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**Developing defined substrates for stem cell culture and
differentiation**

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TITLE

Developing defined substrates for stem cell culture and differentiation

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RUNNING TITLE

Laminins for primary cell culture

SUMMARY

Over the past few decades, a variety of different reagents for stem cell maintenance and differentiation have been commercialized. These reagents share a common goal in facilitating the manufacture of products suitable for cell therapy whilst reducing the amount of non-defined components. Lessons from developmental biology have identified signalling molecules that can guide the differentiation process *in vitro*, but less attention has been paid to the extra-cellular matrix used. With the introduction of more biologically relevant and defined matrices, that better mimic specific cell niches, researchers now have powerful resources to fine-tune their *in vitro* differentiation systems, that may allow the manufacture of therapeutically relevant cell types. In this review article, we revisit the basics of the extracellular matrix, and explore the important role of the cell-matrix interaction. We focus on laminin proteins since they help to maintain pluripotency and drive cell fate specification.

Keywords:

Defined substrates, human recombinant laminins, human pluripotent stem cells, cell culture, cell therapy

INTRODUCTION

Following the derivation of human embryonic stem cells (hESC) in 1998¹, and with the development of human induced pluripotent stem cells (hiPSC) in 2007^{2,3}, stem cell research is rapidly progressing from preclinical studies to the clinical arena with first in-human trials already begun. Continued growth in the cell therapy industry and the commercialization of cell therapies has led to an increased awareness for the need of specialized and defined materials.

Current practices for the maintenance and expansion of undifferentiated human pluripotent stem cells (hPSCs) typically depend on the support of undefined culture systems, such as feeder cells (often mouse embryonic fibroblasts) or undefined basement membrane (BM) extracts such as MatrigelTM and Geltrex[®] containing laminin-111, entactin, collagen, heparin sulfate proteoglycans and growth factors purified from Engelbreth-Holm-Swarm mouse sarcoma. Besides the risk of transmitting pathogens and the introduction of tumor-derived growth factors, culture conditions that depend on these undefined support systems limit experimental reproducibility and the ability to interpret mechanistic studies due to lot-to-lot variations. Ultimately, they hinder the transition of hPSC-derived products into a clinical setting.

Over the past decade, several feeder-free, extracellular matrix (ECM) protein culture matrices have appeared on the market that are defined and xeno-free⁴⁻⁶. However, not all ECM proteins are suitable for hPSC culture since they cannot maintain an undifferentiated⁷ or allow clonal survival of hPSCs without the use of apoptosis inhibitors or undefined additives⁸. Moreover, hPSCs were often passaged as clumps on these substrates, which significantly induced the risk of spontaneous differentiation⁸. High production costs, labour intensive cell passaging and the limited

1
2
3 scale-up potential associated with many of these ECM substrates have driven
4
5 researchers to seek alternative substrates in the synthetic polymer arena. For example,
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7 Synthemax™, an acrylate surface with deposits of various peptide-polymer conjugates,
8
9 has been developed for the adhesion and expansion of hESC⁹. Synthetic scaffolds
10
11 composed of poly(desaminotyrosyl tyrosine ethyl ester carbonate)¹⁰, a tyrosine
12
13 derived polycarbonate polymer¹¹, have also been utilised to study the effects of
14
15 geometry on hESC survival and self-renewal. Carlson *et al.* have shown that poly-D-
16
17 lysine pre-treated scaffolds support hESC survival and colony formation. However,
18
19 the authors also illustrated the importance of cell-ECM interactions for cell
20
21 functionality and concluded that the endogenous cell production of laminin was an
22
23 essential factor for adhesion and survival of the hESCs¹⁰.

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25
26 A lack of biologically relevant signals from the matrix increases the risk for
27
28 mixed cell populations and genetic and phenotypic drift *in vitro*. Compared to many
29
30 other feeder-free matrices on the market, full-length laminin-521 better mimics the
31
32 natural environment for hPSCs in culture^{7, 8, 19}. Due to their biologically relevant
33
34 interactions with cells, laminin-based substrates have significantly advanced the stem
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36 cell research field and are therefore the focus of this article. Over the last decade,
37
38 laminins have become a preferred cell culture substrate both within basic research as
39
40 well as for researches and companies with a therapeutic focus⁴⁵.

