

α -5 Laminin Synthesized by Human Pluripotent Stem Cells Promotes Self-Renewal

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<http://dx.doi.org/10.1016/j.stemcr.2015.06.009>

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SUMMARY

Substrate composition significantly impacts human pluripotent stem cell (hPSC) self-renewal and differentiation, but relatively little is known about the role of endogenously produced extracellular matrix (ECM) components in regulating hPSC fates. Here we identify α -5 laminin as a signature ECM component endogenously synthesized by undifferentiated hPSCs cultured on defined substrates. Inducible shRNA knockdown and Cas9-mediated disruption of the *LAMA5* gene dramatically reduced hPSC self-renewal and increased apoptosis without affecting the expression of pluripotency markers. Increased self-renewal and survival was restored to wild-type levels by culturing the *LAMA5*-deficient cells on exogenous laminin-521. Furthermore, treatment of *LAMA5*-deficient cells with blebbistatin or a ROCK inhibitor partially restored self-renewal and diminished apoptosis. These results demonstrate that endogenous α -5 laminin promotes hPSC self-renewal in an autocrine and paracrine manner. This finding has implications for understanding how stem cells dynamically regulate their microenvironment to promote self-renewal and provides guidance for efforts to design substrates for stem cell bioprocessing.

INTRODUCTION

The extracellular matrix (ECM) is known to play an integral role in human pluripotent stem cell (hPSC) culture and maintenance. In the absence of appropriate substrate cues, hPSCs spontaneously differentiate, which has led to the development of numerous culture approaches and substrates intended to maintain pluripotency in hPSC cultures (Domogatskaya et al., 2008; Evseenko et al., 2009). Efforts in recent years (Chen et al., 2011; Mei et al., 2010; Melkounian et al., 2010; Rodin et al., 2014b; Saha et al., 2011; Villa-Diaz et al., 2013) have specifically focused on the development of fully defined, xeno-free culture substrates as alternatives to the use of mouse embryonic fibroblast (MEF) feeder cells (Thomson et al., 1998) or Matrigel (Ludwig et al., 2006). Use of a defined, xeno-free culture environment is important for the performance of controlled in vitro investigations and for the clinical or commercial application of hPSCs (Azarin and Palecek, 2010; Chen et al., 2011).

Both natural and synthetic approaches have been used to generate fully defined hPSC culture substrates. ECM proteins such as vitronectin and laminins 511 and 521 have been shown to maintain hESC pluripotency and self-renewal (Braam et al., 2008; Domogatskaya et al., 2012; Miyazaki et al., 2012; Rodin et al., 2010, 2014a, 2014b; Yurchenco, 2011). However, not all ECM proteins are suitable for hPSC maintenance because collagens and fibronectin are not able to support an undifferentiated hPSC

population (Azarin and Palecek, 2010; Braam et al., 2008; Evseenko et al., 2009; Miyazaki et al., 2012; Rodin et al., 2010; Villa-Diaz et al., 2013). Other fully defined hPSC culture environments have been produced by modification of substrates with recombinant E-cadherin (marketed as StemAdhere) (Nagaoka et al., 2010) or engineered peptide coatings (marketed as Synthemax) (Melkounian et al., 2010).

Although many studies have applied exogenous ECM components to create hPSC culture substrates, relatively little attention has been given to the role of the endogenously produced ECM in hPSC self-renewal. Specifically, it is unknown whether there exists a common ECM “signature” produced by hPSCs cultured on substrates known to support the maintenance of undifferentiated hPSC culture. The identification of such an ECM signature—i.e., specific ECM components that are commonly produced across undifferentiated hPSCs maintained in different culture environments—could lead to a better understanding of how hPSCs regulate self-renewal.

In this study, we first identified α -5 laminin as a predominant ECM component produced endogenously by undifferentiated hPSCs—both human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs)—cultured on various defined substrates and then employed two different genetic manipulation strategies to disrupt α -5 laminin production to investigate the role of α -5 laminin in hPSC self-renewal and pluripotency. Our findings implicate α -5 laminin as a critical autocrine and paracrine factor that regulates hPSC survival and self-renewal.

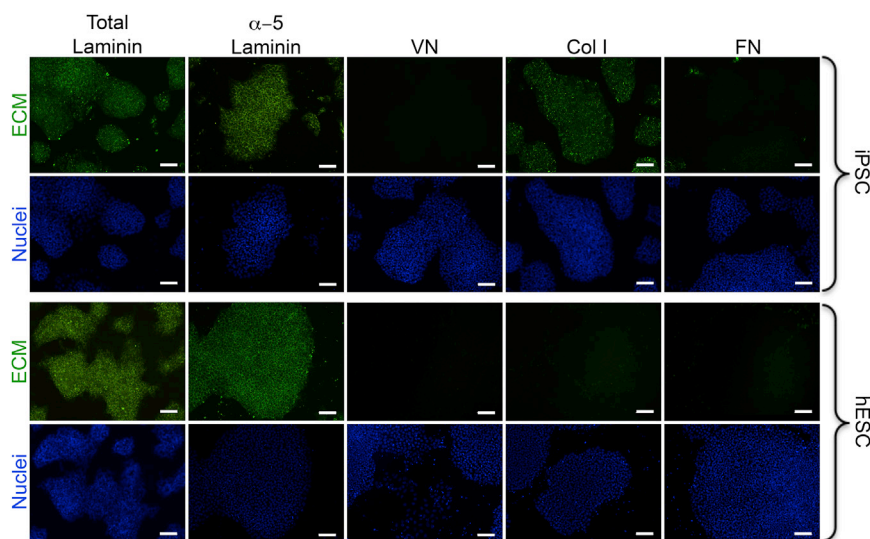


Figure 1. Immunofluorescent Staining of ECM Proteins Deposited by hPSCs Cultured under Fully Defined Conditions

H9 hESCs and 19-9-11 iPSCs were cultured on Synthemax for 5 days in E8 medium and stained for the indicated ECM proteins. Production of laminin (and, in particular, α -5 laminin) was the only common ECM feature across all substrates and both cell lines. Scale bars, 100 μ m. See also Figures S1 and S6. VN, vitronectin; Col I, collagen type I; FN, fibronectin.

RESULTS

α -5 Laminin Is Produced by hPSCs under Defined Culture Conditions

The ECM deposition profile of undifferentiated hPSCs cultured under defined conditions was evaluated by culturing H9 hESCs and 19-9-11 iPSCs on Synthemax, E-cadherin (StemAdhere), or recombinant human vitronectin in E8 medium for 5 days, followed by immunofluorescence detection of laminin, collagen I, fibronectin, and vitronectin (Figure 1; Figures S1A and S1B). Of these ECM proteins, only the production of laminin and, specifically, α -5 laminin was common across all substrates and both hPSC lines (Figure 1).

Generation and Characterization of Inducible shRNA and Cas9 α -5 Laminin Knockout Lines

Two different genetic manipulation strategies were implemented to investigate the role of α -5 laminin in hPSC maintenance. In one approach, H9 and 19-9-11 cell lines were transduced with an inducible short hairpin RNA (shRNA) sequence (Table S1) targeting α -5 laminin (ishLAMA5) to achieve doxycycline (Dox)-induced knockdown of α -5 laminin production (Figure 2A; Figures S2A–S2D; Lian et al., 2013). Dox treatment of ishLAMA5 cell lines resulted in a 60% reduction in α -5 laminin expression (Figure 2A), and the karyotype of the 19-9-11 ishLAMA5 cell line was found to be normal (Figure S2E). Pluripotency characteristics were maintained during LAMA5 knockdown, as indicated by no significant change in the expression of the pluripotency marker Nanog (Figure 2B; Figures S2F and S2G).

In a separate approach, CRISPR-Cas9 gene editing was used in both H9 and 19-9-11 cell lines to create genetic

mutations that resulted in loss of function of the LAMA5 gene (Ran et al., 2013b). A set of guide RNAs (gRNA) targeting LAMA5 (Figure 2C; Table S2) was transfected into the hPSCs concurrently with a Cas9-2A-GFP plasmid that encodes both Cas9 and GFP. Single GFP-positive cells were expanded into colonies, and the gRNA target sites amplified from the genomic DNA of each colony were Sanger-sequenced to confirm LAMA5 gene disruption (Figure 2C). From this screening process, we identified an H9 line containing a heterozygous mutation (Figures S3A and S3B) and a 19-9-11 line containing a homozygous deletion/frameshift mutation (Figure 2C). All mutated lines maintained expression of the pluripotency marker Nanog while exhibiting significantly reduced expression of α -5 laminin (Figures 2D and 2E; Figures S3C–S3E). The homozygous 19-9-11 knockout cell line was found to exhibit a normal human karyotype (Figure S3F).

