Bacterial Cellulose Shifts Transcriptome and Proteome of Cultured Endothelial Cells Towards Native Differentiation*

Gerhard Feil‡, Ralf Horres§, Julia Schulte‡, Andreas F. Mack¶¶, Svenja Petzoldt||, Caroline Arnold***, Chen Meng‡‡, Lukas Jost§§, Jochen Boxleitner‡‡, Nicole Kiessling-Wolf‡, Ender Serbest‡, Dominic Helm||, Bernhard Kuster‡‡§§, Isabel Hartmann‡, Thomas Korff§§, and Hannes Hahne¶¶

Preserving the native phenotype of primary cells in vitro is a complex challenge. Recently, hydrogel-based cellular matrices have evolved as alternatives to conventional cell culture techniques. We developed a bacterial cellulose-based aqueous gel-like biomaterial, dubbed Xellulin, which mimics a cellular microenvironment and seems to maintain the native phenotype of cultured and primary cells. When applied to human umbilical vein endothelial cells (HUVEC), it allowed the continuous cultivation of cell monolayers for more than one year without degradation or dedifferentiation. To investigate the impact of Xellulin on the endothelial cell phenotype in detail, we applied quantitative transcriptomics and proteomics and compared the molecular makeup of native HUVEC, HUVEC on collagen-coated Xellulin and collagen-coated cell culture plastic (polystyrene).

Statistical analysis of 12,475 transcripts and 7831 proteins unveiled massive quantitative differences of the compared transcriptomes and proteomes. K-means clustering followed by network analysis showed that HUVEC on plastic upregulate transcripts and proteins controlling proliferation, cell cycle and protein biosynthesis. In contrast, HUVEC on Xellulin maintained, by and large, the expression levels of genes supporting their native biological functions and signaling networks such as integrin, receptor tyrosine kinase MAP/ERK and PI3K signaling pathways, while decreasing the expression of proliferation associated proteins. Moreover, CD34—an endothelial cell differentiation marker usually lost early during cell culture - was re-expressed within 2 weeks on Xellulin but not on plastic. And HUVEC on Xellulin showed a significantly stronger functional responsiveness to a prototypic pro-inflammatory stimulus than HUVEC on plastic.

Taken together, this is one of the most comprehensive transcriptomic and proteomic studies of native and propagated HUVEC, which underscores the importance of the morphology of the cellular microenvironment to regulate cellular differentiation, and demonstrates, for the first time, the potential of Xellulin as versatile tool promoting an in vivo-like phenotype in primary and propagated cell culture. Molecular & Cellular Proteomics 16: 10.1074/mcp.RA117.000001, 1563–1577, 2017.

Primary cells cultured in vitro are well-established model systems to analyze distinct cellular responses under defined experimental conditions to investigate disease mechanisms and to identify putative therapeutic options. However, primary cell culture is still subject to biological as well as experimental constraints. Conventional culture of primary cells is almost always associated with culture conditions stimulating proliferation and cellular activity. As a result, the phenotypes may be unstable and promote dedifferentiation of primary cells upon repeated propagation in culture (1, 2). In addition, cellular differentiation is also modulated through the mechanical properties of the in vivo microenvironment (3, 4), and conventional cell culture of primary cells on rigid and hydrophobic plastic surfaces may therefore evoke phenotypic alterations (5), ultimately contributing to the discrepancy of in vivo and in vitro biological models. A multitude of technologies emerged to better mimic the mechanical and regulatory properties of the cellular microenvironment for two- and three-dimensional...

From the ‡Xellutec GmbH, Eichenstraße 15, 82061 Neuried, Germany; §§GenXPro GmbH, Altenhoferallee 3, 60438 Frankfurt am Main, Germany; †Institute of Clinical Anatomy and Cell Analysis, University of Tübingen, Österbergstraße 3, 72074 Tübingen, Germany; ||OmicScouts GmbH, Emil-Erlenmeyer-Forum 5, 85354 Freising, Germany; **Institute of Physiology and Pathophysiology, Division of Cardiovascular Physiology, University of Heidelberg, Im Neuenheimer Feld 326, 69120 Heidelberg, Germany; ‡‡Chair of Proteomics and Bioanalytics, Technische Universität München, Emil-Erlenmeyer-Forum 5, 85354 Freising, Germany; §§Bavarian Center for Biomolecular Mass Spectrometry (BayBioMS), Technische Universität München, Gregor-Mendel-Straße 4, 85354 Freising, Germany.