41 42 43 44 45 46 **THE EXTRACELLULAR MATRIX AND THE IMPORTANCE OF** 47 48 **BIOLOGICAL RELEVANCE FOR CELL CULTURE** 49

50
51 Most organized and stationary cells of the body grow on specialized, sheet-like ECM
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53 structures called basement membranes (BM). Different cells and tissues require a
54
55 specific ECM composition for survival and proper function¹², and many cells, such as
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3 fibroblasts¹³, endothelial cells¹⁴ and cardiomyocytes¹⁵, themselves produce and
4 deposit ECM proteins. The BM contains specific, highly conserved proteins, and
5 consists mostly of laminin, type IV collagen, agrin, perlecan, fibronectin and
6
7 nidogen^{12, 16} (Figure 1a).
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11 Over the past 40 years, several adhesion molecules (fibronectin, vitronectin,
12 laminin-111, or a placenta-derived mixture of laminins) have been discovered and
13 used to improve cell cultures^{17, 18}. The concept of adhesive matrix molecules has
14 gradually improved: adhesion is not only for anchoring cells in place but is also
15 crucial for cell survival and phenotype maintenance. Cells which lack their relevant
16 ECM cues undergo anoikis, controlled suicide, which is a natural mechanism that
17 allows for the correct organization of tissues and for the selection of specific cell
18 types. Laminin and type IV collagen form networks that have a structural role,
19 influence cell migration and regulate the differentiation processes of associated cells.
20 Nidogens are sulfated glycoproteins that link the BM proteins together, and the
21 glycosaminoglycan-containing perlecan and agrin bind growth factors and contribute
22 to the matrix volume¹⁹. Laminins are the most abundant component of the BM. In
23 addition to their structural functions, laminins play an essential role in the structural
24 organization of the BM and the regulation of cell behaviour²⁰.
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42 Laminins are multidomain, heterotrimeric glycoproteins, composed of three
43 different subunits; an α -chain, β -chain and γ -chain, combined and expressed in at least
44 16 different isoforms in the human body^{16, 19}. The physical, topological, and
45 biochemical expression of the different laminin isoforms in the BM is heterogeneous
46 and laminin expression changes during development. Laminin-111 is widely
47 expressed during embryogenesis, but its tissue distribution after birth is restricted to
48 only a few tissues, such as the brain and kidney²¹. After birth, the α 5-laminins are
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3 most common²². Without the right combination of laminin isoforms cells and tissues
4 becomes dysfunctional. While epithelial cells need laminin-332 together with laminin
5 -511/521 for proper function, muscle and nerve cells require laminin -211, -221 and
6 laminin -511/521, and endothelial cells prefer a combination of laminin -411/421 and
7 laminin -511/521¹⁹.
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13 The molecular structure of laminins is cross-shaped with the N-terminal ends
14 forming three short arms, containing globular laminin N-terminal domains and a
15 globular laminin IV domain^{23, 24}. Each of these domains are separated by rod-like,
16 epidermal-growth-factor-like domains, harbouring nidogen binding sites²⁵. Nidogen
17 and perlecan in turn, allow the laminin network to be connected to collagen IV. The
18 globular N-terminal domains also bind to syndecans which regulate cell adhesion and
19 motility^{26, 27} (Figure 1b). Cells bind to laminins via cell-surface receptors, such as
20 integrins, which regulate vital cellular responses, such as proliferation and
21 differentiation, migration, phenotype stability, and resistance to apoptosis^{19, 28, 29}. The
22 α 5-laminins (especially laminin -511 and laminin -521) exhibit the broadest degree of
23 integrin binding interactions²². There are many binding sites on the full-length
24 laminin molecule which can interact with cell membrane receptors, however, the
25 molecular mechanism underlying the laminin-mediated signalling to integrins and
26 other cell surface receptors is still not fully elucidated. Studies indicate that the LG
27 domains in C-terminal region of laminins are highly involved in laminin recognition
28 by integrins^{30, 31}. Integrins, α -dystroglycan, perlecan and sulphated glycolipids also
29 interact with some of the globular N-terminal laminin domains^{16, 32}. Fractionated
30 laminin molecules isolated from tissue and truncated laminin molecule derivatives lack
31 most of the laminin domains that are required for the formation of a proper
32 extracellular network. Moreover, full-length recombinant laminins with all their
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3 native biological activities intact are essential for biomimetic *in vitro* function³³ and
4 for the stimulation of authentic cellular signal transductions. Emphasizing biology and
5 mimicking the natural matrix proteins is one of the most important aspects to create a
6 biologically relevant milieu for the cells, resulting in phenotypically stable cell
7 cultures and reproducible protocols.
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13 14 15 **BIOLOGICALLY RELEVANT CELL CULTURE MATRICES ENABLE** 16 **CLINICAL TRANSLATION OF RESEARCH PROTOCOLS** 17

18
19 Advancing a PSC-derived cell therapy from pre-clinical studies to a Phase 1 clinical
20 trial requires a demonstration of a well-controlled production process and a safe and
21 efficacious product to the regulatory agencies. The development of a differentiation
22 protocol that generates the target cell type at a sufficient quantity and purity, with
23 phenotypic maturity and appropriate cellular functions is arguably challenging. Due to
24 their validated functionality and biological properties, human recombinant laminins in
25 conjunction with streamlined differentiation protocols, offer exciting prospects for
26 regenerative medicine. This has been highlighted in a number of high-impact
27 scientific articles in the past two years and a few examples are described below:
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40 **Pluripotent stem cell derivation, maintenance and safety**

