Experimental Cell Research 353 (2017) 16–25

Contents lists available at ScienceDirect

Experimental Cell Research

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

# The formation of quiescent glomerular endothelial cell monolayer in vitro is strongly dependent on the choice of extracellular matrix coating

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ARTICLE INFO

*Keywords:*
Laminin-521
Quiescent
Endothelial
Permeability
VE-cadherin
Glomerular

ABSTRACT

*Background and aims:* Nephropathy involves pathophysiological changes to the glomerulus. The primary glomerular endothelial cells (GEnCs) have emerged as an important tool for studying glomerulosclerotic mechanisms and in the screening process for drug-candidates. The success of the studies is dependent on the quality of the cell model. Therefore, we set out to establish an easy, reproducible model of the quiescent endothelial monolayer with the use of commercially available extracellular matrices (ECMs).

*Methods:* Primary hGEnCs were seeded on various ECMs. Cell adhesion was monitored by an impedance sensing system. The localization of junctional proteins was assessed by immunofluorescence and the barrier function by passage of fluorescent dextrans and magnitude of VEGF response.

*Results:* All ECM matrices except recombinant human laminin 111 (rhLN111) supported comparable cell proliferation. Culturing hGEnCs on rhLN521, rhLN511 or fibronectin resulted in a physiologically relevant barrier to 70 kDa dextrans which was 82% tighter than that formed on collagen type IV. Furthermore, only hGEnCs cultured on rhLN521 or rhLN511 showed plasma-membrane localized zonula occludens-1 and vascular endothelial cadherin indicative of proper tight and adherens junctions (AJ).

*Conclusion:* We recommend culturing hGEnCs on the mature glomerular basement membrane laminin - rhLN521 – which, as the only commercially available ECM, promotes all of the characteristics of the quiescent hGEnC monolayer: cobblestone morphology, well-defined AJs and physiological perm-selectivity.

## 1. Introduction

The glomerular filtration barrier (GFB) is the highly specialized blood filtration unit in the kidney, which allows unrestricted passage of small and medium size molecules while preventing filtration of large proteins such as albumin. GFB integrity and functionality is maintained in strict cooperation between its three major components – the glomerular endothelial cells (GEnCs) on the luminal side, podocytes on the apical side and the extracellular matrix (ECM)-rich glomerular basement membrane (GBM) separating the two cell types [1]. The glomerulus contains the thickest basement membrane in the body and provides the main physical barrier to passage of proteins. On the urinary side podocytes provide filtration by the formation of slit diaphragms between adjacent cells and this is essential for filtration. On the opposing side the endothelial cells perform their sieving function through the creation of fenestrations which allow selective passage. Glomerular endothelium has been neglected in kidney research for many years in favour of the podocyte. The reasons for the podocentric view of kidney disease are complex, yet the clear genetic involvement of podocyte-specific genes in hereditary renal disease [2], historical difficulty in primary hGEnC culture as well as the, only recently challenged, belief that glomerular endothelium does not contribute to GFB perm-selectivity are by far the major reasons. In recent years hGEnCs have become commercially available. Moreover, advances in our understanding of the pathophysiology of renal disease show that glomerular endothelium is crucial for proper GFB perm-selectivity [3] and damage to GEnCs without overt injury of podocytes causes proteinuria [4,5]. Research interest in GEnCs is now reviving

*Abbreviation list:* AF, Attachment factor; AJ, adherens junction; AUC, area under the curve; CDH5, cadherin 5; CI, cell index; COL4A1-5, collagen type IV alpha 1–5; hCOLIV, (human) collagen type IV; DAPI, 4′,6-diamidino-2-phenylindole; DPBS, Dulbecco's phosphate buffered saline; ECIS, electric cell-substrate impedance sensing; ECM, extracellular matrix; hFN, (human) fibronectin; GBM, glomerular basement membrane; GFB, glomerular filtration barrier; hGEnC, (human) glomerular microvascular endothelial cells; rhLN, (recombinant human) laminin; MMP, matrix metalloproteinase; qPCR, real-time quantitative PCR; RPL27, ribosomal protein L27; RPS13, ribosomal protein S13; RT, room temperature; $P_{D70}$, diffusional permeability unit [cm/s] for 70 kDa dextran; TIMP, tissue inhibitor of metalloproteinase; TJ, tight junction; TJP1, tight junction protein 1; VE-cadherin, vascular endothelial cadherin; VEGF, vascular endothelial growth factor; ZO-1, zonula occludens-1

