by measuring the absorbance at 280 nm (extinction coefficient: 1.51). Fetal bovine serum (FBS; #172012–500ML), bovine serum albumin (BSA; #A7906), and thrombin (#T4393) from human plasma were from Sigma-Aldrich (St. Louis, MO, USA). Thrombin powder was dissolved in HEPES-buffered saline pH 7.4 (HBS (7.4): 20 mM HEPES-NaOH, pH 7.4, containing 137 mM NaCl).

Construction of expression vectors

pcDNA3.4-based expression vectors for recombinant E8 fragments of human LM α5 (Ala^{2534}-Ala^{3327}), LM β1 (Leu^{1561}-Leu^{1786}), and LM γ1 (Asn^{1362}-Pro^{1609}) (pcDNA3.4-α5E8, pcDNA3.4-β1E8, and pcDNA3.4-γ1E8) were prepared as follows. cDNA fragments encoding human LM α5E8, β1E8, and γ1E8 were excised from pSecTag2B-based expression vectors for LM α5E8, β1E8, and γ1E8 using NheI and NotI [21]. The resulting cDNA fragments were ligated into NheI/NotI-cleaved pcDNA3.4 (Thermo Fisher Scientific) containing the multicloning site derived from pSecTag2A (pcDNA3.4+MCS) [22]. His_6, HA, and FLAG tags were added to the recombinant LM α5E8, β1E8, and γ1E8 chains, respectively. An expression vector for human LM α5E8 (Ala^{2534}-Ala^{3310}) with a His_{10} tag at the C-terminal end was prepared as follows. A His_{10}