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42 In the developing embryo, laminins containing the α 1- and α 5-chain are the first ECM
43 proteins to be expressed. They are essential for early embryogenesis and initiation of
44 morphogenesis²⁰. α 5-chain laminins (i.e. laminin-521 and laminin-511) are produced
45 by and surround the cells in the inner cell mass of the blastocyst which gives rise to
46 all embryonic tissues^{12, 19, 34} (Figure 1c). α 5 laminin is also produced endogenously by
47 pluripotent hPSCs cultured *in vitro* and is a critical autocrine and paracrine factor that
48 regulates hPSC survival and self-renewal. Knockdown and disruption of the *LAMA5*
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3 gene dramatically reduces hPSC self-renewal and increases apoptosis⁷. LN-521 thus
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5 constitutes the relevant niche for pluripotent stem cells when cultured *in vitro*.
6
7 Laminin-111 is mostly expressed in the Reichert's membrane, which supports the
8
9 outer extra-embryonic layer of trophoblasts and is widely expressed during
10
11 embryogenesis^{12, 35} (Figure 1d). Hence, using cell culture substrates that contain
12
13 laminin-111 (e.g. Matrigel or Cultrex) is suboptimal for reliable survival and
14
15 expansion of hESCs and hiPSCs^{8, 19, 28}. Human ESCs dissociated into single-cell
16
17 suspension and plated on Matrigel, or different human recombinant laminin (LN)
18
19 substrates, LN-111, LN-121, LN-511 and LN-521, in the presence of apoptosis
20
21 inhibitor (ROCK inhibitor, Y-27632), survive on all surfaces. However, in the
22
23 absence of ROCK inhibitor, hESCs do not survive well on Matrigel, LN-111 or LN-
24
25 121, whereas they readily adhere and spread on LN-511 and LN-521⁸. By culturing
26
27 cells on the laminin isoforms that they normally adhere to *in vivo in vitro*, the
28
29 biologically relevant niche is better recapitulated by the medium and the matrix.
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33 In 2014, Rodin *et al.* first described an efficient xeno-free and chemically
34
35 defined protocol for monolayer culturing of hPSC on LN-521³⁶ (Figure 2a). Via the
36
37 interaction to specific hPSC cellular receptors, LN-521 triggers authentic cell
38
39 signalling pathways, which promotes high survival (Figure 2b) and robust long-term
40
41 expansion of single-cell cultured hESC and hiPSC (Figure 2c), even without the
42
43 addition of ROCK inhibitor or any other inhibitors of anoikis. LN-521 binds to $\beta 1$
44
45 integrin receptors, primarily $\alpha 6\beta 1$, which plays a pivotal role in the stem cell-matrix
46
47 interaction. Human PSCs predominantly express the $\alpha 6\beta 1$ integrin, which interacts
48
49 strongly with LN-521 globular domains inducing the PI3K/Akt pathway^{8, 37, 38}. The
50
51 inactivation of focal adhesion kinase (FAK) signalling pathway via the $\alpha 6\beta 1$ integrin
52
53 is also linked the hPSC self-renewal and expression of pluripotency³⁹. The motility of
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3 hPSCs on LN-521 is higher than that of the cells on other matrices, which correlates
4
5 to the high survival of the cells⁸.
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7 Human PSCs in single-cell suspension plated on LN-521 quickly attach,
8
9 migrate and form monolayer colonies which rapidly expand (Figure 2a). Human PSCs
10
11 cultured in thick multilayer colonies often require manual, arbitrary removal of
12
13 spontaneously differentiated parts. In contrast, hPSCs cultured on LN-521 grow as a
14
15 homogenous monolayer, with minimal risk of spontaneous differentiation or genetic
16
17 abnormalities⁴⁰. The biologically relevant culture environment generates hPSC
18
19 cultures with a more uniform gene expression between different hPSC lines⁴¹ and
20
21 enhances cell maturation, polarization and functional organization in many cell
22
23 applications^{42, 43, 44}.
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26 The LN-521 matrix also allows efficient clonal derivation, clonal survival and
27
28 long-term self-renewal of hESCs. Human ESC lines can even be derived from a
29
30 single blastomere under chemically defined and xeno-free conditions^{8, 45}, which
31
32 circumvents the ethical issues associated with embryo destruction for hESC line
33
34 derivation. The biologically relevant culture environment provided by the LN-521
35
36 substrate allows for flexible culture protocols that support weekend-free feeding⁴⁰,
37
38 ultra-low seeding density (>5,000 cells/cm²) and high confluence culture.
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43 **Stem cell derived dopamine progenitors from a high-yield GMP-adapted** 44 **differentiation protocol** 45

46 Parkinson's disease (PD) is an interesting target for cell replacement therapies due to
47
48 its focal degeneration of midbrain dopamine (DA) neurons. Proof-of-concept has
49
50 been achieved in clinical trials using human foetal ventral mesencephalic (VM)
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52 tissue⁴⁶. However, the low availability of such tissue limits its use, and a renewable
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3 source of cells is required in order to develop a therapy that is accessible to a large
4
5 number of patients.