Disrupted α -5 Laminin Production Increases Apoptosis, Causing Impaired Self-Renewal

Under defined culture conditions on the Synthemax substrate, treatment of ishLAMA5 hPSC lines with Dox resulted in an immediately apparent reduction in cell number relative to both untreated and ishRNA sequence-scrambled controls (Figure 3A; Figure S4A). Several other factors related to cell self-renewal were then examined to identify the cause of this disparity in cell number. Production of Ki-67, a marker present during active progression through the cell cycle (Gerdes et al., 1984), was not significantly altered by α -5 laminin knockdown in either H9 (Figure S4B) or 19-9-11 (Figure 3B) cells. Cell-cycle distribution in α -5 laminin knockdown and untreated cells was also quantified and found not to be significantly affected by the disruption in α -5

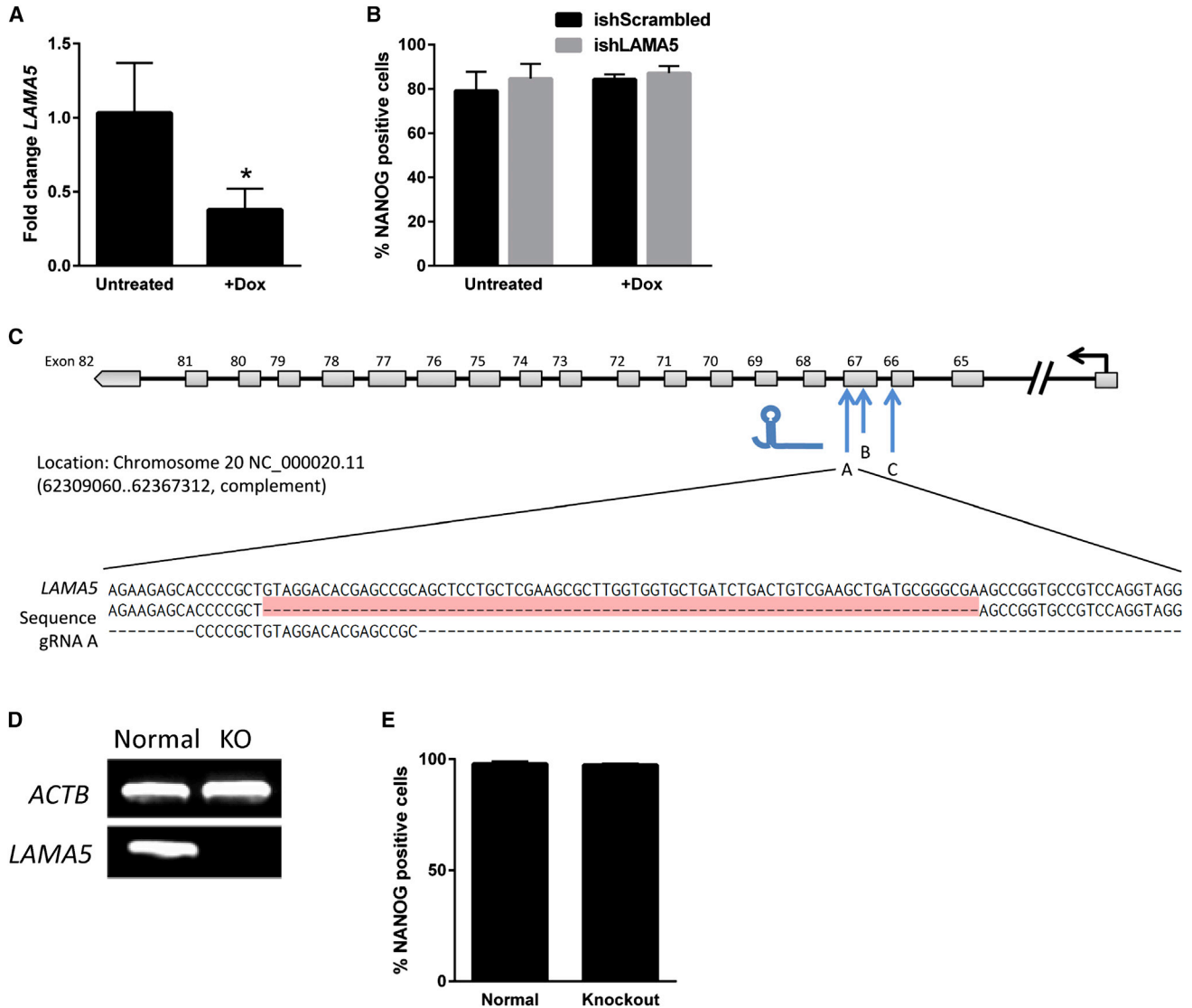


Figure 2. Generation of hPSC Knockdown and Knockout Lines

(A) Doxycycline treatment of the Dox-inducible *LAMA5* 19-9-11 shRNA cell line resulted in decreased *LAMA5* expression relative to untreated ish*LAMA5* cells, as measured by qRT-PCR after 3 days of culture on Synthemax (n = 3 independent samples/condition).
 (B) Expression of the pluripotency marker Nanog was detected via flow cytometry and found to be maintained in ish*LAMA5* 19-9-11 cells and scrambled shRNA controls cultured on Synthemax for 5 days (n = 3 independent samples/condition).
 (C) Schematic of the human *LAMA5* locus showing gRNA targeting locations for Cas9-mediated gene disruption. Sanger sequencing results for the 19-9-11 KO line are shown below, with the red section indicating a homozygous deletion of the *LAMA5* DNA sequence.
 (D) Detection of *LAMA5* in normal and homozygous knockout 19-9-11 iPSCs via RT-PCR using a primer within the deleted region of the knockout line. Lack of amplification in the KO line verifies homozygous deletion of the gene as detected by Sanger sequencing.
 (E) Nanog expression by normal and KO 19-9-11 iPSCs as measured by flow cytometry after 5 days of culture on Synthemax (n = 3 independent samples/condition).

*p < 0.01. Data are presented as mean ± SD. See also Figures S2 and S3.

laminin production (Figures S4C and S4D). H9 and 19-9-11 cells subjected to Cas9-mediated knockout of α -5 laminin function behaved similarly as the shRNA knockdown cell lines, exhibiting dramatically reduced cell

numbers relative to control hPSCs cultured on Synthemax (Figure 3C; Figure S4E).

Apoptosis in H9 and 19-9-11 cultures with disrupted α -5 laminin production was evaluated to further investigate

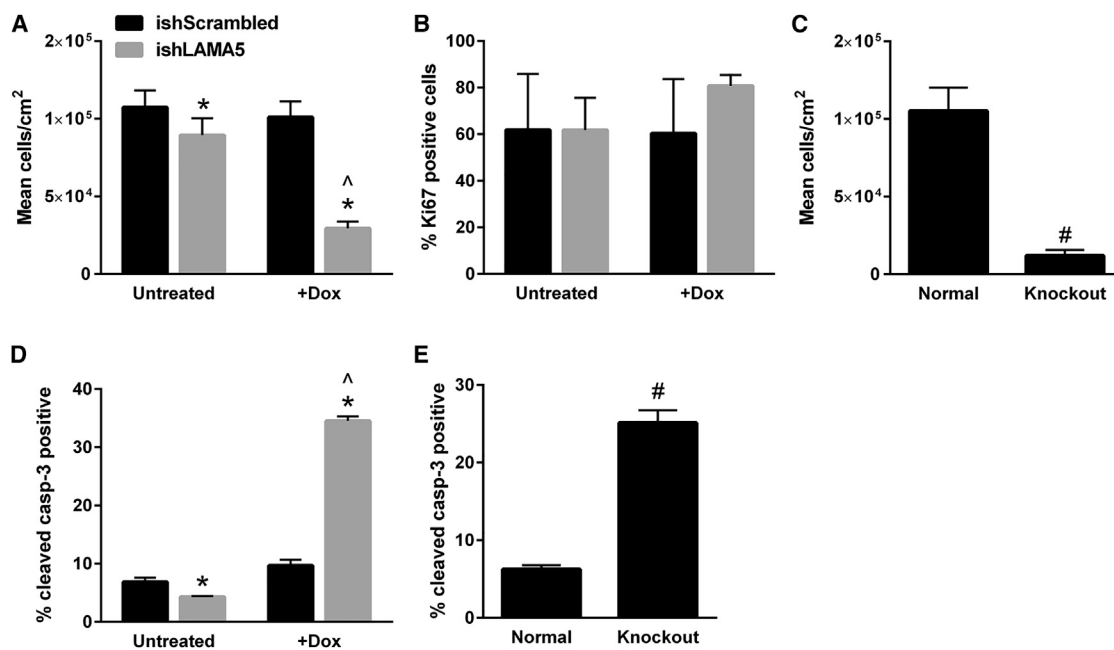


Figure 3. Disruption of α -5 Laminin Production Impacts 19-9-11 Cell Number and Apoptosis

(A) Dox treatment of ishLAMA5 19-9-11 cells cultured on Synthetmax for 5 days resulted in decreased cell density compared with untreated or scrambled ishRNA controls. Cells were initially seeded at 6,600 cells/cm².

(B) Expression of the proliferation marker Ki67 as measured via flow cytometry after 5 days of culture on Synthetmax.

(C) Cas9-mediated knockout of *LAMA5* in 19-9-11 cells also resulted in decreased cell density.

(D) Dox treatment significantly increased apoptosis in 19-9-11 ishLAMA5 cells cultured for 5 days on Synthetmax, as indicated by measurement of caspase-3 via flow cytometry.

(E) Cas9-mediated knockout of *LAMA5* in 19-9-11 cells increased apoptosis relative to wild-type (normal) 19-9-11 iPSCs cultured on Synthetmax for 5 days.

* $p < 0.001$ versus untreated cells, ^ $p < 0.001$ versus Dox-treated scrambled control cells, # $p < 0.001$ versus wild-type 19-9-11 iPSCs ($n = 3$ independent samples/condition). Data are presented as mean \pm SD. See also [Figures S4](#) and [S5](#).

the cause of the observed decline in cell number in α -5 laminin knockdown and knockout hPSC lines. Increased production of both cleaved caspase-3 (Li et al., 2014) and annexin V (van Engeland et al., 1998; Zhang et al., 1997) was found in both H9 and 19-9-11 inducible knockdown cell lines relative to scrambled controls (Figure 3D; Figure S4F). H9 and 19-9-11 α -5 laminin knockout cell lines also exhibited significantly increased expression of cleaved caspase-3 and annexin V relative to unmodified hPSCs (Figure 3E; Figures S5A and S5B).

Rescue of hPSC Self-Renewal via Culture on Laminin-521

hPSCs possessing the Cas9-mediated disruption to *LAMA5* were cultured on exogenously supplied laminin-521 to determine whether the noted decrease in cell number and increase in apoptosis could be reversed by the presentation of α -5 laminin to the cells. Indeed, culture of knockout H9 and 19-9-11 cells on exogenous laminin-521 restored cell expansion to levels near those found

in normal hPSC controls (Figures 4A and 4B; Figures S5C–S5E). The extent of rescue was dependent on the laminin-521 coating density (Figure S5F). Although the expansion of normal hPSCs was improved by culture on exogenous vitronectin relative to Synthetmax, use of vitronectin as a culture substrate was unable to fully rescue the expansion deficit resulting from *LAMA5* knockout. This finding indicates that rescue of the knockout cells was likely specific to α -5 laminin and not simply due to the presence of an exogenous ECM component that supports undifferentiated hPSC culture. Laminin-111 was not used as a comparison condition because pure human laminin-111 does not support the attachment of normal hPSCs (Figure S6A).