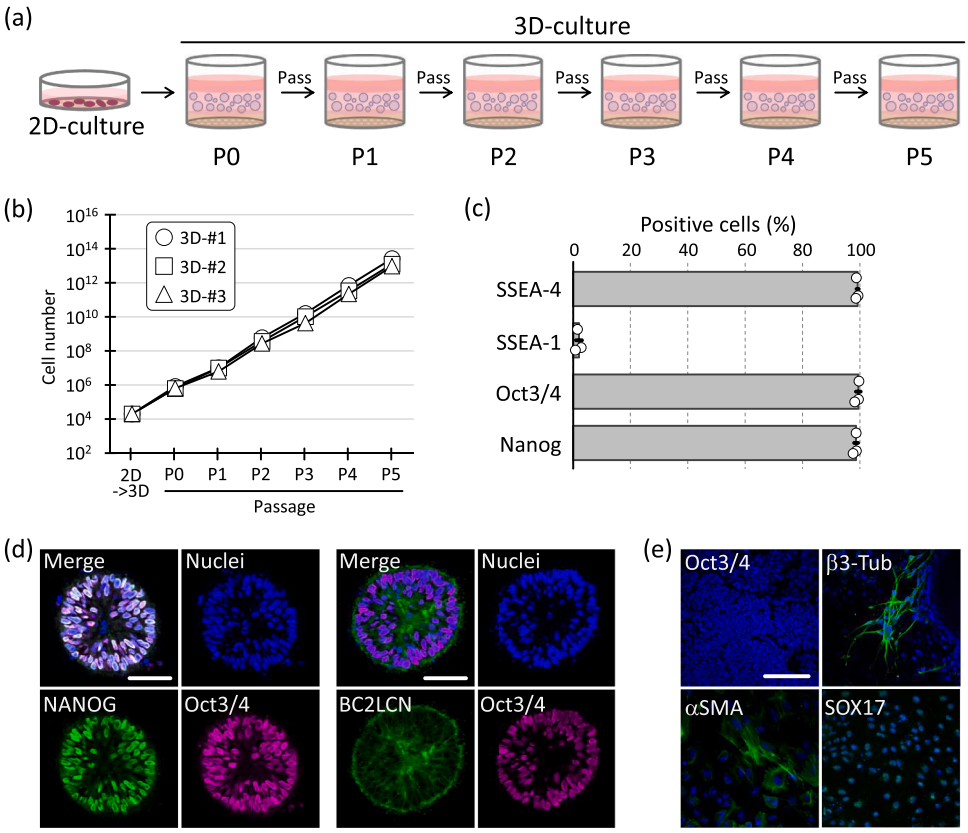


Fig. 4. Long-term culture of hiPSCs in Chimera-511-functionalized fibrin gels. (***a***) Schematic diagram showing the passaging schedule. (***b***) Growth curves of hiPSCs cultured in fibrin gels functionalized with 50 nM Chimera-511. At each passage, the fibrin gels containing hiPSC spheroids were digested with Accumax, and the cell numbers were counted. Cell growth was expressed as the cumulative cell numbers ($N = 3$). (***c***) Flow cytometric analysis of hiPSCs after five passages. Dissociated cells were fixed, blocked, and incubated with monoclonal antibodies against SSEA-1, SSEA-4, Oct3/4, and NANOG. The percentages of positive cells were determined as described in *Experimental procedures*. The *bars* show the mean ± standard deviation ($N = 3$). (***d***) Multiple immunofluorescence staining of cryosections of hiPSC spheroids after five passages. Fibrin gels containing hiPSC spheroids were fixed, frozen, and sectioned as described in *Experimental procedures*. Cryosections were immunostained with antibodies against NANOG (*green*) and OCT3/4 (*magenta*) (*left*), and BC2LCN (*green*) and OCT3/4 (*magenta*) (*right*). Nuclei were stained with Hoechst 33342 (*blue*). *Scale bar*, 50 μm. (***e***) Trilineage differentiation potential of 3D-P5 hiPSCs. hiPSCs were plated on dishes coated with LM-511E8 and cultured for 14 days in medium lacking growth factor supplementation to induce trilineage differentiation. The cells were fixed, permeabilized, blocked, and incubated with the following antibodies: anti-Oct-3/4 mAb, anti-tubulin β3 mAb, anti-α-smooth muscle actin (αSMA) mAb, or SOX17 pAb. Bound antibodies were visualized with Alexa Fluor 488-conjugated secondary antibodies (*green*). Nuclei were stained with Hoechst 33342 (*blue*). *Scale bar*, 100 μm.

tag was introduced by extension PCR using pcDNA3.4-α5E8 (Ala^{2534}-Ala^{3327}) as a template. The resulting PCR product was digested with AscI and NotI, followed by ligation into the AscI/NotI sites of pcDNA3.4-LMα5E8 (Ala^{2534}-Ala^{3327}) (pcDNA3.4-α5E8/His_{10}).

Expression vectors for the human fibrinogen Aα (Fbg Aα; Met^{1}-Pro^{644}), Bβ (Fbg Bβ; Met^{1}-Gln^{491}), and γ (Fbg γ; Met^{1}-Val^{437}) chains were prepared as follows. A human liver cDNA library was created using ReverTra Ace -α- (Toyobo, Osaka Japan; #FSK-101F) with human liver total RNA (TaKaRa Bio, Shiga, Japan; #Z6531N) as a template. Full-length cDNAs of human fibrinogen Aα, Bβ, and γ were cloned by PCR using the human liver cDNA library as a template. The resulting PCR products were digested with NheI/NotI, followed by ligation into the NheI/NotI sites of pcDNA3.4-MCS (pcDNA3.4/FbgAα, pcDNA3.4/FbgBβ, and pcDNA3.4/Fbgγ).

Expression vectors for chimeric proteins Fbg Bβ (Met^{1}-Asn^{194})–LM α5 (Ile^{2716}-Ala^{3310}-His_{10}), Fbg Aα (Met^{1}-His^{151})–LM β1 (Leu^{1761}-Leu^{1786}), and Fbg γ (Met^{1}-Ser^{132})–LM γ1 (Ile^{1579}-Pro^{1609}) were constructed as follows. cDNAs encoding Fbg Bβ (Met^{1}-Asn^{194})–LM α5 (Ile^{2716}-Ala^{3310}-His_{10}), Fbg Aα (Met^{1}-His^{151})–LM β1 (Leu^{1761}-Leu^{1786}), and Fbg γ (Met^{1}-Ser^{132})–LM γ1 (Ile^{1579}-Pro^{1609}) were amplified by PCR using pcDNA3.4/FbgBβ and pcDNA3.4/α5E8(His_{10}), pcDNA3.4/FbgAα and pcDNA3.4/β1E8, and pcDNA3.4/Fbgγ and pcDNA3.4/γ1E8 as templates. Each pair of PCR products was assembled by overlap extension PCR, followed by ligation into the NheI/NotI sites of pcDNA3.4-MCS (pcDNA3.4/FbgBβ–LMα5(His_{10}), pcDNA3.4/FbgAα–LMβ1, and pcDNA3.4/Fbgγ–LMγ1). The secretion signal peptides of the three expression vectors were substituted with those of the mouse Igk-chain derived from pSecTag2 (Thermo Fisher Scientific) by overlap extension PCR using pcDNA3.4/FbgBβ–LMα5(His_{10}), pcDNA3.4/FbgAα–LMβ1, and pcDNA3.4/Fbgγ–LMγ1 as templates (pcDNA3.4/Igκ-FbgBβ (Gln^{31}-Asn^{194})–LMα5(His_{10}) (pcDNA3.4/Chimera-α5), pcDNA3.4/Igκ-FbgAα (Ala^{20}-His^{151})–LMβ1 (pcDNA3.4/Chimera-β1), and pcDNA3.4/Igκ-Fbgγ (Tyr^{27}-Ser^{132})–LMγ1 (pcDNA3.4/Chimera-γ1)). Expression vectors for Igκ-Fbg Aα (Ala^{20}-His^{151})–LM α5(His_{10}), Igκ-Fbg γ (Tyr^{27}-Ser^{132})–LM α5(His_{10}), Igκ-Fbg Bβ (Gln^{31}-Asn^{194})–LM β1, Igκ-Fbg γ (Tyr^{27}-Ser^{132})–LM β1, Igκ-Fbg Aα (Ala^{20}-His^{151})–LM γ1, and Igκ-Fbg Bβ (Gln^{31}-Asn^{194})–LM γ1 were prepared by overlap extension PCR using the same procedure as the construction of pcDNA3.4/Chimera-α5, pcDNA3.4/Chimera-β1, and pcDNA3.4/Chimera-γ1. An expression vector for pcDNA3.4/Chimera-γ1 with substitution of Glu^{1607} by Gln was prepared by overlap extension PCR using pcDNA3.4/Chimera-γ1 and pSecTag2B-γ1E8(Glu1607Gln) as templates [21]. Expression vectors for pcDNA3.4/Chimera-α5 with Arg18Ser and pcDNA3.4/Chimera-β1 with Arg20Ser were prepared by overlap extension PCR using pcDNA3.4/Chimera-α5 and pcDNA3.4/Chimera-β1 as templates.