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culture of primary cells, including natural, synthetic and semisynthetic extracellular matrices, synthetic or biological hydrogels (6, 7). Also, a variety of approaches have been developed using scaffolds and matrices to facilitate cell attachment and to create bioartificial tissues in vitro (8, 9). An emerging material for biomedical applications, such as skin and cartilage repair (10–14), is bacterial cellulose (BC)1. This exopolysaccharide, consisting of linear β-D-glucose molecules linked by β(1–4) glycosidic bonds and synthesized by Gluconacetobacter xylinus, is pure and exhibits mechanical strength, porosity, biocompatibility, and noninherent biodegradability in vivo (10, 15).

To assess the potential of BC in cell culture, we have developed a BC-based hydrological biomaterial as cell culture support, dubbed Xellulin, and obtained cell cultures on Xellulin for a variety of cell types. Given the significance of endothelial cells for physiological and pharmacological investigations of (tumor-associated) angiogenesis, coagulation, inflammation, and other processes in endothelia (16, 17), we consequently investigated the differentiation of human umbilical vein endothelial cells (HUVEC) on Xellulin to exemplary showcase the versatility of this BC for cell culture purposes.

Here we demonstrate that the transcriptomic and proteomic phenotype of propagated HUVEC cultured on collagen-coated Xellulin preserves important features of the native phenotype, including reduced expression of proliferation associated proteins, and sustained expression of integrin and receptor tyrosine kinase signaling, MAP/ERK and PI3K signaling pathways, and ELK, MEF, and NFAT transcription factors. Our studies also revealed that collagen-coated Xellulin induces the re-expression of the endothelial differentiation marker CD34 and that the quiescent HUVEC monolayers may be stimulated to acquire an activated phenotype on Xellulin. These results indicate the potential of BC in general, and Xellulin in particular, as versatile cell culture support promoting an in vivo-like phenotype in primary and propagated cell culture.

EXPERIMENTAL PROCEDURES

Preparation of Xellulin from Bacterial Cellulose—Xellulin is a natural polymer based on BC. This exopolysaccharide is synthesized by authentic cultures derived from strains of Gluconacetobacter xylinus (Leibniz-Institut DSMZ - Deutsche Sammlung Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, DSM 2325). Xellulin is produced in flat-bed bioreactors in a modified process according to Hofinger et al. (18). Harvested Xellulin sheets are cleaned in 0.2 M NaOH (Carl Roth, Karlsruhe, Germany) and then washed in distilled water. Discs are cut out from thin Xellulin sheets and mounted on frames. These so-called Xell-Discs fit to 6-well and 24-well cell culture plate formats, and Xell-Discs in the 6-well format were used as cell culture support for HUVEC in this study. Xellulin was examined using scanning electron microscopy (S.E.) as described previously by Schulte et al. (19).

Isolation and Culture of Human Umbilical Vein Endothelial Cells— investigations with human umbilical vein endothelial cells (HUVEC) were performed with written informed consent from six paras and following ethical guidelines. Umbilical cords were collected after birth and stored at 2–8 °C. HUVEC were isolated with prewarmed dispase solution (3.6–3.8 U/ml; Thermo Fisher Scientific, Damstadt, Germany) within one to four hours after birth. The obtained HUVEC were propagated in cell culture flasks. HUVEC passage 1 were seeded at a density of 3.8 × 104 cells/cm² on Xell-Discs (Xellutec GmbH, Neuried, Germany) that had been coated with 0.5 ml Cellovations® rat tail (type I) collagen solution (750 µg/ml in 0.1% acetic acid; PELOBiotech GmbH, Martinsried, Germany). HUVEC were cultured in complete endothelial cell growth medium (PromoCell, Heidelberg, Germany) supplemented with 1% fetal bovine serum (FBS; Thermo Fisher Scientific). After 3 weeks and 6 weeks, respectively, HUVEC were detached with collagenase type IV (500 U/ml; Thermo Fisher Scientific). For standard cell culture HUVEC passage 1 were seeded with a density of 2.2 × 104 cells/cm² on 6-well cell culture plates (Techno Plastic Products AG, Trasadingen, Switzerland) coated with 1 ml Cellovations® rat tail (type I) collagen solution. The HUVEC were fed with complete endothelial cell growth medium supplemented with a total of 1% or 5% FBS. Confluent monolayers were harvested 2 days after cell seeding (details are given in supplementary Material).