Culture on exogenously supplied laminin-521 was also able to inhibit apoptosis in *LAMA5* knockout H9 (Figures S6A and S6B) and 19-9-11 (Figures 4C and 4D) cells. Specifically, culture of knockout cells on laminin-521 resulted in a full reduction of the apoptosis markers cleaved caspase-3 and annexin V to levels observed in wild-type H9 and

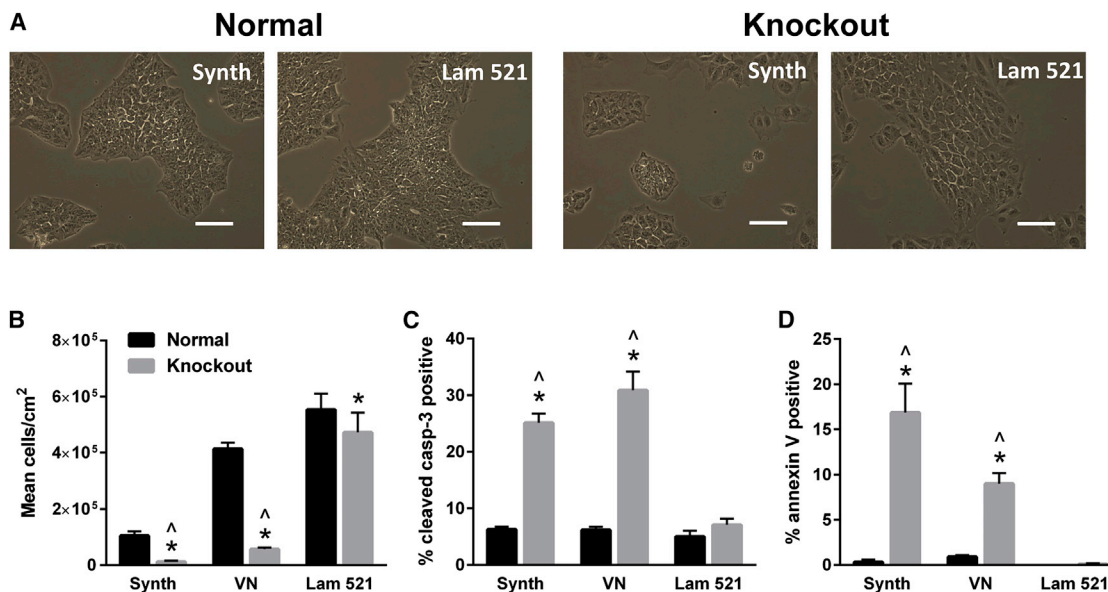


Figure 4. Culture of 19-9-11 *LAMA5* Knockout Cells on Laminin-521, but Not Vitronectin, Results in Rescue of Cell Number

(A) Bright-field images of wild-type and *LAMA5* KO 19-9-11 iPSCs cultured on laminin-521 (Lam 521) or Synthemax (Synth) for 3 days, illustrating differences in cell density.

(B) Culture of Cas9-mediated *LAMA5* KO 19-9-11 iPSCs on laminin-521 rescued cell number, as indicated by analysis of mean cell density after 5 days of culture on Synthemax, vitronectin, or laminin-521. Cells were initially seeded at 6,600 cells/cm².

(C and D) Apoptosis of 19-9-11 *LAMA5* KO cells was also restored to wild-type levels by culture on laminin-521, as indicated by measurement of caspase-3 (C) and annexin V (D) via flow cytometry after 5 days of culture.

Scale bars, 100 μ m. * $p < 0.001$ versus wild-type 19-9-11 iPSCs cultured on the same substrate, ^ $p < 0.001$ versus knockout cells cultured on laminin-521 ($n = 3$ independent samples/condition). Data are presented as mean \pm SD. See also Figure S5.

19-9-11 cells. Culture on vitronectin was not able to reduce apoptosis markers in the knockout lines.

Apoptotic Response Arises from Cell Blebbing and Is Partly Reversed by Contractile Inhibition

The results reported in Figures 3 and 4 suggest that increased apoptosis is at least partly responsible for the decreases in cell expansion observed upon disruption of α -5 laminin production. Additionally, a significant increase in cell blebbing was observed in knockout cells cultured on Synthemax (Figures 5A and 5B; Figure S6B), indicating a role for cytoskeletal contractility in regulating apoptosis under these conditions. Cell survival was not improved by the addition of soluble laminin-521 to suspension cultures of *LAMA5* knockout 19-9-11 hPSCs (Figure S6C), suggesting that binding of α -5 laminin to cell surface receptors alone is not sufficient for cell rescue but that the presentation of α -5 laminin on the substrate surface may be needed to provide appropriate ECM contacts for regulating cytoskeletal tension. Therefore, we next investigated mechanisms linking α -5 laminin to this apoptotic response by applying to H9 and 19-9-11 knockout lines a panel of small molecule inhibitors targeting a range of apoptotic pathway components as well as elements of the cytoskel-

eton (Table S3), followed by evaluation of cell expansion and apoptosis. In both the H9 and 19-9-11 cell lines with the *LAMA5* knockout, treatment with either a Rho kinase (ROCK) inhibitor (Y27632) or the myosin inhibitor blebbistatin was able to increase cell expansion, resulting in partial rescue. These levels of expansion remained lower than those observed in normal hPSCs cultured on Synthemax (Figure 5C; Figure S6D). This partial rescue of cell expansion was accompanied by a decrease in the expression of apoptosis markers relative to the untreated knockout population (Figure 5D; Figure S6E). These results suggest that relieving cell tension can at least partly restore hPSC expansion by inhibiting apoptosis in the absence of α -5 laminin.

DISCUSSION

In this study, we first identified laminin as a prevalent signature component of the ECM produced endogenously by undifferentiated H9 hESCs and 19-9-11 iPSCs cultured on three different defined substrates and subsequently examined the role of endogenously produced α -5 laminin in regulating the self-renewal and pluripotency marker

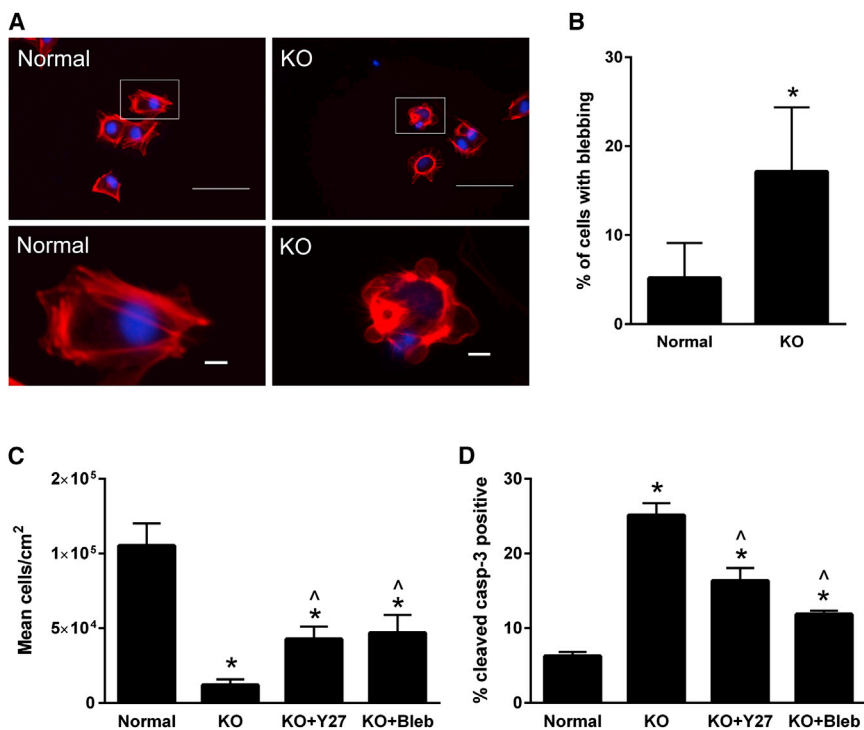


Figure 5. Partial Rescue of Cell Self-Renewal by Treatment with Inhibitors of Cell Contractility

(A) F-actin staining of normal and KO 19-9-11 cultures after 48 hr on Synthemax. White boxes indicate regions examined at a higher magnification. Scale bars, 100 μm (top) and 10 μm (bottom).

(B) Percentage of observed blebbing cells in normal and KO 19-9-11 cultures, calculated as the number of cells with observed blebs over the total number of cells per image field.

(C) Treatment with inhibitors of cell contractility (blebbistatin [Bleb] and Y27632 [Y27]) was able to partially rescue cell number for *LAMA5* KO 19-9-11 iPSCs cultured on Synthemax for 5 days. Cells were initially seeded at 6,600 cells/cm².

(D) Treatment with blebbistatin or Y27632 was also able to decrease apoptosis relative to untreated *LAMA5* KO 19-9-11 iPSCs, as measured by flow cytometric analysis of cleaved caspase-3 after 5 days of culture on Synthemax.

*p < 0.001 versus wild-type 19-9-11 iPSCs, ^p < 0.001 versus *LAMA5* knockout iPSCs. n = 3 independent samples/condition. Data are presented as mean ± SD. See also Figure S6.

expression of these stem cells. We targeted the α -5 chain specifically because the majority of integrin binding activity in the laminin molecule is thought to be conferred by the C-terminal globular domains encoded on the α strand (Beck et al., 1990; Deutzmann et al., 1990; Ido et al., 2006; Li et al., 2002; Miner and Yurchenco, 2004). Furthermore, hPSCs predominantly express the $\alpha_6\beta_1$ integrin, which interacts strongly with these laminin globular domains (Evseenko et al., 2009; Li et al., 2002; Meng et al., 2010; Miyazaki et al., 2012; Rodin et al., 2010). Using two different techniques to disrupt α -5 laminin production in both hESC and iPSC lines, we found that hPSC expression of the pluripotency marker Nanog was not strongly dependent on α -5 laminin production but that hPSC self-renewal diminished as a result of apoptosis in the absence of this specific ECM component. Importantly, this apoptosis could be reduced by supplying the knockout cells with an exogenous source of α -5 laminin, such as culturing them on a laminin-521 substrate (Rodin et al., 2014b).