An expression vector for EGFP-tagged Chimera-α5 was prepared by overlap extension PCR using pcDNA3.4/Chimera-α5 and pEGFP-N1

(TaKaRa Bio; #6085–1) as templates. EGFP with a His_6 tag was added to the C-terminus of Igκ-Fbg Bβ (Gln^{31}-Asn^{194})-LM α5. The Ala206Lys mutation was introduced to minimize any unexpected dimerization of EGFP (the first Met residue of EGFP was numbered as 1).

The DNA sequences of all expression vectors constructed in the study were verified using an ABI PRISM 3130xl genetic analyzer (Thermo Fisher Scientific).

An expression vector for the extracellular domain of the human integrin α6 subunit with an acidic helical domain and a FLAG tag at the C-terminus was prepared as reported previously [35]. An expression vector for the extracellular domain of the human integrin β1 subunit with a basic helical domain and a His_6 tag at the C-terminus was kindly provided by Junichi Takagi (Institute for Protein Research, the University of Osaka) [36].

Determination of the chain combinations of LM-511 and fibrinogen

FreeStyle™ 293-F cells (Thermo Fisher Scientific; #R79007) were simultaneously transfected with expression vectors for the three combinations of fibrinogen-laminin chimeric proteins and cultured for 3 days. After removal of the cells and debris by centrifugation, the conditioned media were applied to 5 %–20 % gradient polyacrylamide gels (ATTO, Tokyo, Japan; #E-T520L). After electrophoresis, the separated proteins were transferred onto polyvinylidene difluoride membranes (Merck Millipore; #IPVH00010), probed with antibodies against PentaHis (Qiagen) or human fibrinogen (GeneTex), and visualized using ECL™ Western Blotting Detection Reagents (Cytiva; #RPN2109).

Expression and purification of recombinant proteins

Chimera-511 and its derivatives were produced using a FreeStyle™ 293 expression system (Thermo Fisher Scientific; #K900001). The resulting conditioned media were loaded onto cOmplete™ His-Tag Purification Resin (Roche, Basel, Switzerland; #5893801001), followed by washing with HEPES-buffered saline pH 8.0 (HBS [8.0]: 20 mM HEPES-NaOH pH 8.0, containing 137 mM NaCl) and elution of the bound proteins with HBS (8.0) containing 250 mM imidazole. The peak fractions were pooled and concentrated using Amicon Ultra-15 Centrifugal Filter Units 10K-cut (Merck Millipore; #UFC901024) and further purified on a Superose 6 10/300 GL column (Cytiva; #29091596) using HBS (7.4) as a running buffer. Fractions containing Chimera-511 were collected, passed through a 0.22-μm Millex-GV Syringe Filter Unit (Merck Millipore; #SLGVJ13SL), and stored at −75°C until use.

Recombinant LM-511E8 and α6β1 integrin were expressed using the FreeStyle™ 293 Expression System and purified as described previously [23,35].

The protein concentrations of all recombinant products were determined using a BCA protein assay kit (Thermo Fisher Scientific; #23227) using BSA as a standard.

The purified Chimera-511 was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 5–20 % gradient polyacrylamide gel (Tokyo, Japan; #2331830) under nonreducing and reducing conditions. The separated proteins were visualized by Coomassie Brilliant Blue staining.

Solid-phase binding assays

Solid-phase integrin binding assays were performed as follows. Briefly, ninety-six-well Nunc MaxiSorp™ flat-bottom plates (Thermo Fisher Scientific; #442,404) were coated with 10 nM LM-511E8 or 5 nM Chimera-511 overnight at 4°C. After washing with Tris-buffered saline without divalent cations (TBS(-): 20 mM Tris–HCl, pH 7.4, containing 137 mM NaCl) supplemented with 0.02 % (v/v) Tween-20 (Sigma; #P1379) and 0.1 % (w/v) BSA (Sigma; #P7906) (0.1 % BSA/TBS-$T_{0.02}$), the wells were blocked with TBS-$T_{0.02}$ containing 1 % (w/v) BSA (Sigma) for 1 h at room temperature. After washing with 0.1 % BSA/TBS-$T_{0.02}$, the wells were incubated with increasing concentrations of recombinant α6β1 integrin for 3 h at room temperature in the presence of 1 mM $MnCl_2$ or 10 mM EDTA. Bound integrins were detected by incubation with 1.5 μg/mL biotinylated anti-ACID/BASE pAb for 30 min, followed by incubation with 0.53 μg/mL HRP-conjugated streptavidin for 15 min. Integrin-antibody complexes were quantified by measuring the absorbance at 490 nm after incubation with 0.4 mg/mL o-phenylenediamine (OPD) and 0.04 % (v/v) hydrogen peroxide (H_2O_2). The apparent dissociation constants were determined as described previously [37].