6
7 A new differentiation protocol that efficiently and rapidly patterns hESCs to
8
9 midbrain DA neurons using extrinsic patterning agents has been developed in recent
10
11 years^{47, 48}. These stem cell derived DA neurons function *en par* with fetal midbrain
12
13 DA neurons⁴⁹. Kirkeby *et al.* have developed a fully defined and xeno-free protocol.
14
15 Even so, a number of steps were required to develop a Good Manufacturing Practice
16
17 (GMP) version compliant with use in clinical trials^{44, 50}. One important step was to
18
19 switch from an initial suspension culture step to a fully attached protocol. Matrigel
20
21 had previously been used for this purpose³² but is not ideal for GMP manufacturing
22
23 and more suitable substrates, such as recombinant laminins were required for GMP
24
25 production.
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28
29 Seven different recombinant laminin isoforms were screened for their ability
30
31 to replace Matrigel/free floating suspension cultures and 4 of them were found to
32
33 efficiently support adherent differentiation of VM progenitors (LN-111, LN-421, LN-
34
35 511 and LN-521). It has previously been reported that LN-511 and LN-521 efficiently
36
37 support growth of hPSCs⁸, making them less ideal in this differentiation protocol. In
38
39 contrast, undifferentiated hESCs detach from LN-111-coated culture dishes when
40
41 kept in pluripotency medium but efficiently attach in neural differentiation medium
42
43 making it an ideal substrate to move forward with.
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47 When implementing this in the GMP protocol, the differentiation on LN-111
48
49 resulted in robust and reproducible differentiation of midbrain DA progenitors with
50
51 minimal variation between batches^{44, 50}. Moreover, the yield was >40 times higher
52
53 than the original research grade differentiation protocol^{44, 48} (Figure 3a). This high
54
55 yield means that it is possible to produce >3.8 x 10⁸ transplantable progenitor cells in
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3 16 days when starting from just 1×10^6 undifferentiated hESCs⁴⁴ (Figure 3b). This
4
5 would provide more than 500 doses of cryopreserved DA neurons from one manual
6
7 manufacturing batch at a relatively small scale.
8
9

10 **Recombinant laminins support the differentiation potential of satellite cell-** 11 **derived myoblasts during long-term culture** 12 13

14 Every tissue in the body originates from a stem cell during development but there are
15
16 a number of tissues where stem cells exist to facilitate tissue regeneration in the adult.
17
18 In skeletal muscle, that stem cell is called the satellite cell and it remains quiescent
19
20 unless stimulated by muscle damage⁵¹. Upon damage, it enters a program to
21
22 asymmetrically expand to generate a transit amplifying cell and another satellite cell⁵²,
23
24
25 ⁵³. The transit amplifying cell undergoes several rounds of division to create a
26
27 population of cells called myoblasts that can fuse together to create new muscle fibers
28
29 or fuse to existing fibers to repair membrane lesions.
30
31

32 Large-scale expansion of muscle stem cells *in vitro* is critical for a variety of
33
34 purposes. Large quantities of satellite cells are required for cell engineering, cell
35
36 therapy and to support skeletal muscle drug discovery campaigns. Relatively small
37
38 numbers of primary satellite cells must be scalable to millions, even billions of cells,
39
40 while maintaining the ability to differentiate into mature myotubes. While multiple
41
42 substrates have been commonly used for growth of satellite cells, long term effects of
43
44 culturing on different substrates has not been well characterized. Matrigel is
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46 commonly utilized, however, it is a complex substrate exhibiting significant lot-to-lot
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48 variation, and variable amounts of growth factors that may skew experimental
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50 outcomes and mitigates reliable translation of protocols from the literature to drug
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52 discovery applications⁵⁴. Additionally, Matrigel implementation varies largely from
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54 lab to lab making it difficult to reliably translate protocols from the literature for drug
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3 discovery applications. In order to develop an optimized and defined culture platform,
4 an in-depth comparison of long-term satellite cell activity between multiple defined
5 extracellular matrices and Matrigel has been performed. It was shown that LN-521
6 dramatically enhances the proliferation and differentiation of satellite cells and
7 extends their culture potential well past previously established passaging limits⁴³.
8
9 Following eight passages *in vitro*, LN-521 expanded satellite cells form more mature
10 myotubes containing high numbers of nuclei and better organization compared to the
11 other substrates (Figure 3c). In contrast, both LN-111 and Matrigel expanded cultures
12 contain smaller immature myotubes with small numbers of nuclei (Figure 3d), an
13 effect observed with both human and mouse derived satellite cells. Overall, it was
14 demonstrated that LN-521 provides a good substrate that enables large-scale
15 expansion in a shorter time while maintaining a differentiation performance that better
16 resembles freshly isolated cells⁴³.
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31 Additional work from Penton and colleagues has demonstrated that cells
32 grown on LN-521 show enhanced engraftment into skeletal muscle niches *in vivo*
33 (unpublished). Although there have been many publications that suggest the limited
34 utility of satellite cells for cell therapy, those experiments may have been flawed since
35 they employed satellite cells that had already lost some of their expansion and
36 differentiation potential *in vitro*. Interestingly, satellite cell markers including pax7
37 and integrin alpha 7 were expressed at similar levels across substrates suggesting that
38 LN-521 is regulating satellite cell activity through novel mechanisms (unpublished).
39 Plating satellite cells on LN-521 immediately after isolation by fluorescence activated
40 cell sorting (FACS) appears to fundamentally protect the cells from the loss of the
41 expansion and differentiation abilities. Penton and colleagues are investigating
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3 additional approaches that should permit enhanced expansion and engraftment to
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5 develop advanced therapeutic approaches for patients with muscular dystrophies.
6