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http://dx.doi.org/10.1016/j.yexcr.2017.02.039
Received 11 October 2016; Received in revised form 12 February 2017; Accepted 22 February 2017
Available online 22 February 2017
0014-4827/ 

and there is a need for good, physiologically relevant, reproducible in vitro GEnC models.

Traditionally, the ECM was viewed merely as support for adherent cells, which are attached via integrins. However, it is clear that ECM is also a tightly controlled reservoir of growth factors and that integrins commonly associate with growth factor receptors, modulating cellular responses to the outside microenvironment [6]. There is compelling evidence that the choice of ECM coating affects cell characteristics in vitro [7]. Attempts to provide more complex and tissue-specific ECM have been made [8,9,10]. Although these methods are scientifically very interesting and address important cell culture problems, they are technically challenging and hard to quality control in a regular laboratory.

Most commonly, primary GEnCs are cultured either on gelatin (irreversibly hydrolysed collagen type I) or plasma-derived fibronectin (FN) coated surfaces, yet neither of the substrates are a major component of the healthy mature GBM [11]. To our knowledge no comprehensive study on the effect of ECM on primary GEnC characteristics in vitro has been performed. The findings from the few reports on the effect of ECM on other endothelial cells cannot be extrapolated to GEnCs as it is now well recognized that endothelial cells are not a homogenous cell type and their characteristics differ significantly depending on their original location in the body [12,13]. Secondly, the GBM is unique among other BMs in terms of its collagen IV (COLIV) and laminin (LN) composition. LN and COLIV undergo a series of strictly regulated isoform switches during glomerulogenesis. GEnC and podocyte derived LN111 and COLIV α1α1α2 dominate the immature GBM, while primarily podocyte-derived LN521 and COLIV α3α4α5 uniquely feature in mature healthy GBM. The intermediate GBM contains LN511 and predominantly podocytic COLIV α1α1α2 [14]. One can easily imagine that such strict temporal and spatial control over ECM molecule expression must be of profound importance for GEnC and podocyte phenotype and behaviour. Indeed, reversion to immature GBM in Alport's syndrome mice causes GFB cell dedifferentiation and profound proteinuria [15].

The aim of this study was two-fold. We set out to establish a simple, reproducible model of quiescent hGEnC monolayer in vitro by determining the best commercially available ECM substrate. The quiescent hGEnC model should be characterized by well-defined adherens junctions, cobblestone morphology and appropriate permeability. Secondly, we compared the effect of the mature GBM hLN521, of the intermediate GBM hLN511, and of the main LN in foetal GBM – hLN111.

## 2. Materials and methods

All reagents were from ThermoFisher Scientific (Waltham, MA, US) unless stated otherwise.

### 2.1. Culture surface coating

Culture surfaces were pre-coated overnight at 4 °C. The coating material included the proprietary, predominantly collagen I – based Attachment Factor which is supplied together with hGEnCs (AF; Cell Systems Corporation (CSC), Kirkland, WA, US), human collagen type IV (hCOLIV; #354245, Corning Inc., Corning, NY, US), recombinant human laminin 521, 511, 111 (rhLN; Biolamina, Sundbyberg, SE) or human plasma-derived fibronectin (hFN; #F0895, Sigma Aldrich, Saint Louise, MO, US). AF was used as supplied. Human COLIV was diluted in 10 mM acetic acid, while all the other coating solutions were prepared in Dulbecco's phopshate buffered saline (DPBS) containing calcium and magnesium to give a final coating density of 1 μg/cm$^2$. Whenever possible, the effect of the commercial ECMs was compared to hGEnC monolayer grown on uncoated tissue plastic.