The fibrinogen binding assay for LM-511E8 and Chimera-511 was performed as described below. Briefly, ninety-six-well Nunc MaxiSorp™ flat-bottom plates (Thermo Fisher Scientific) were coated with 100 nM human fibrinogen (Enzyme Research Laboratories) diluted with HBS (7.4) overnight at room temperature under gentle shaking. The fibrinogen-coated wells were blocked with TBS(-) containing 0.1 % (v/v) Tween-20 (Sigma) and 1 % (w/v) skim milk (Nacalai Tesque; #31149–75) (1 % skim milk /TBS-$T_{0.1}$). After one wash with TBS-$T_{0.1}$, 0.25 NIH U/mL thrombin was added to the wells together with increasing concentrations of Chimera-511 (0.16, 0.31, 0.63, 1.25, 2.5, 5, and 10 nM) for 1 h at room temperature. HBS (7.4) was used as the negative control for thrombin treatment. Bound Chimera-511 was detected using mAb 4C7 against human LM α5 or recombinant human α6β1 integrin. For the 4C7 binding assay, the mAb was allowed to react with Chimera-511 bound to the thrombin-treated fibrinogen at room temperature for 1 h. The bound mAb was detected using HRP-conjugated donkey anti-mouse IgG pAb, followed by measurement of the absorbance at 490 nm after incubation with OPD and H_2O_2. For the α6β1 integrin binding assay, the plate was reacted with 30 nM α6β1 integrin at room temperature for 1 h. Bound integrin was detected after sequential incubation with biotinylated anti-ACID/BASE pAb and HRP-conjugated streptavidin. The amount of bound integrin was quantified by measuring the absorbance at 490 nm after incubation with OPD and H_2O_2.

The fibrinogen binding assay for Chimera-511 and its mutants was performed as described below. Ninety-six-well Nunc MaxiSorp™ flat-bottom plates were coated with 100 nM human fibrinogen diluted with HBS (7.4) overnight at room temperature under gentle shaking. The fibrinogen-coated wells were blocked with 1 % (w/v) skim milk /TBS-$T_{0.1}$. After one wash with HBS (7.4), 0.25 NIH U/mL thrombin was added to the wells together with increasing concentrations of Chimera-511 (0.005, 0.05, 0.5, 5, 50, and 500 nM) for 1 h at room temperature. Bound Chimera-511 and its mutants were detected using mAb 4C7 against human LMα5 or recombinant human α6β1 integrin as described above, except that HRP-conjugated streptavidin used was from Proteintech.

Chimera-511^{EGFP}-functionalized fibrin gel

The fibrin gel containing Chimera-511^{EGFP} was prepared as follows. A 100-μL aliquot of HBS (7.4) containing 4.75 mg/mL fibrinogen and 0.25 mg/mL Alexa Fluor™ 647-conjugated fibrinogen was mixed with an equal volume of 100 nM Chimera-511^{EGFP} and 1 U/mL thrombin solution in HBS (7.4), followed by transfer of 100 μL of the mixture onto a glass-bottom 24-well plate (Greiner, Frickenhausen, Germany; #662892) to prepare the fibrin gel. After incubation in a CO_2 incubator for 10 min, the fibrin gel was washed twice with HBS (7.4) and observed under a Fluoview FV1200 laser scanning confocal microscope (Olympus, Tokyo, Japan).

Maintenance of hiPSCs

The hiPSC line 201B7 was obtained from the RIKEN BioResource Research Center [38]. The hiPSCs were routinely cultured on 0.25 μg/cm^2 iMatrix-511 (Matrixome, Osaka, Japan; #892) in StemFit AK02N culture medium (Ajinomoto, Tokyo, Japan) in an uncoated

manner [39] and passaged every 3 or 4 days as single cells after dissociation with Accumax™ (Nacalai Tesque; #17087–54) diluted three-fold with Dulbecco's phosphate-buffered saline (D-PBS(-)) without divalent cations containing 0.5 mM EDTA (0.5 mM EDTA/D-PBS(-)) and 10 μM Y-27632 (Nacalai Tesque). The cells were seeded onto a Falcon 6-well plate (Corning, New York, NY, USA; #353046) at a density of 5000 or 10,000 cells/cm^2 for 3 or 4 days of culture, respectively.