To link this apoptotic response to an ECM-dependent mechanism, we tested heterozygous and knockout *LAMA5* lines against several small-molecule inhibitors and identified blebbistatin and the ROCK inhibitor Y27632 as being able to reduce the apoptotic response of the knockout lines cultured under defined conditions.

Blebbistatin and Y27632 are known to act in pluripotent stem cells by preventing membrane blebbing, a phenomenon that was observed during culture of α -5 laminin knockout cells under defined conditions. These agents prevent blebbing via inhibition of contractility (Chen et al., 2010, 2014; Ohgushi et al., 2010; Ohgushi and Sasai, 2011; Watanabe et al., 2007), which may reduce anoikis, a type of apoptotic cell death that occurs because of a lack of cell-cell and cell-ECM contacts (Chen et al., 2010; Kovács et al., 2004; Ohgushi et al., 2010; Ohgushi and Sasai, 2011; Watanabe et al., 2007). Although contractility inhibition did reduce apoptosis in α -5 laminin-deficient cells, the restoration of hPSC self-renewal was only partial, which does suggest the involvement of additional signaling mechanisms in the hPSC response to α -5 laminin.

The specificity of the hPSC apoptotic response to a lack of proper ECM contacts highlights the importance of substrate interactions in regulating hPSC survival. Work by our own group suggests that differentiating pluripotent stem cells produce an ECM that is synergistic with the differentiation cues given to the cells (Laperle et al., 2015). Meanwhile, recent work by Meng et al. (2012) suggests that matrices, media, and the soluble growth factors to which hPSCs are exposed during culture have a synergistic

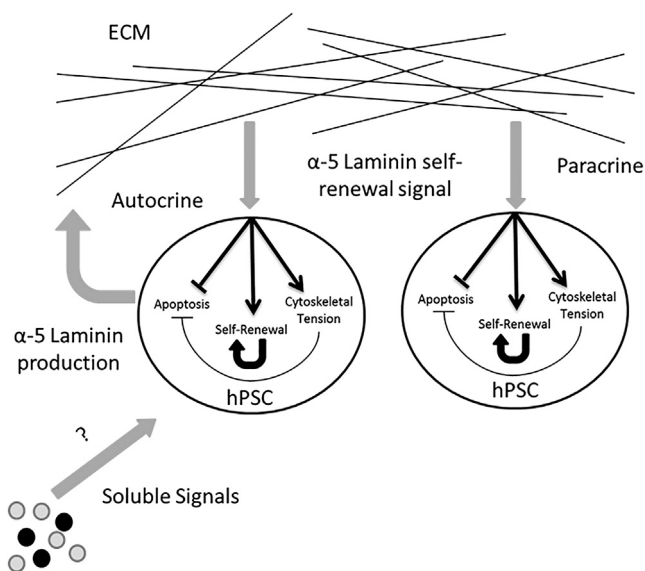


Figure 6. Proposed Autocrine and Paracrine Positive Feedback Loops in α -5 Laminin Self-Renewal Signaling

Undifferentiated hPSCs attach to a surface and receive soluble self-renewal cues such as TGF- β . As part of the cellular response to these maintenance cues, the hPSCs begin to produce an ECM enriched in α -5 laminin. Signaling from this ECM microenvironment, in turn, synergizes with the existing soluble cues and enables efficient self-renewal of cells in contact with the endogenous ECM.

effect on the growth and attachment of hPSCs under defined culture conditions. For instance, in mouse mesangial and early endoderm progenitor cells, transforming growth factor β (TGF- β) signaling has been shown to drive laminin production and increase the production of other ECM components (Jiang et al., 2005; Sugiyama et al., 2013; Tonary and Carnegie, 1996; Wells and Discher, 2008). It is possible that the α -5 laminin self-renewal signal, combined with other soluble components, drives hPSCs to produce a matrix that synergizes with these self-renewal signals. The hPSCs subsequently produce a matrix rich in α -5 laminin and receive supportive self-renewal signals from that matrix in an autocrine and paracrine signaling loop (Figure 6). Although this endogenous ECM may be necessary for efficient self-renewal, it is unlikely to be sufficient to fully support this process on its own.

In this work, we developed multiple tools to interrogate this autocrine and paracrine signaling loop involving α -5 laminin production by hPSCs. The use of the inducible short hairpin lines allowed us to control the dynamics of α -5 laminin production in a reversible manner. However, the interpretation of results is complicated by the action of the inducer, doxycycline, itself on cells; it is known to inhibit matrix remodeling proteinases (Gajbhiye et al.,

2014; Nip et al., 1993). In addition, knockdown was partial and variable among hairpins from cell line to cell line, with our best hairpin providing only \sim 60% knockdown efficiency. The Cas9-mediated knockout lines were developed to address these drawbacks with the hairpin system and provide a complete removal of the globular domains of α -5 laminin in hPSCs. The Cas9 system can easily be used, by simple modification of the guide RNA, to remove other domains of α -5 laminin and, in general, provides unprecedented precision in disrupting molecular signaling domains in large, complex proteins like ECM components. Application of the Cas9 tool in hPSCs offers a powerful way to study stem cell signaling, particularly in multicellular autocrine and paracrine contexts that involve reprogramming and differentiation.

Together, the findings reported here have implications with respect to not only understanding how existing substrates maintain hPSC self-renewal but also for designing new, defined substrates for hPSC culture. α -5 laminin is known to be present in numerous widely used but undefined hPSC culture systems. For instance, the functionality of MEF feeder cells in maintaining hPSCs is based on MEF production of laminin 511 (Hongisto et al., 2012). Additionally, α -5 laminin has been detected in Matrigel, perhaps the most widely used substrate for hPSC propagation (Hughes et al., 2010). Based on the results reported in this work, we put forth the hypothesis that the ability of many fully defined substrates to support hPSC self-renewal (Amit et al., 2011; Azarin and Palecek, 2010; Braam et al., 2008; Celiz et al., 2014; Chen et al., 2011; Evseenko et al., 2009; Klim et al., 2010; Mei et al., 2010; Melkounian et al., 2010; Miyazaki et al., 2012; Rodin et al., 2010, 2014b; Saha et al., 2011; Villa-Diaz et al., 2010, 2013; Weber et al., 2010; Zweigerdt et al., 2011) is likely due, in part, to their support of α -5 laminin deposition by the cultured cells. An illustrative example of such a substrate is a peptide coating based on repeats of a heparin binding motif that is designed to promote stem cell attachment via binding of extracellular glycosaminoglycan (GAG) domains on the stem cell surface (Klim et al., 2010). However, because of the high-affinity interactions that occur between heparin and the globular domains of α -5 laminin (Hozumi et al., 2009; Katagiri et al., 2014), it is also likely that these GAG-binding domains are able to bind secreted ECM proteins, specifically laminins. In another example, polymer substrate screening studies have identified specific chemical functionalities, namely carboxylic acid and ester residues, as critical polymer features for the support of hPSC growth (Mei et al., 2010; Saha et al., 2011). These same residues, along with substrate surface potential, are thought to control the adsorption and conformation of laminin molecules on a surface (Lin et al., 2014; Rodríguez Hernández



et al., 2007), suggesting that the ability of these materials to support hPSC culture may be at least partly related to laminin deposition. Synthetic polymer substrates such as poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl) ammonium hydroxide] (PMEDSAH) (Qian et al., 2014; Villa-Diaz et al., 2010) may also support hPSC expansion via a similar mechanism because we have observed the production of α -5 laminin by hESCs cultured on this substrate (Figure S6F).

We conclude that α -5 laminin endogenously produced by hPSCs is a critical component of the undifferentiated pluripotent stem cell ECM and that it is necessary for the survival and expansion of these cells. This work describes an autocrine and paracrine mechanism of self-renewal signaling through the endogenous ECM of hPSCs and is important in understanding the signals regulating self-renewal and hPSC/substrate interactions. Our findings have implications for the design and optimization of hPSC culture substrates and scaffolds, whose performance in supporting undifferentiated hPSCs may be related to the ability of the environment to support the deposition and production of α -5 laminin.

EXPERIMENTAL PROCEDURES

hPSC Culture

H9 hESCs, 19-9-11 iPSCs, and all genetically modified lines derived from these parental lines (WiCell) were maintained in E8 medium on tissue culture polystyrene (TCPS) plates (BD Falcon, Corning) coated with Matrigel (WiCell) as described previously (Chen et al., 2011). Briefly, hESCs were cultured in 6-well plates (BD Falcon) coated with Matrigel (83 μ g/well) in 2.5 ml of E8 medium/well. Cells were split at a ratio of 1:12 every 5 days with Versene solution (Life Technologies). For matrix production studies, cells were singularized with Accutase (Innovative Cell Technologies) and seeded in E8 medium, with 5 μ M Y27632 (Stemgent) present for the first 24 hr. In subsequent experiments involving genetically modified cell lines, cells were singularized with Accutase and seeded using mTeSR1 medium (WiCell), with 5 μ M Y27632 present for the first 24 hr. H1 and CHB10 hESCs were cultured on PMEDSAH as described by Villa-Diaz et al. (2010).

ECM Coating Preparation

Six- and 12-well TCPS plates were coated with a range of defined substrates according to the manufacturer's recommendations. Vitronectin (provided by the lab of Dr. Jamie Thomson, University of Wisconsin-Madison) or laminin-521 (BioLamina) were diluted in PBS and coated onto TCPS plates at a density of 0.61 μ g/cm² overnight at 37°C. Synthemax (Corning) was coated onto TCPS at a density of 3.3 μ g/cm² in PBS and incubated overnight at 37°C. StemAdhere coatings were achieved by diluting 240 μ l StemAdhere (STEMCELL Technologies) in 6 ml of StemAdhere dilution buffer, with 1 ml of this solution (1.02 μ g/cm²) placed into each well of a 6-well non-tissue culture polystyrene plate (BD

Falcon) and incubated overnight at 37°C. Prior to cell seeding, all plates were washed with sterile PBS to remove any non-adsorbed coating.