7 Previous studies have shown that transplanted hPSC-derived myogenic cells
8
9 can fuse with host myofibers and improve muscle function⁵⁵. However, it is unknown
10
11 if hPSC-derived myotubes alone can generate 3D functional skeletal muscle since
12
13 comparison studies suggest they are more developmentally immature than primary
14
15 myotubes. By trying to mimic the tissue architecture both with biologically relevant
16
17 matrix molecules and different 3D approaches, it is possible to generate functional
18
19 biomimetic skeletal muscle tissues entirely from hPSC-derived myogenic cells⁵⁶.
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24 **Recombinant laminins drive the differentiation and self-organization of hESC-** 25 **derived hepatocytes** 26 27

28 There has been tremendous progress over the last 15 years in generating pure
29
30 populations of endodermal cells, including hepatocytes, for biomedical application⁵⁷.
31
32 While cell-based models improve the ability to model human liver biology ‘in the
33
34 dish’ and may be suitable for cell-based therapy, most approaches rely on undefined
35
36 materials in the differentiation process⁵⁸. This severely limits technology
37
38 reproducibility and scale up, acting as a barrier to technology translation.
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42 In recent years, serum free and chemically defined differentiation procedures
43
44 have been developed⁵⁹ that have been validated by the pharmaceutical industry to be
45
46 as good as their current gold standard⁶⁰. While this marked progress at the time, the
47
48 early prototype systems still relied on poorly defined ECMs, such as Matrigel, which
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50 resulted in batch-to-batch variation. Since these studies, a differentiation system has
51
52 been designed using polymer library screening to identify new polyurethane
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54 substrates for cell culture^{61, 62}.
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3 More recently, differentiation has been performed using recombinant
4 laminins⁴². This enabled a highly reproducible and transformative differentiation
5 process, which has been semi-automated to permit large scale manufacture.
6
7 Importantly, cells generated in this manner express proteins, HNF4a, MRP1 and
8 albumin, found in hepatocyte (Figure 3e and f). An essential part of the stem cell
9 scale-up is the ability to use highly defined ‘off the shelf reagents’.
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18 **FROM HERE TO THE FUTURE: ENABLING CELL THERAPY**

19
20 Pluripotent stem cells, whether they are derived from human embryos or
21 reprogrammed adult or foetal somatic cells, have an intrinsic capability for unlimited
22 self-renewal and the ability to make all the cells in the body. They are therefore an
23 ideal candidate to be used as starting material for cell therapies. Much effort has been
24 put into creating methods for hPSC expansion that are defined, robust, simple and
25 safe⁶³. Having a defined method ensures low variance, high-quality, traceability and
26 control over reagents, all required for manufacture of clinical grade cells. The
27 robustness of the method allows consistency of results and predictability. A simple
28 and controlled method reduces cost, minimizes the risk for mistakes, and will more
29 likely be adapted by users. Ultimately, the most important aspect of the hPSC culture
30 method is to create a safe cell source. With extended time in culture, cells may begin
31 to drift genetically, resulting in chromosomal abnormalities. Although hPSCs have an
32 impressive ability to maintain genomic integrity over time, chromosomal
33 abnormalities often occur at higher passages and/or as a result of suboptimal culture
34 conditions^{40, 64, 65}.
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55 **Cell therapy grade cell culture substrates**

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3 The stem cell therapy field is still in its infancy. Translational researchers are now
4 looking more intensely at the quality and character of both the cell products and the
5 culture products used for primary, stem and differentiated cells. So far, FDA and
6
7 EMA have not demanded GMP for phase 1 studies but it is evident that the methods
8
9 employed today will not be sustainable for commercial therapies. As a consequence,
10
11 the conversion of optimized, scalable, and standardized culture methods into GMP-
12
13 compatible protocols will become another challenge. Each and every reagent needs to
14
15 be high quality, traceable, controlled and preferably manufactured according to GMP.
16
17 To address these issues a cell therapy grade (CTG) LN-521 substrate has been
18
19 developed to enable cell therapy fast forward to an affordable price. The CTG LN-
20
21 521 substrate is stably expressed and produced by CAP[®]Go cell, a suspension-based
22
23 platform already used for therapeutic proteins in human clinical trials, and is a
24
25 validated source of protein of ethically accepted origin. A qualified GMP master cell
26
27 bank (MCB) together with a scalable and regulatory compliant production process of
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29 CTG LN-521, serve as the foundation for translational research and cell manufacture.
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38 **Cell culture at the appropriate scale and with automation**