3D in-gel culture of hiPSCs within fibrin gels

To prepare the composite fibrin gel containing Chimera-511 for 3D culture of hiPSCs, a two-layered gel structure was employed to prevent direct contact of cells with the surface of the culture plate. First, a bottom layer of fibrin gel (without Chimera-511) was formed by mixing equal volumes of 5 mg/mL fibrinogen in StemFit AK02N medium supplemented with 10 μM Y-27632 (SF-Y) and 1 U/mL thrombin in SF-Y. A 250-μL aliquot of this mixture was transferred to each well of a 24-well plate (Falcon, Corning; #353047) and allowed to gel. After gelation, 1 mL of SF-Y containing 1 mM tranexamic acid was overlaid on the fibrin gel. On the following day, hiPSCs maintained under standard 2D culture conditions were detached using a dissociation solution, suspended in SF-Y, and mixed with thrombin and Chimera-511 at final concentrations of 1 U/mL and 100 nM, respectively. This cell suspension was then combined with an equal volume of 5 mg/mL fibrinogen in SF-Y. A 250-μL aliquot of the resulting mixture containing 2×10^4 hiPSCs and 50 nM Chimera-511 was layered onto the preformed bottom gel to form the upper layer of the composite gel. After gelation, 1 mL of SF-YT was added to each well. The culture medium was replaced with fresh StemFit AK02N containing 1 mM tranexamic acid on days 1, 4, 5, and 6 after cell seeding.

The hiPSC spheres grown in fibrin gels were passaged after 7 days of culture. Briefly, after two washes with 0.5 mM EDTA/D-PBS(-), the fibrin gel was dissolved with Accumax™ containing 10 μM Y-27632 at 37°C with rotation (120 rpm). After centrifugation of the gel solution at 300 × *g* for 5 min at 25°C, the cell pellet was resuspended in D-PBS(-) containing 10 μM Y-27632. The cell suspension was centrifuged at 300 × *g* for 5 min at 25°C, and the cell pellet was resuspended in SF-Y. After the cell number was counted, the cell suspension was supplemented with thrombin and Chimera-511 at concentrations of 1 U/mL and 100 nM, and added to an equal volume of 5 mg/mL fibrinogen in SF-Y. A 250-μL aliquot of the mixture was transferred onto the preformed bottom gel and used as the cell culture layer (2×10^4 cells, 50 nM Chimera-511, 2.5 mg/mL fibrinogen, and 0.5 U/mL thrombin). After gelation, 1 mL of SF-YT was added to each well. The medium was replaced with fresh StemFit AK02N containing 1 mM tranexamic acid at 1, 4, 5, and 6 days after cell seeding.

For whole-mount immunostaining, hiPSC spheres were prepared as described above, except that a 40-μL aliquot of the mixture containing 3.2×10^3 cells, 50 nM Chimera-511, 2.5 mg/mL fibrinogen, and 0.5 U/mL thrombin was transferred onto a glass-bottom 24-well plate (Matteck, Ashland, MA, USA; #P24G-0–13-F) without the bottom gel. After gelation, 0.6 mL of SF-YT was added gently to each well. The medium was replaced with StemFit AK02N containing 3 μM Y-27632 and 1 mM tranexamic acid on day 1, followed by StemFit AK02N culture medium containing 1 mM tranexamic acid on days 4, 5, and 6 after cell seeding.

Flow cytometric analysis

3D-P5 hiPSCs were prepared using the methods described above. Dissociated cells were suspended in DMEM containing 10 % (v/v) FBS and passed through a Falcon 40 μm cell strainer (Corning; #352340). The cells were counted using a hemocytometer and then fixed with 3.7 % formaldehyde in D-PBS(-) at 4°C for 10 min. After three washes with D-PBS(-), the cells were resuspended in a washing solution (D-PBS(-) containing 2 % (v/v) FBS). Next, the cells were incubated with the following antibodies at 4°C for 60 min; Alexa Fluor 488-conjugated mouse anti-human Oct3/4 mAb, mouse anti-human Nanog mAb, FITC-conjugated mouse anti-SSEA-1 mAb, or mouse anti-SSEA-4 mAb. FITC-labeled mouse IgG3 and IgM and Alexa Fluor 488-conjugated mouse IgG1 were used as the isotype controls. After three washes with washing solution, the cells were passed through a Falcon 35 μm cell strainer (Corning; #352235). Data were collected using a FACSCelesta™ flow cytometer and analyzed using the BD FlowJo™ v10 software.

Immunostaining

hiPSCs cells serially passaged five times under 3D gel conditions were seeded on a Cell Desk LF1 (Sumitomo Bakelite, Tokyo, Japan; #MS-92132Z) coated with 0.5 μg/cm^2 iMatrix-511. After culture in StemFit AK02N culture medium for 5 days, the cells were fixed with 4 % (w/v) paraformaldehyde in D-PBS(-) for 10 min at room temperature. The fixed cells were permeabilized with 0.1 % (v/v) Triton X-100 in D-PBS(-) for 15 min at room temperature, blocked with 1 % BSA (w/v) in D-PBS(-) for 30 min, and incubated with primary antibodies, mouse anti-human Oct-3/4 mAb, rabbit anti-human Nanog mAb, or rabbit anti-SOX2 pAb, at 4°C overnight. After three washes with D-PBS(-), the bound antibodies were visualized with Alexa Fluor 488-conjugated secondary antibodies and Hoechst 33342. The stained samples were mounted on glass slides with Fluoro-KEEPER Antifade Reagen and observed under a BZ-X710 fluorescence microscope (Keyence, Osaka, Japan).