Generation and Validation of Inducible shRNA hPSC Lines

An inducible knockdown vector targeting the α -5 chain of laminin (LAMA5) was constructed by combining a small interfering RNA (siRNA) sequence (LAMA5, catalog no. s8066, Life Technologies) with an existing Tet-pLKO-puro backbone (Addgene, plasmid 21915). These vectors were co-transfected with the helper plasmids psPAX2 and pMD2.G (Addgene, plasmids 12260 and 12259) into HEK293TN cells (System Biosciences) for lentivirus particle production. Virus-containing media were collected 48, 72, 96, and 120 hr after transfection. The supernatant was first concentrated using Lenti-X™ Concentrator (Clontech Laboratories) according to the manufacturer's instructions for storage at -80°C, and then used for hPSC infection in the presence of 6 μ g/ml polybrene (Sigma). Lentiviral transduction was performed on hPSCs by plating 100,000 cells/well in a 6-well plate with 2 ml mTeSR and incubating with 25 μ l viral supernatant overnight. Transduced cells were cultured in mTeSR1 medium on Matrigel for at least 3 days and then selected and clonally isolated in mTeSR1 based on resistance to 1 μ g/ml puromycin (Lian et al., 2013).

Screening for knockdown efficiency was conducted via RT-PCR and qRT-PCR against the human LAMA5 gene. For RT-PCR, total RNA was extracted using DNA/RNA Shield with Quick-RNA MiniPrep (Zymo Research) according to the manufacturer's instructions. cDNA was generated from 1 μ g of RNA using Omniscript reverse transcriptase (QIAGEN) and Oligo-dT(20) primers (Life Technologies). RT-PCR was performed using GoTaq Green Master Mix (Promega) using primers (Table S4) from Integrated DNA Technologies, and 2% agarose gel electrophoresis was performed to visualize the PCR products. GAPDH and β -actin (ACTB) were used as endogenous controls. qRT-PCR for LAMA5 was performed as described in a subsequent section. This approach was used to produce the H9 ishLAMA5 (WiCell line WIP01e-H9ishLAMA5), H9 ishSCR (WiCell line WIP02e-H9ishSCR), 19-9-11 ishLAMA5 (WiCell line WIP03i-IPShLAMA5), and 19-9-11 ishSCR (WiCell line WIP04i-IPShSCR) cell lines. The 19-9-11 ishLAMA5 cell line was submitted to WiCell for karyotyping.

Generation and Validation of Cas9 Gene-Disrupted Lines

Cas9-mediated knockout (KO) of LAMA5 in hESCs and iPSCs was performed according to the protocol published by Ran et al. (2013b). H9 and 19-9-11 cells were first singularized with Accutase (Innovative Cell Technologies) and electroporated using a Bio-Rad Gene Pulser XL with 2 μ g of each guide RNA (Table S1), 2 μ g Cas9 plasmid (Addgene, plasmid 44719), and 1 μ g of GFP plasmid per one million cells. Three million cells were used in each electroporation. Electroporated cells were plated (100,000 cells/cm²) onto Matrigel-coated plates in mTeSR1 medium containing 5 μ M Y27632 (Stemgent) and cultured for 48 hr. After 48 hr, cells were again singularized with Accutase and sorted for GFP expression



via fluorescence-activated cell sorting (FACS) (BD FACSAria). Sorted cells were again re-plated on Matrigel at a low density of 1000 cells/cm² and allowed to expand for 7–10 days in mTeSR medium. Individual colonies were isolated using an AMG EVOS XL core cloning scope and expanded on Matrigel (H9) or laminin-521 (19-9-11) for another 7-14 days, after which genomic DNA was isolated from each clonal sample for an assay of *LAMAS* disruption.

To assay target gene disruption, 500 ng of genomic DNA was isolated from cells using the QIAGEN DNeasy blood and tissue kit and combined with genomic DNA primers (final concentration, 0.2 μM) from IDT (Table S5) and AccuPrime high-fidelity Taq polymerase (Life Technologies) at the recommended annealing temperatures for 35 cycles on an Eppendorf Mastercycler Nexus GSX1. PCR products were purified using a QIAGEN QIAquick PCR purification kit. Genomic sequencing was performed by mixing 500 ng of purified genomic PCR product with 1 μl of the genomic primer used to amplify that segment, and purified water was added to bring the total volume to 25 μl. Samples were submitted to the University of Wisconsin Biotechnology Center DNA Sequence Facility for Sanger sequencing. Sequencing results were visualized using Benchling software.

Heterozygous mutation of *LAMAS* and gene disruption efficiency were assessed using a T7 endonuclease I assay as described previously (Cho et al., 2013; Ran et al., 2013a, 2013b; Yang et al., 2013). Briefly, 1 μg of purified genomic DNA was hybridized in NEB buffer II (New England Biolabs), followed by addition of 1 μl of T7 endonuclease to each T7 digestion and incubation at 37°C for 15 min prior to addition of 2.1 μl stop solution. Purified genomic DNA, hybridization product, and T7 digest product were visualized on a 2% agarose gel and imaged using a Bio-Rad Gel Doc XR+. This approach was used to generate the H9 Cas9 Het (WiCell line WIP07e-H9Cas9Het) and 19-9-11 Cas9 KO (WiCell line WIP05i-IPSCas9KO) cell lines, and the latter was submitted for karyotype analysis (WiCell).

Immunocytochemical Detection of ECM

The deposition of endogenous ECM proteins onto uncoated and ECM-coated TCPS plates by H9 hESCs and 19-9-11 iPSCs was assessed via immunocytochemical staining. Samples were fixed in 10% formalin (Sigma) and permeabilized using 1% Triton X-100 (Sigma) in PBS. Primary antibodies against human fibronectin (Santa Cruz Biotechnology, catalog no. SC80559), laminin (Abcam, catalog no. ab30320), α-5 laminin (Millipore, catalog no. MAB1924), vitronectin (Santa Cruz Biotechnology, catalog no. SC74485), collagen type IV (Santa Cruz Biotechnology, catalog no. SC52317), and collagen type 1 (Sigma, catalog no. C2456) were diluted 1:500 in 3% BSA in PBS and allowed to incubate overnight at 4°C, followed by rinsing in PBS and incubation with Alexa Fluor 488- and 568-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (Life Technologies, catalog nos. A10667 and A11031, respectively) in 3% BSA at room temperature for 1 hr. Cell nuclei were counterstained with 1 μg/ml DAPI (4',6-diamidino-2-phenylindole dihydrochloride, Life Technologies, catalog no. D1306), and fluorescent images were captured using a Nikon IX51 epifluorescence microscope.

Apoptosis, Cell Cycle, and Pluripotency Analysis via Flow Cytometry

hPSCs cultured on the aforementioned coatings were singularized with Accutase and fixed in cold 90% methanol. Primary antibodies against annexin V (1:500, Santa Cruz Biotechnology, catalog no. SC74458), Nanog (1:500, Cell Signaling Technology, catalog no. 4893), cleaved caspase-3 (1:250, Cell Signaling Technology, catalog no. 9664), Ki67 (1:500, Abcam, 15580), and an isotype control (1:500 and 1:250, mouse immunoglobulin G₁ [IgG₁], BD Biosciences, catalog no. 550878) were prepared in 0.3% BSA in PBS and incubated with samples overnight at 4°C. After washing, samples were incubated for 1 hr at room temperature with Alexa Fluor 488- or 568-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (Life Technologies, catalog nos. A10667 and A11031, respectively) diluted 1:500 in 0.3% BSA. Samples were analyzed on a BDCanto flow cytometer using BD FACSDiva software, and negative control gating was performed on samples treated with an isotype control primary antibody and a secondary antibody identical to that used for samples. For cell-cycle analysis, samples were stained with propidium iodide (Life Technologies), and cell-cycle distribution was determined using the ModFit LT version 4 software package from Verity Software House.

qRT-PCR

mRNA was isolated from cultured cells using the QIAGEN RNeasy Plus mini kit. cDNA was produced using the Applied Biosystems high-capacity cDNA reverse transcription kit. Gene expression was assessed via qRT-PCR using the *NANOG*, and *LAMAS* human TaqMan gene expression assays from Applied Biosystems. Samples were measured using a StepOne real-time PCR system (Applied Biosystems), and data analysis was performed using the ΔΔCt method relative to *GAPDH*.

Statistical Analysis

Statistical analysis was performed via one-way ANOVA with a Tukey honest significant difference (HSD) post hoc test using the KaleidaGraph software package. *p* Values less than or equal to 0.05 were considered statistically significant. Data are presented as mean ± SD. All experiments were repeated a minimum of two times with three independent samples per condition each time.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and five tables and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2015.06.009>.

ACKNOWLEDGMENTS

This work was supported by NIH grants R01 EB007534 and R01 HL093281. We would like to thank Nicholas Propson and Dr. James Thomson from the Regenerative Biology Group at the Morgridge Institute for Research for providing recombinant human vitronectin and for the use of their flow cytometry and FACS facilities. We also thank Jared Carlson-Stevermer for assistance with guide RNA design and protocols. We acknowledge



generous financial support from the Wisconsin Institute for Discovery (to K.S.), NSF (CBET-1350178, to K.S.), and the Society in Science Foundation (to K.S.).