39 Generation of hESC and hiPSC lines is laborious. To decrease the amount of work
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41 needed, increase success rates and minimize hazardous deviations, the process needs
42
43 to be standardized. In addition, an automated process is desirable to avoid subjective,
44
45 operator-dependent mistakes and to cut costs.
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48 Generally, upscaling is achieved by manual expansion in traditional culture
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50 vessels or through scalable planar flask cultures, which all involve multi-layered
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52 stacked-plate systems. Automation of traditional culture vessels, such as plates and
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54 flasks, can be achieved by pipetting (semi or whole) closed system robotics. Another
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3 approach is packed-bed bioreactors which are perfusion-based 3D scaffolds. A further
4 type of bioreactor supported system offering the precise control of the culture
5 environment is microcarrier culture in stirred tanks, ideal systems for achieving
6 required lot sizes. The surface-area-to-volume is greatly increased and thus renders
7 more cells with less medium, reagents and materials⁶⁶.
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14 Stem cell scale-up and subsequent differentiation would ideally be performed
15 in the same, closed, automated and fully controlled bioreactor. Human recombinant
16 laminin can easily be adsorbed by microcarriers (e.g. Corning® Enhanced Attachment
17 Microcarriers) and LN-521 coated microcarriers have been shown to sustain
18 expansion of both hESC and hiPSCs in stirred tank bioreactors, with sequential
19 differentiation to cardiomyocytes⁶⁷ (Figure 2d).
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29 **Importance of biological relevance: spatial, temporal and regional impact**

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31 The development of robust protocols that safely and accurately direct hESC and
32 hiPSC into the desired cell types, mimicking the signalling that occurs during
33 development is difficult to achieve *in vitro*. While many protocols successfully
34 recapitulate the temporal developmental processes via well designed medium
35 formulations, the majority of protocols ignore the role of the ECM and the spatial and
36 tissue specific distribution of the ECM proteins that form the tissue architecture.
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44 One of the biggest and most obvious differences between cells cultured in 2D
45 versus 3D is their morphology. The orientation of the integrin mediated adhesions,
46 guides the orientation and thus the shape of the cells. A 3D microenvironment
47 influences the attachment, spreading, growth, and most importantly cell polarity^{68, 69}.
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52 The limitations of 2D cultures have contributed to poor predictive power of pre-
53 clinical cell-based drug and toxicity screening assays. Human recombinant laminins,
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3 in combination with other key ECM molecules, can be used together with various
4 available 3D solutions. Hydrogels can be covalently linked with proteins and thus
5 serve as a 3D context for the cells. In addition, recombinant silk proteins can be used
6 as scaffolds that can easily be adsorbed with e.g. LN-521 to maintain and differentiate
7 hESCs towards different lineages. In order to mirror the correct *in vivo* tissue
8 assembly *ex vivo*, and to regenerate damage tissue and even repair whole body parts,
9 biologically relevant and physiological matrices in a structure allowing spatial
10 orientation need to be developed.
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20 This article has been designed to create an awareness around the necessity and
21 need for defined materials to improve basic scientific research and to permit the
22 translation of these advances through reliable manufacture of cell-based products at a
23 large scale. Additionally, we emphasize the importance of a close collaboration
24 between the users, suppliers and regulators to facilitate the commercial development
25 of safe and effective cell therapies globally.
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35 **COMPETING INTERESTS**

36 LH and TK are both employed by BioLamina AB and PA and CP by Icagen Inc.
37 DCH is a founder, director and shareholder in Stemnovate Limited. DCH is also a
38 director and shareholder in HigherSteaks Limited.
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FIGURE LEGENDS

Figure 1a-c. Laminins are abundant components of the BMs and have essential roles in structural organization. Via binding to cell-surface receptors, such as integrins, laminins are also key regulators of cell behaviour (a). The molecular structure of laminin isoforms is cross-shaped. The Greek letters indicate the specific chains. The α chains have five globular domains (LG1–5) at the C terminus. The short arms are composed of a globular laminin N-terminal domain (LN), globular laminin IV domain (L4), or laminin four domain (LF). Each of these domains is separated by rod-like regions formed by multiple laminin epidermal-growth-factor-like (LE) domains (b). The $\alpha 5$ -chain laminins are key cell adhesion proteins of the natural stem cell niche. It is naturally expressed and secreted by cells in the inner cell mass ICM of the embryo whereas the $\alpha 1$ -chain laminins are mostly expressed in the Reichert's membrane which supports the outer extra-embryonic layer of trophoblasts (c).

Figure 2a-d. Human PSCs grow as homogenous monolayers on LN-521, exhibiting a high nuclear-to-cytoplasm ratio and prominent nucleoli. Due to the biologically relevant support from the matrix, hESC and hiPSC can be seeded as single cells on LN-521 without the need to use apoptosis inhibitors (e.g. ROCKi) for survival. Within 1 hour after seeding, the cells have attached and are evenly distributed across the surface (a, day 0). The cells show high motility on LN-521, migrate and form small colonies and start dividing (a, day 1). The colonies merge (a, day 3) into a homogenous monolayer without signs of spontaneous differentiation (a, day 4). LN-521 supports higher post-seeding survival (24 h) of single-cell dissociated hPSCs, compared to Matrigel (MG) and non-relevant laminin matrices (b). In addition, human PSCs proliferate faster on LN-521 compared to other feeder-free matrices

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3 (EHS extract; Engelbreth-Holm-Swarm mouse sarcoma extract), with about 10-fold
4 increase every 4 days (c). LN-521 even enables large-scale automated processes for
5 robust hPSC expansion in bioreactors. The cells easily attach, spread evenly on the
6 surface of the microcarriers and gives a 20-fold expansion within 4 days (d).