For the trilineage differentiation assay, 3D-P5 hiPSCs were seeded on a μ-Slide 4 Well (ibidi, Gräfelfing, Germany; #80426) coated with 0.5 μg/cm^2 iMatrix-511 in StemFit AK02N culture medium without solution C. After 14 days of culture, the cells were fixed, permeabilized, and blocked as described above. Next, the cells were incubated with primary antibodies, mouse anti-human Oct-3/4 mAb, mouse anti-tubulin β3 mAb, mouse anti-α-smooth muscle actin mAb or goat anti-SOX17 pAb, at 4°C overnight. After three washes with D-PBS(-), the bound antibodies were visualized using Alexa Fluor 488-conjugated secondary antibodies and Hoechst 33342. The stained cells were washed three times with D-PBS(-) and then covered with Fluoro-KEEPER Antifade Reagent. The stained samples were observed under a Fluoview FV1200 laser scanning confocal microscope.

Immunostaining of the Chimera-511-fibrin composite gel was performed as follows. After 7 days of 3D gel culture, the fibrin gels containing cell spheres were washed with D-PBS(-), fixed with 4 % (w/v) paraformaldehyde in D-PBS(-) for 1 h at 4°C, washed with D-PBS(-), and soaked in 30 % (w/v) sucrose in D-PBS(-) until the samples sank. The samples were further incubated with 30 % sucrose/FSC 22 Frozen Section Media (Leica Biosystems, Nußloch, Germany; #FC-22) (volume ratio, 1:2) overnight at 4°C. Finally, the gels were embedded in 100 % FSC 22 Frozen Section Media and snap-frozen onto a metal block precooled with liquid nitrogen. Cryosections (7-μm thickness) were washed with D-PBS(-), blocked with D-PBS(-) containing 3 % (w/v) BSA and 0.1 % (v/v) Tween-20 for 30 min, and incubated with primary antibodies, mouse anti-human Oct-3/4 mAb and rabbit anti-human Nanog mAb, at 4°C overnight. After three washes with D-PBS(-), the bound antibodies were visualized with Alexa Fluor 488- or 647-conjugated secondary antibodies, rBC2LCN-FITC, and Hoechst 33342. After another three washes with D-PBS(-), the sections were mounted with Fluoro-KEEPER Antifade Reagent and observed under a Fluoview FV1200 laser scanning confocal microscope.

Whole-mount immunostaining was performed as follows. After

culture for 7 days, the Chimera-511-functionalized fibrin gels were washed with D-PBS(-), fixed with 4 % (w/v) paraformaldehyde in D-PBS (-), and blocked with D-PBS(-) containing 3 % (w/v) BSA and 0.1 % (v/v) Tween-20 at 37°C for 3 h. Next, the gels were incubated with antibodies against human integrin α6, human integrin β1, and LM-γ1 at 37°C overnight. After washing with D-PBS(-) containing 0.1 % (v/v) Tween-20 (DPBS-T), the gels were incubated with secondary antibodies, Alexa Fluor™ 405-conjugated mouse IgG, Alexa Fluor™ 488-conjugated rat IgG, and Alexa Fluor™ 546-conjugated rabbit IgG, at 37°C overnight. After washing with DPBS-T, the gels were mounted with Fluoro-KEEPER Antifade Reagent and observed under a Fluoview FV1200 laser scanning confocal microscope.

Funding

This work was supported by KAKENHI Grant-in-Aid for Transformative Research Area (A) 23721401 to K.S.

Author contributions

Y.T. and K.S. wrote the manuscript. M.T. performed the molecular design of Chimera-511. Y.T., M.T., A.H., and A.I. prepared the recombinant proteins and performed the binding assays and cell-based assays. All authors read and commented on the manuscript.

Declaration of competing interest

K.S. is a cofounder and shareholder of Matrixome, Inc. Y.T. holds a position as a project leader at Matrixome, Inc. A.H. and A.I. hold positions as Staff Scientists at Matrixome, Inc.

Acknowledgments

We thank Dr. Junichi Takagi (Institute for Protein Research, The University of Osaka) for providing the expression vector for recombinant human integrin β1. We also thank Dr. Fumi Ebisu and Kohei Minobe (Institute for Protein Research, The University of Osaka) for maintenance of the hiPSCs. Finally, we thank Alison Sherwin, PhD, from Edanz (https://jp.edanz.com/ac) for editing a draft of this manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.matbio.2025.10.003.

Data availability

Data will be made available on request.