Received: November 12, 2014

Revised: June 24, 2015

Accepted: June 30, 2015

Published: July 30, 2015

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Stem Cell Reports, Volume 5

Supplemental Information

α -5 Laminin Synthesized

by Human Pluripotent Stem Cells

Promotes Self-Renewal

Alex Laperle, Cheston Hsiao, Michael Lampe, Jaime Mortier, Krishanu Saha, Sean P. Palecek, and Kristyn S. Masters

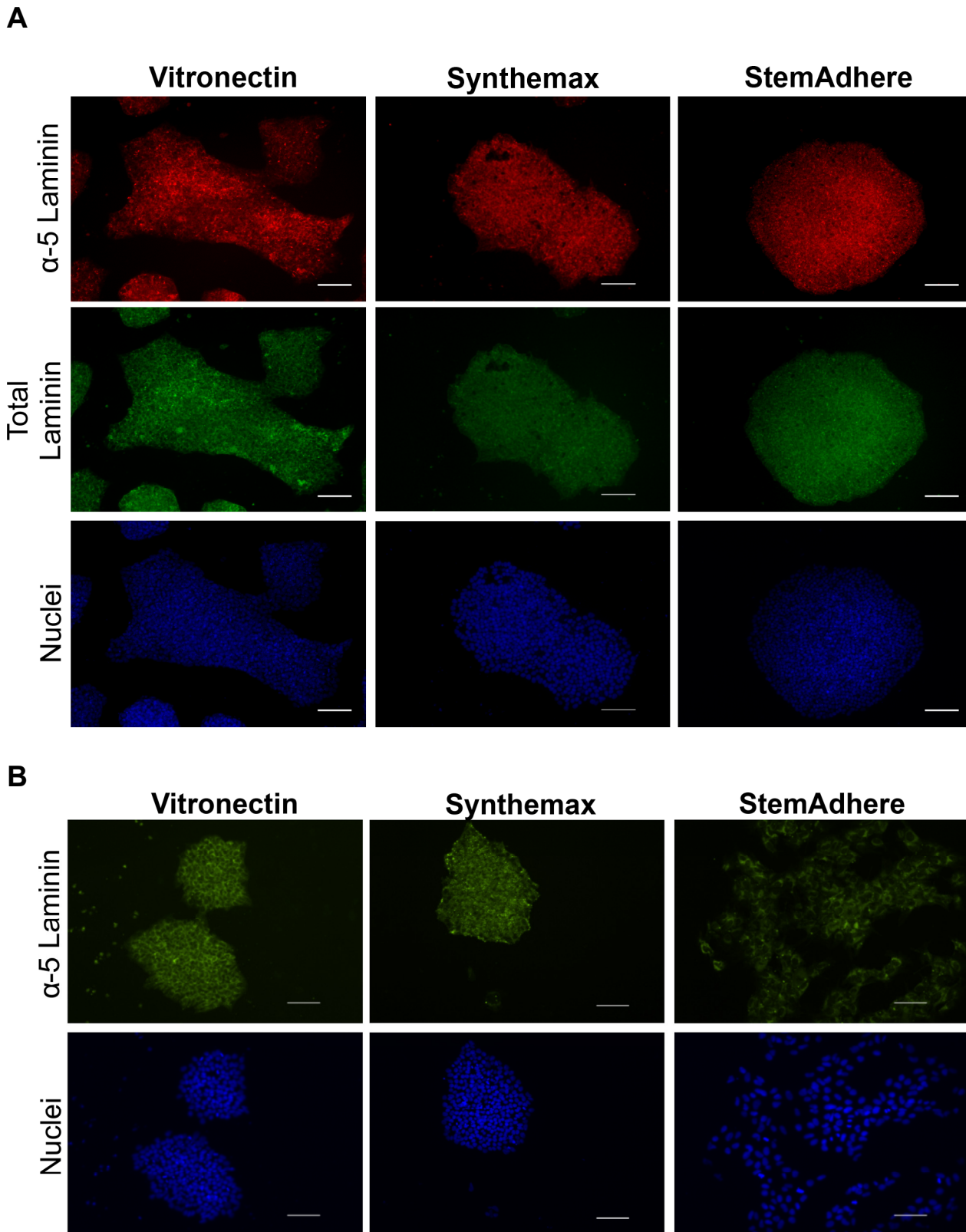


Figure S1: ECM deposition by hPSCs on three defined substrates. (A) H9 hESCs and (B) 19-9-11 iPSCs were cultured on the indicated substrates for 5 days in E8 medium. Aberrant morphology on StemAdhere is due to the low seeding density (25,000 cells per well) employed in this assay and is no longer present when cells are plated at higher (>50,000 cells per well) densities. Scale bars = 100 μ m. n=3 independent samples/condition

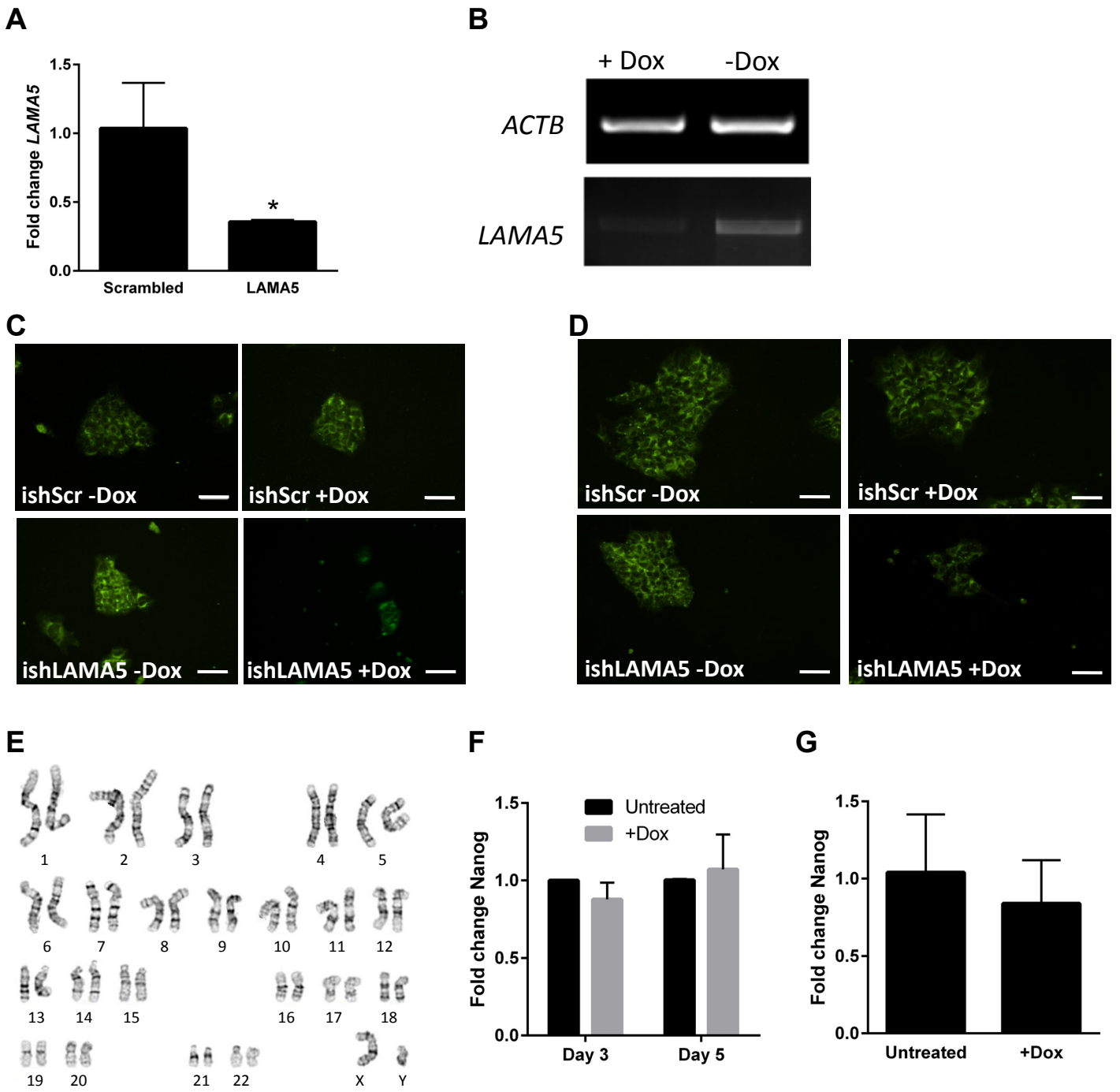


Figure S2: Generation of H9 and 19-9-11 doxycycline (dox) inducible lines. (A) *LAMA5* expression by H9s transfected with *LAMA5* siRNA at 48 hrs post-transfection. (B) RT-PCR showing reduction in *LAMA5* expression upon addition of 5 μ M dox. (C) α -5 laminin (green) deposition by H9 *LAMA5* knockdown (ishLAMA5) and scrambled (ishScr) cells. (D) α -5 laminin (green) deposition by 19-9-11 *LAMA5* knockdown (ishLAMA5) and scrambled (ishScr) cells. (E) Karyotype analysis of 19-9-11 ishLAMA5 cells. (F) qRT-PCR demonstrating *NANOG* expression by H9 ishLAMA5 cells cultured on Synthemax. (G) qRT-PCR demonstrating *NANOG* expression by 19-9-11 ishLAMA5 cells cultured on Synthemax. * $p < 0.05$ vs. Scrambled. Scale bars = 50 μ m. $n = 3$ independent samples/condition