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13 **Figure 3a-f.** Differentiation of hESCs on LN-111 towards DA cells results in more
14 than 40-fold increase of transplantable DA progenitors at day 16 (a) and the human
15 progenitors innervate the striatum, become tyrosine hydroxylase (TH+) neurons and
16 contribute to complete behavioural recovery after transplantation into animals
17 modelling PD (b; scale bar represents 1.5mm). LN-521 improves muscle cell
18 proliferation and differentiation, giving larger myotubes, higher amounts of nuclei per
19 myotube (express myosin heavy chain, MHC) and more consistent differentiation in
20 long-term cultures (c; MHC in green) compared to cells on Matrigel (d; DAPI in blue).
21 Human ESCs differentiated on LN-521 and LN-111 demonstrate efficient hepatocyte
22 maturation with significant improvements in cell function⁴². The hepatocyte-like cells
23 are highly organized on LN-521, express transporter protein MRP1 (e; green) and
24 HNF4a (red), and a majority of the cells are albumin+ (f; green) Cell nuclei are
25 visualized with DAPI (blue).

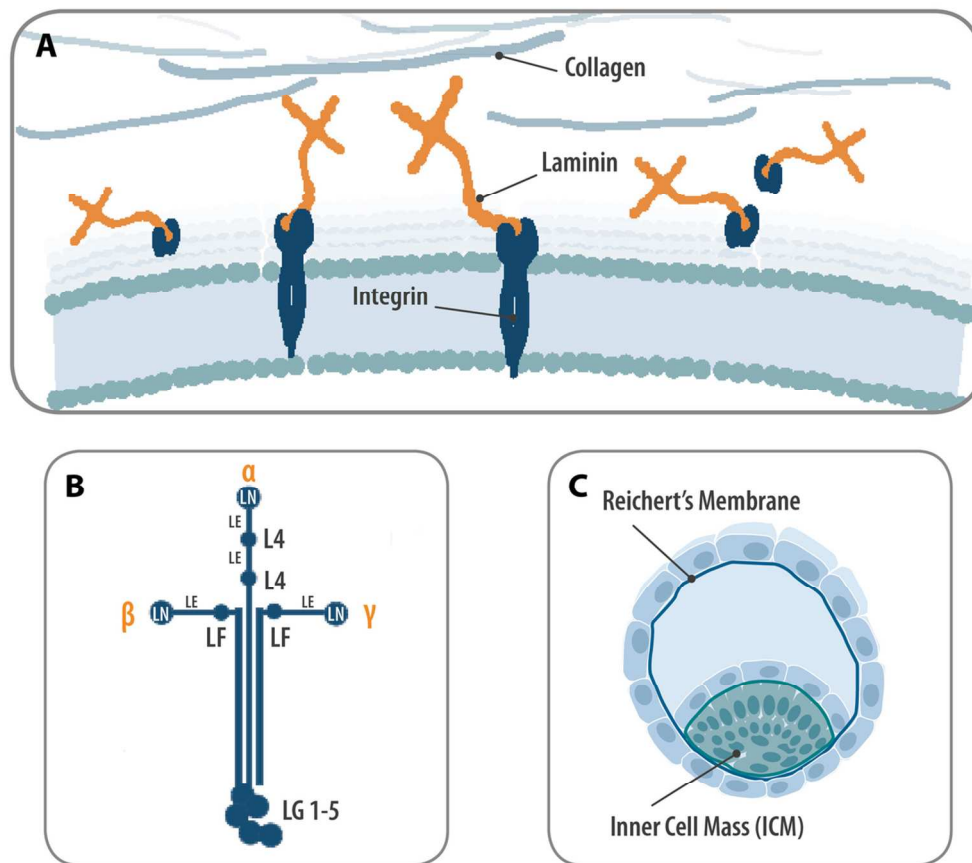


Figure 1a-c. Laminins are abundant components of the BMs and have essential roles in structural organization. Via binding to cell-surface receptors, such as integrins, laminins are also key regulators of cell behaviour (a). The molecular structure of laminin isoforms is cross-shaped. The Greek letters indicate the specific chains. The α chains have five globular domains (LG1–5) at the C terminus. The short arms are composed of a globular laminin N-terminal domain (LN), globular laminin IV domain (L4), or laminin four domain (LF). Each of these domains is separated by rod-like regions formed by multiple laminin epidermal-growth-factor-like (LE) domains (b). The α 5-chain laminins are key cell adhesion proteins of the natural stem cell niche. It is naturally expressed and secreted by cells in the inner cell mass ICM of the embryo whereas the α 1-chain laminins are mostly expressed in the Reichert's membrane which supports the outer extra-embryonic layer of trophoblasts (c).