References

[1] Y. Shao, J. Fu, Engineering multiscale structural orders for high-fidelity embryoids and organoids, Cell Stem. Cell 29 (2022) 722–743.
[2] J. Kim, B.-K. Koo, J.A. Knoblich, Human organoids: model systems for human biology and medicine, Nat. Rev. Mol. Cell Biol. 21 (2020) 571–584.
[3] B.N. Kharbikar, P. Mohindra, T.A. Desai, Biomaterials to enhance stem cell transplantation, Cell Stem. Cell 29 (2022) 692–721.
[4] S.R. Caliari, J.A. Burdick, A practical guide to hydrogels for cell culture, Nat. Methods 13 (2016) 405–414.
[5] S. Rezakhani, N. Gjorevski, M.P. Lutolf, Extracellular matrix requirements for gastrointestinal organoid cultures, Biomaterials 276 (2021) 121020.
[6] A.C. Brown, T.H. Barker, Fibrin-based biomaterials: modulation of macroscopic properties through rational design at the molecular level, Acta Biomater 10 (2014) 1502–1514.
[7] J.J. Rice, M.M. Martino, L. De Laporte, F. Tortelli, P.S. Briquez, J.A. Hubbell, Engineering the regenerative microenvironment with biomaterials, Adv. Healthc. Mater. 2 (2013) 57–71.
[8] E.A. Aisenbrey, W.L. Murphy, Synthetic alternatives to Matrigel, Nat. Rev. Mater. 5 (2020) 539–551.
[9] I.V. Roberts, D. Bukhary, C.Y.L. Valdivieso, N. Tirelli, Fibrin matrices as (Injectable) biomaterials: formation, clinical use, and molecular engineering, Macromol. Biosci. 20 (2020) e1900283.
[10] M.W. Mosesson, Fibrinogen and fibrin structure and functions, J. Thromb. Haemost. 3 (2005) 1894–1904.
[11] P. de la Puente, D. Ludeña, Cell culture in autologous fibrin scaffolds for applications in tissue engineering, Exp. Cell Res. 322 (2014) 1–11.
[12] E. Dejana, M.G. Lampugnani, M. Giorgi, M. Gaboli, P.C. Marchisio, Fibrinogen induces endothelial cell adhesion and spreading via the release of endogenous matrix proteins and the recruitment of more than one integrin receptor, Blood 75 (1990) 1509–1517.
[13] J.C. Schense, J. Bloch, P. Aebischer, J.A. Hubbell, Enzymatic incorporation of bioactive peptides into fibrin matrices enhances neurite extension, Nat. Biotechnol. 18 (2000) 415–419.
[14] K. Nam, J.P. Jones, P. Lei, S.T. Andreadis, O.J. Baker, Laminin-111 peptides conjugated to fibrin hydrogels promote formation of Lumen containing parotid gland cell clusters, Biomacromolecules 17 (2016) 2293–2301.
[15] E. Hohenester, Structural biology of laminins, Essays Biochem. 63 (2019) 285–295.
[16] T. Arimori, N. Miyazaki, E. Mihara, M. Takizawa, Y. Taniguchi, C. Cabañas, K. Sekiguchi, J. Takagi, Structural mechanism of laminin recognition by integrin, Nat. Commun. 12 (2021) 4012.
[17] M. Aumailley, L. Bruckner-Tuderman, W.G. Carter, R. Deutzmann, D. Edgar, P. Ekblom, J. Engel, E. Engvall, E. Hohenester, J.C.R. Jones, H.K. Kleinman, M. P. Marinkovich, G.R. Martin, U. Mayer, G. Meneguzzi, J.H. Miner, K. Miyazaki, M. Patarroyo, M. Paulsson, V. Quaranta, J.R. Sanes, T. Sasaki, K. Sekiguchi, L. M. Sorokin, J.F. Talts, K. Tryggvason, J. Uitto, I. Virtanen, K. von der Mark, U. M. Wewer, Y. Yamada, P.D. Yurchenco, A. simplified laminin nomenclature, Matrix Biol 24 (2005) 326–332.
[18] M. Yamada, K. Sekiguchi, Molecular basis of laminin-integrin interactions, Curr. Top. Membr. 76 (2015) 197–229.
[19] T. Miyazaki, S. Futaki, H. Suemori, Y. Taniguchi, M. Yamada, M. Kawasaki, M. Hayashi, H. Kumagai, N. Nakatsuji, K. Sekiguchi, E. Kawase, Laminin E8 fragments support efficient adhesion and expansion of dissociated human pluripotent stem cells, Nat. Commun. 3 (2012) 1236.
[20] M. Nakagawa, Y. Taniguchi, S. Senda, N. Takizawa, T. Ichisaka, K. Asano, A. Morizane, D. Doi, J. Takahashi, M. Nishizawa, Y. Yoshida, T. Toyoda, K. Osafune, K. Sekiguchi, S. Yamanaka, A novel efficient feeder-free culture system for the derivation of human induced pluripotent stem cells, Sci. Rep. 4 (2014) 3594.
[21] H. Ido, A. Nakamura, R. Kobayashi, S. Ito, S. Li, S. Futaki, K. Sekiguchi, The requirement of the glutamic acid residue at the third position from the carboxyl termini of the laminin γ chains in integrin binding by laminins, J. Biol. Chem. 282 (2007) 11144–11154.
[22] M. Takizawa, T. Arimori, Y. Taniguchi, Y. Kitago, E. Yamashita, J. Takagi, K. Sekiguchi, Mechanistic basis for the recognition of laminin-511 by α6β1 integrin, Sci. Adv. 3 (2017) e1701497.
[23] Y. Taniguchi, M. Takizawa, S. Li, K. Sekiguchi, Bipartite mechanism for laminin-integrin interactions: identification of the integrin-binding site in LG domains of the laminin α chain, Matrix Biol 87 (2020) 66–76.
[24] R. Deutzmann, M. Aumailley, H. Wiedemann, W. Pysny, R. Timpl, D. Edgar, Cell adhesion, spreading and neurite stimulation by laminin fragment E8 depends on maintenance of secondary and tertiary structure in its rod and globular domain, Eur. J. Biochem. 191 (1990) 513–522.
[25] U. Sung, J.J. O'Rear, P.D. Yurchenco, Cell and heparin binding in the distal long arm of laminin: identification of active and cryptic sites with recombinant and hybrid glycoprotein, J. Cell Biol. 123 (1993) 1255–1268.
[26] L. Medved, J.W. Weisel, Fibrinogen and factor XIII subcommittee of scientific standardization committee of international society on thrombosis and haemostasis, recommendations for nomenclature on fibrinogen and fibrin, J. Thromb. Haemost. 7 (2009) 355–359.
[27] R. Nishiuchi, J. Takagi, M. Hayashi, H. Ido, Y. Yagi, N. Sanzen, T. Tsuji, M. Yamada, K. Sekiguchi, Ligand-binding specificities of laminin-binding integrins: a comprehensive survey of laminin-integrin interactions using recombinant α3β1, α6β1, α7β1 and α6β4 integrins, Matrix Biol. 25 (2006) 189–197.
[28] S. Futaki, I. Nakano, M. Kawasaki, N. Sanzen, K. Sekiguchi, Molecular profiling of the basement membrane of pluripotent epiblast cells in post-implantation stage mouse embryos, Regen Ther. 12 (2019) 55–65.
[29] T. Miyazaki, S. Futaki, K. Hasegawa, M. Kawasaki, N. Sanzen, M. Hayashi, E. Kawase, K. Sekiguchi, N. Nakatsuji, H. Suemori, Recombinant human laminin isoforms can support the undifferentiated growth of human embryonic stem cells, Biochem. Biophys. Res. Commun. 375 (2008) 27–32.
[30] J. Gu, A. Fujibayashi, K.M. Yamada, K. Sekiguchi, Laminin-10/11 and fibronectin differentially prevent apoptosis induced by serum removal via phosphatidylinositol 3-kinase/akt- and MEK1/ERK-dependent pathways, J. Biol. Chem. 277 (2002) 19922–19928.
[31] C. Duval, A. Profumo, A. Aprile, A. Salis, E. Millo, G. Damonte, J.S. Gauer, R.A. S. Ariëns, M. Rocco, Fibrinogen αc-regions are not directly involved in fibrin polymerization as evidenced by a "Double-Detroit" recombinant fibrinogen mutant and knobs-mimic peptides, J. Thromb. Haemost. 18 (2020) 802–814.
[32] A.S.C. Soon, S.E. Stabenfeldt, W.E. Brown, T.H. Barker, Engineering fibrin matrices: the engagement of polymerization pockets through fibrin knob technology for the delivery and retention of therapeutic proteins, Biomaterials 31 (2010) 1944–1954.
[33] N. Polisetti, L. Sorokin, N. Okumura, N. Koizumi, S. Kinoshita, F.E. Kruse, U. Schlötzer-Schrehardt, Laminin-511 and -521-based matrices for efficient ex