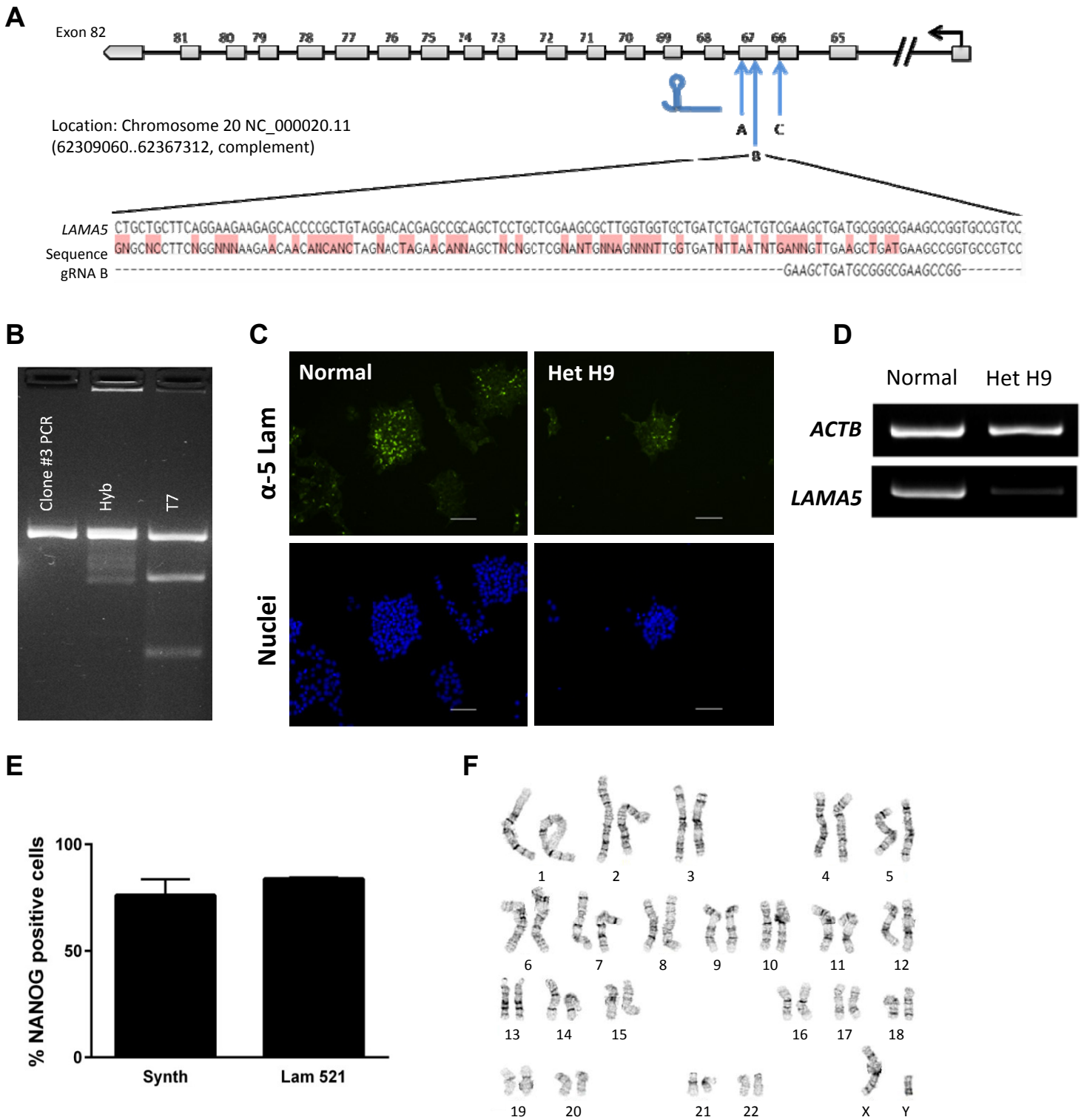


Figure S3: Generation of H9 Cas9 heterozygous *LAMA5* knockout line. (A) Schematic showing gRNA locations and Sanger sequence for Het H9 line. (B) T7 Assay demonstrating heterozygosity of the Het H9 line. (C) Immunocytochemical staining for α -5 laminin deposition after 3 days of H9 and Het H9 culture on Synthemax. (D) *LAMA5* expression by Het H9 cells on Synthemax, as measured by RT-PCR. (E) NANOG expression by Het H9 cells, as measured by flow cytometry after 5 days of culture on the indicated substrates. (F) Karyotype analysis of homozygous *LAMA5* 19-9-11 knockout line. Scale bars = 100 μ m. n=3 independent samples/condition

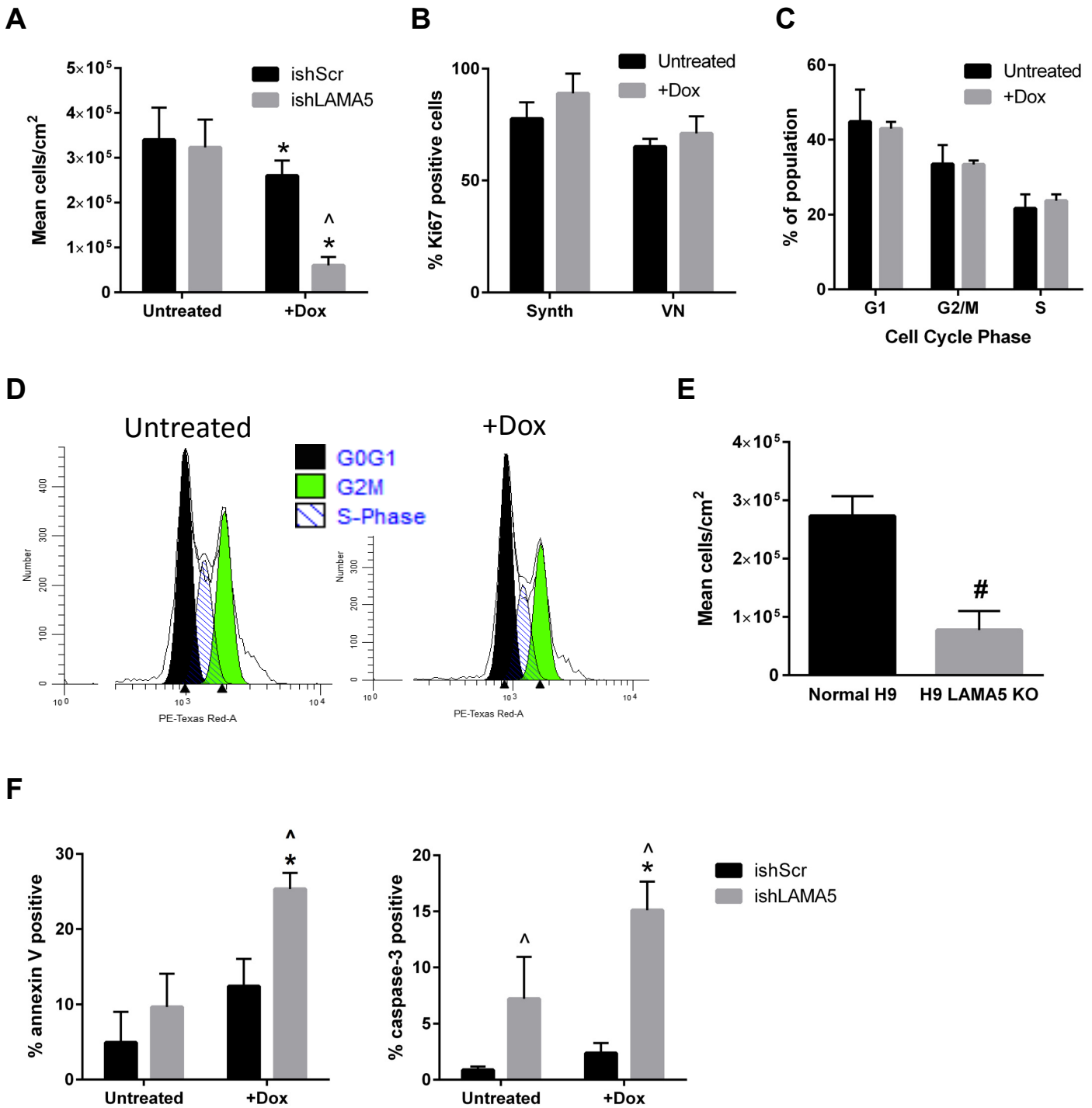


Figure S4: Characterization of cell number and apoptosis in H9 and 19-9-11 knockout lines. (A) Mean cell density of ishLAMA5 and ishScr H9 cell lines after 5 days of culture on Synthemax. Cells were seeded at 2,630 cells/cm². (B) Ki67 expression by H9 ishLAMA5 cells, as measured by flow cytometry after 5 days of culture on the indicated substrates. (C) Cell cycle distribution of 19-9-11 ishLAMA5 cells cultured on Synthemax for 3 days. (D) Example distribution fits for cell cycle analysis. DNA content was measured using propidium iodide and distribution was determined using ModFit LT software. (E) Normal and Het H9 mean cell density after 5 days of culture on Synthemax. Cells were seeded at 2,630 cells/cm². (F) Annexin V and cleaved caspase 3 expression by ishLAMA5 and ishScr H9 cell lines, as measured by flow cytometry after 5 days of culture on Synthemax. *p<0.001 vs. untreated; ^p<0.001 vs. ishScr; #p<0.001 vs. Normal H9. n=3 independent samples/condition