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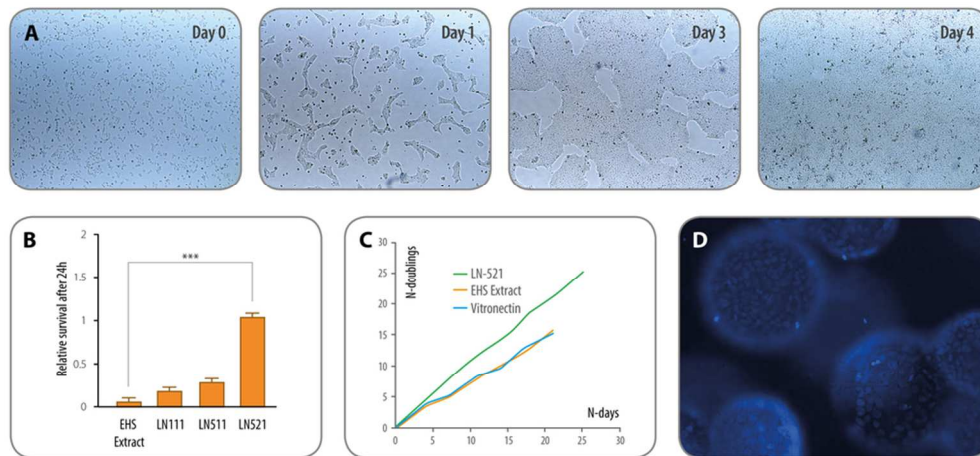


Figure 2a-d. Human PSCs grow as homogenous monolayers on LN-521, exhibiting a high nuclear-to-cytoplasm ratio and prominent nucleoli. Due to the biologically relevant support from the matrix, hESC and hiPSC can be seeded as single cells on LN-521 without the need to use apoptosis inhibitors (e.g. ROCKi) for survival. Within 1 hour after seeding, the cells have attached and are evenly distributed across the surface (a, day 0). The cells show high motility on LN-521, migrate and form small colonies and start dividing (a, day 1). The colonies merge (a, day 3) into a homogenous monolayer without signs of spontaneous differentiation (a, day 4). LN-521 supports higher post-seeding survival (24 h) of single-cell dissociated hPSCs, compared to Matrigel (MG) and non-relevant laminin matrices (b). In addition, human PSCs proliferate faster on LN-521 compared to other feeder-free matrices (EHS extract; Engelbreth-Holm-Swarm mouse sarcoma extract), with about 10-fold increase every 4 days (c). LN-521 even enables large-scale automated processes for robust hPSC expansion in bioreactors. The cells easily attach, spread evenly on the surface of the microcarriers and gives a 20-fold expansion within 4 days (d).

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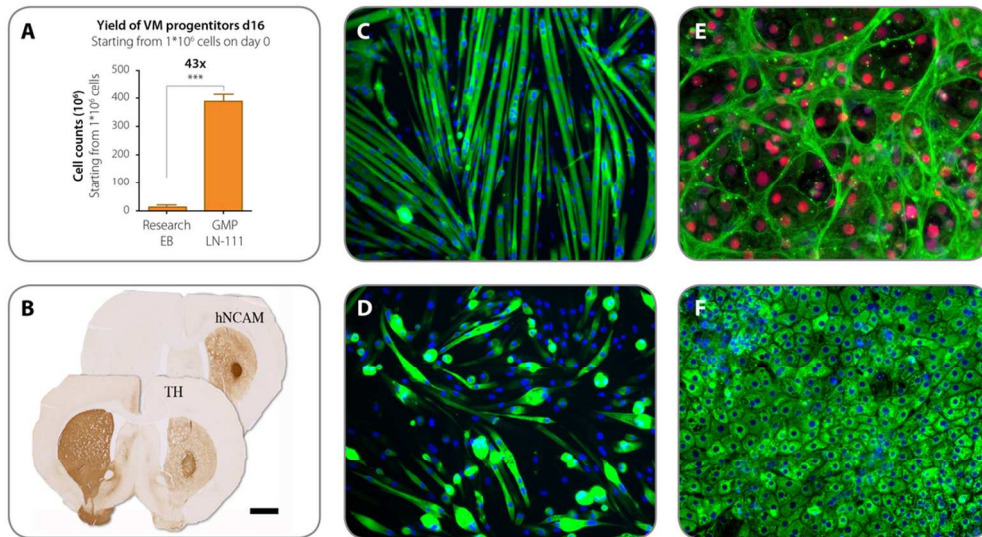


Figure 3a-f. Differentiation of hESCs on LN-111 towards DA cells results in more than 40-fold increase of transplantable DA progenitors at day 16 (a) and the human progenitors innervate the striatum, become tyrosine hydroxylase (TH⁺) neurons and contribute to complete behavioural recovery after transplantation into animals modelling PD (b; scale bar represents 1.5mm). LN-521 improves muscle cell proliferation and differentiation, giving larger myotubes, higher amounts of nuclei per myotube (express myosin heavy chain, MHC) and more consistent differentiation in long-term cultures (c; MHC in green) compared to cells on Matrigel (d; DAPI in blue). Human ESCs differentiated on LN-521 and LN-111 demonstrate efficient hepatocyte maturation with significant improvements in cell function⁴². The hepatocyte-like cells are highly organized on LN-521, express transporter protein MRP1 (e; green) and HNF4a (red), and a majority of the cells are albumin⁺ (f; green) Cell nuclei are visualized with DAPI (blue).

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