vivo-expansion of human limbal epithelial progenitor cells, Sci. Rep. 7 (2017) 5152.
[34] A. Saraswathibhatla, D. Indana, O. Chaudhuri, Cell-extracellular matrix mechanotransduction in 3D, Nat. Rev. Mol. Cell Biol. 24 (2023) 495–516.
[35] H. Ido, K. Harada, Y. Yagi, K. Sekiguchi, Probing the integrin-binding site within the globular domain of laminin-511 with the function-blocking monoclonal antibody 4C7, Matrix Bio. 25 (2006) 112–117.
[36] J. Takagi, H.P. Erickson, T.A. Springer, C-terminal opening mimics "inside-out" activation of integrin α5β1, Nat. Struct. Biol. 8 (2001) 412–416.
[37] R. Nishiuchi, O. Murayama, H. Fujiwara, J. Gu, T. Kawakami, S. Aimoto, Y. Wada, K. Sekiguchi, Characterization of the ligand-binding specificities of integrin α3β1 and α6β1 using a panel of purified laminin isoforms containing distinct α chains, J. Biochem. 134 (2003) 497–504.
[38] K. Takahashi, K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, S. Yamanaka, Induction of pluripotent stem cells from adult human fibroblasts by defined factors, Cell 131 (2007) 861–872.
[39] T. Miyazaki, T. Isobe, N. Nakatsuji, H. Suemori, Efficient adhesion culture of Human pluripotent stem cells using laminin fragments in an uncoated manner, Sci. Rep. 7 (2017) 41165.