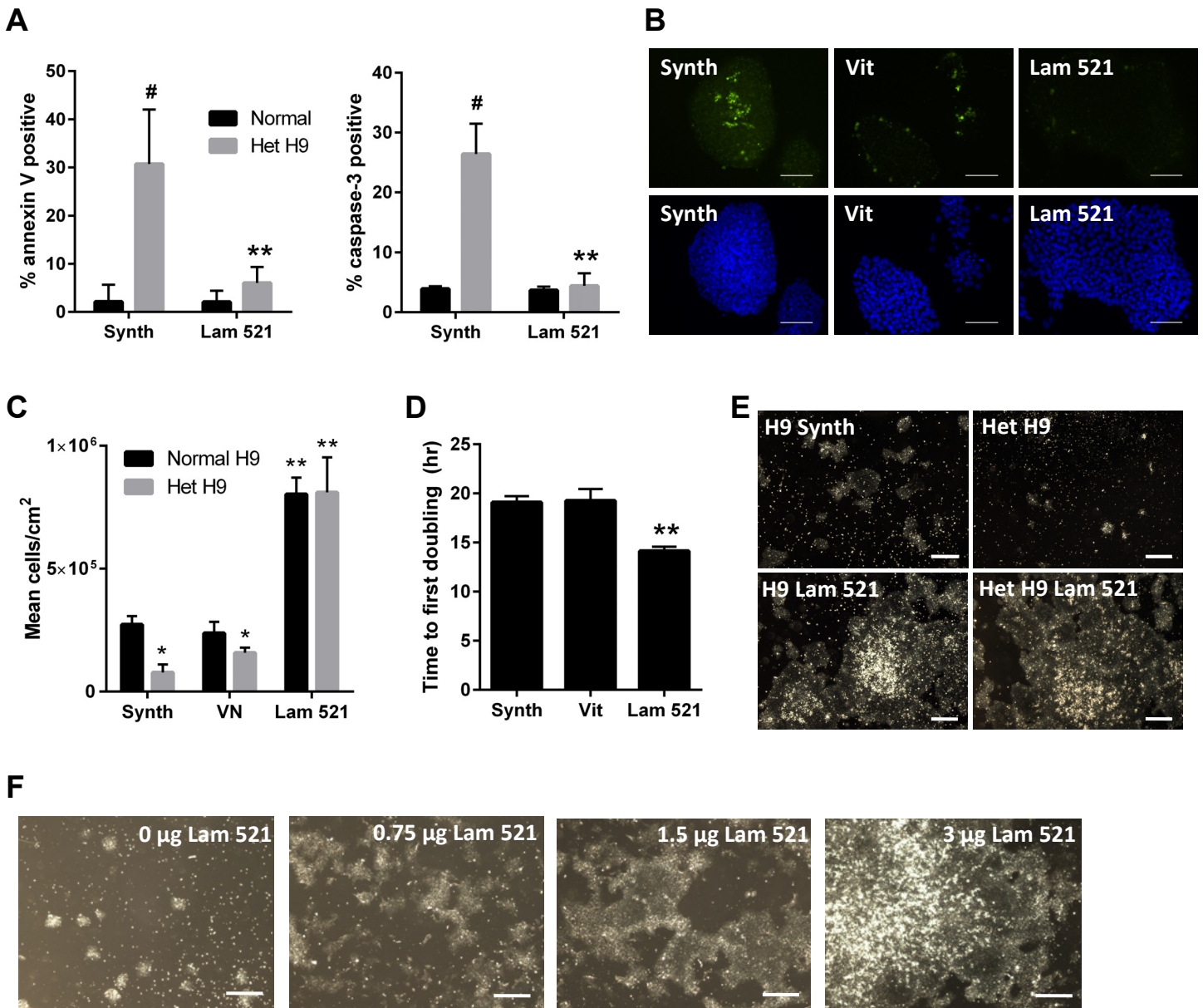


Figure S5: Partial restoration of self-renewal in Het H9s via culture on exogenous laminin 521. (A) Annexin V and cleaved caspase 3 expression by normal and Het H9 cells, as measured by flow cytometry after 5 days of culture on the indicated substrates. (B) Immunocytochemical staining showing cleaved caspase-3 expression (green) in Het H9 cells on the indicated substrates after 3 days of culture; scale bar = 100 μm. (C) Normal and Het H9 mean cell density after 5 days of culture on indicated substrates. Cells were seeded at 2,630 cells/cm². (D) Average time from seeding to first observed doubling in Het H9 cells cultured on the indicated substrates. Cells were synced with nocodazole 24 hrs prior to seeding. (E) Brightfield images of normal and Het H9 cells cultured on Synthemax or laminin 521 for 3 days; scale bar = 500 μm. (F) Brightfield images of Het H9 cells cultured on increasing densities of laminin-521 for 5 days, demonstrating dose-response of cell rescue by exogenous laminin-521; scale bar = 100 μm. *p<0.001 vs. Normal H9; #p<0.001 vs. Normal H9; **p<0.001 vs. same cells on other surface(s). n=3 independent samples/condition

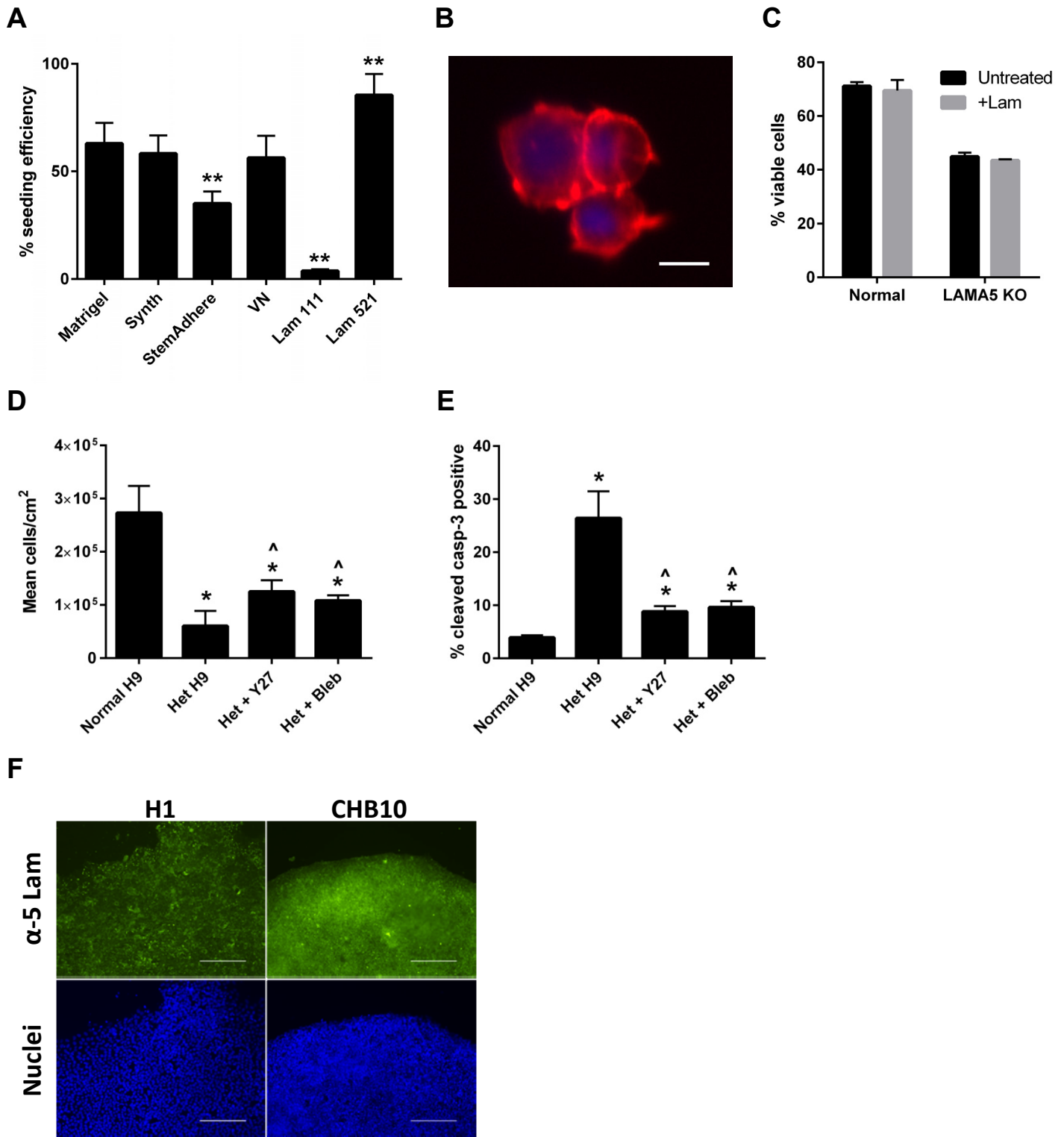


Figure S6: Partial restoration of self-renewal in Het H9s via inhibition of contractility. (A) Attachment efficiency (expressed as % of initially seeded cells) of normal 19-9-11 cells seeded on indicated substrates in the presence of 5 μ M Y27639. (B) F-actin immunocytochemistry illustrating blebbing in Het H9 cells cultured on Synthemax for 48 hrs; scale bar = 10 μ m. (C) Viability of normal and KO 19-9-11 cells cultured in suspension for 3 hours with or without soluble laminin 521. (D) Mean cell density and (E) cleaved caspase-3 expression for Het H9 cells cultured on Synthemax with the indicated inhibitors. (F) α -5 laminin deposited by H1 and CHB10 hESCs after 5 days of culture on TCPS coated with the defined synthetic polymer PMEDSAH. Scale bars = 100 μ m. * p <0.001 vs. Normal H9; ^ p <0.001 vs. untreated Het H9 condition; ** p <0.001 vs. culture on Synthemax. n =3 independent samples/condition

Supplemental Table S1: Sequences of siRNA against LAMA5 and shRNA LAMA5 construct cloned into Addgene plasmids 12260 and 12259

RNAi	Sequence
siRNA	
Sense	5' ACAGGGCAGTGCTACTGTAtt 3'
Antisense	5' TACAGTAGCACTGCCCTGTga 3'
shRNA	
Sense	5'CCGGACAGGGCAGTGCTACTGTATTCTCGAGAATACAGTAGCACTGCCCTGTTTTTG 3'
Antisense	5'AATTCAAAAACAGGGCAGTGCTACTGTATTCTCGAGAATACAGTAGCACTGCCCTG 3'

Supplemental Table S2: Sequences and chromosomal locations of all Cas9 *LAMA5* guide RNAs used

Guide RNAs	
gRNA A	
Chromosome location	chr20:60888167-60888189
Gene name	LAMA5
23 bp Target Sequence	GCGGCTCGTGTCTACAGCGGGG
gRNA B	
Chromosome location	chr20:60888231-60888253
Gene name	LAMA5
23 bp Target Sequence (no spaces)	GAAGCTGATGCGGGCGAAGCCGG
gRNA C	
Chromosome location	chr20:60888373-60888395
Gene name	LAMA5
23 bp Target Sequence (no spaces)	GGCCTTGTGCCCCGGTGCCTGTGG

Supplemental Table S3: Inhibitors tested against *LAMA5* KO cells

Name	Target	Concentration used
Bax inhibiting peptide	Bax	200 μ M
PD 98059	MEK	1 μ M
SU 5402	VEGFR and FGFR	2 μ M
Y27632	ROCK	5 μ M
Blebbistatin	Myosin contractility	10 μ M
Z-IETD-FMK	Caspase 8	40 μ M
NS 3694	Caspase 9	50 μ M

Supplemental Table S4: Sequences of all *LAMA5* mRNA primers used for *LAMA5* detection

LAMA5 mRNA Primers	Sequence (5'→3')
Primer pair 1	
Forward primer	CAGGCTAAGGAGGAGCTGGA
Reverse primer	TGCTTGTCTCGTCTGTGTCC
Product length	535
Primer pair 2	
Forward primer	AGGCTAAGGAGGAGCTGGA
Reverse primer	AGCATGGCCTCTTCTAGTGC
Product length	385
Deletion Detection Primer	
Forward primer	GCCAGCCCCAAAGTCATACA
Reverse primer	CGCATCAGCTTCGACAGTCA
Product length	242

Supplemental Table S5: Sequences of all genomic *LAMA5* primers used to detect and sequence Cas9-induced mutations

Genomic DNA Primers	Sequence (5'→3')
Primer pair 1	
Forward primer	CAGAAACAGGGCAGGGTTAGT
Reverse primer	ATCGAGATGGACACGCTGAA
Product length	408
Primer pair 2	
Forward primer	AGAAACAGGGCAGGGTTAGTG
Reverse primer	TCAGTCCGTCCTGCTGTAGT
Product length	540
Primer pair 3	
Forward primer	CCCATCGTTCCATCTCCTCT
Reverse primer	TGGTCAGCCTCTACAACCTCG
Product length	558