### 2.2. Cell culture

Primary human glomerular microvascular endothelial cells (hGEnCs; ACBRI 128) were obtained from CSC. Cells were subcultured up to passage 8 using CSC reagents. Human GEnCs were seeded in pre-coated vessels at a final density of $10^5$ cells/cm$^2$. Cells were allowed to settle for 15 min at room temperature then cultured for 72 h (day3) at 37 °C. Some cells were cultured for 5 or 7 days in order to determine proliferation.

### 2.3. Impedance sensing – xCELLigence

The cumulative effect of cell attachment, spreading and proliferation was monitored in real-time using the xCELLigence RTCA 96well plate system (ACEA Biosciences, Inc, San Diego, CA, US). Cells were seeded in E-Plate 96 which had been pre-coated with various ECM proteins. First, the background impedance without cells was acquired ($R_b$). Subsequently, cells were seeded at $10^5$ cells/cm$^2$ and the impedance of the wells ($R_n$) was monitored real-time at 15 min intervals. The xCELLigence system reports the data as cell index (CI) values over time. CI equals ($R_n$-$R_b$)/15. For statistical analysis of 3–5 independent experiments, the area under the curve (AUC) of each graph was determined in GraphPad Prism 6.0.

### 2.4. Immunocytochemistry

After culture, cells were fixed in 4% paraformaldehyde for 15 min at room temperature (RT) then permeabilised for 15 min at RT in 0.5% Triton-X 100 in DPBS. After 60 min blocking at RT in 3% BSA in DPBS, cells were incubated with primary antibody overnight at 4 °C rotating. The mouse monoclonal anti-zonula occludens-1 (ZO-1) antibody (clone ZO1-1A12) was used at 3 μg/ml, while the goat polyclonal anti-vascular endothelial-cadherin (VE-cadherin) antibody (clone C-19; Santa Cruz Biotechnology, Inc, Dallas, TX, US) at 2 μg/ml. Incubation with secondary antibody took place at RT for 45 min. Donkey anti-mouse AF488 antibody (#A-21202) was used at 20 μg/ml and the rabbit-anti-goat AF488 antibody (#A11078) at 5 μg/ml. All antibodies were diluted in 3% BSA in DPBS. Actin cytoskeleton was visualized with ActinGreen 488 ReadyProbes following manufacturer's instructions. Finally, the cells were counterstained for 2 min at RT with 300 nM DAPI (4′,6-Diamidino-2-Phenylindole, dilactate) and mounted in ProlongGold antifade reagent. Cells were imaged on Cytation3 Cell Imaging Multi-Mode Reader (BioTek, Winooski, VT, US) equipped with a DAPI filter cube (377/447 nm) coupled to LED 365 nm as well as GFP filter cube (469/525 nm) coupled to LED 465 nm using Gen5 2.07 software in the Imager Manual Mode. The LED intensity, integration time and camera gain were kept constant for each fluorophore in a particular experimental set. In order to assess the effect of ECM proteins on hGEnC proliferation over time, the number of DAPI-positive areas in the 4× magnification images was determined using the Visiopharm integrator software (Visiopharm, Hørsholm, DK). The nuclei were counted in the contrast green-blue input band applying square filter. The software was also used to determine the ZO-1 and VE-cadherin signal intensity in cytoplasm and the membranes in 20× magnification images. To this end the intensity inverted RGB-G input band was used. The contrast between the membrane and cytoplasmic features were enhanced with the square and poly local linear filters. The pixel intensities were then classified using a simple threshold analysis. The output consisted of the number of nuclei per image (cell number), pixel intensity of membrane features and pixel intensity of cytoplasmic features in the image. The final data is presented as the membrane to cytoplasm ratio per cell.

### 2.5. Gene expression

Total mRNA was extracted from cells used in xCELLigence experi-