Native HUVEC were freshly isolated from six umbilical cords and then propagated (passage 0). Ten samples HUVEC passage 1 were cultured on Xellulin and twelve samples HUVEC passage 1 in standard plastic cell culture. An overview of the experimental design is given in supplemental Table S1.

Additionally, HUVEC cultivated on Xellulin for one year and for two months, respectively, were detached, propagated on plasticware and then re-seeded on Xellulin as described above.

Scratch Assay of HUVEC Monolayers—To assess the physiological potential of quiescent HUVEC on Xellulin, HUVEC monolayers cultured on collagen-coated Xellulin were injured by a scratch with a 100 µl pipette tip. As control, HUVEC monolayers cultured on collagen-coated culture plates were scratched as well. Cell behavior was monitored for 2 days.

Histology and Immunohistology of Human Umbilical Cord Samples—Tissue samples were fixed and embedded for cryostat, or for paraffin sectioning using standard methods. Paraffin sections were deparaffinized, rehydrated, and then stained histologically with Azan stain according to standard protocols (supplementary Material). Cryosections were incubated with blocking/permeabilizing solution. Afterward, primary antibodies against platelet/endothelial cell adhesion molecule PECAM-1 (CD31) and zonula occludens protein 1 (ZO-1) were applied. Antibodies were visualized by subsequent incubation with goat anti-mouse IgG Alexa Fluor 546, and goat anti-rabbit IgG Alexa Fluor 633 secondary antibodies followed by incubation with SYTOX green nucleic acid stain (supplementary Material).

Immunocytochemistry of HUVEC on Xellulin—Xellulin with HUVEC monolayers was fixed and then stained immunocytochemically for CD31, von Willebrand factor (vWF), or for CD 34, respectively, with antigen-specific primary antibodies (supplementary Material). Bound antibodies were visualized by subsequent incubation with Alexa Fluor 594 goat anti-mouse IgG antibodies, anti-rabbit IgG-FITC antibodies, or Alexa Fluor 488 goat anti-mouse IgG antibodies, respectively. Nuclei were stained with DAPI. To characterize the phenotype of HUVEC seeded on Xellulin, HUVEC passage 1 from the same origin

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1 The abbreviations used are: BC, Bacterial cellulose; BH, Benjaminsmi-Hochberg; DAPI, 4',6-diamidino-2-phenylindole; FCS, Fetal calf serum; FDR, False discovery rate; GSEA, Gene set enrichment analysis; HUVEC, Human umbilical vein endothelial cells; LC-MS/MS, Liquid chromatography tandem mass spectrometry; MACE, Massive Analysis of cDNA Ends; PCA, Principle component analysis; ROS, Reactive oxygen species; TMT, Tandem mass tags; TPM, Tags per million.
were additionally seeded into ibiTreat 8-well μ-Slides (ibidi, Martin-sried, Germany). Chamber slide cultures were fixed and stained for von Willebrand Factor (vWF) and CD31 (supplementary Material).

For comparison to umbilical vein sections stained immunohistologically against CD31 and ZO-1 (see above), HUVEC on Xellulin were fixed and then incubated with blocking/permeabilizing solution. Subsequently, primary antibodies against CD31 and ZO-1 were applied in cocktails. Bound antibodies were detected by goat anti-rabbit Cy3, or goat anti-mouse Cy5 secondary antibodies followed by incubation with SYTOX green nucleic acid stain (supplementary Material).

**Microscopy**—Images of HUVEC both on plastic and Xellulin stained immunologically for CD31, vWF, and CD34 were acquired with a phase contrast and fluorescence microscope (Axio Observer, Z1, Carl Zeiss) and taken with a digital microscope camera. Images of human umbilical cord sections and HUVEC on Xellulin stained immunologically for CD31, ZO-1, CD34, vWF, VE-Cadherin and Angiopoietin were acquired with a confocal laser scanning microscope (LSM 510 META, Carl Zeiss, Jena, Germany) using sequential scans and appropriate filter sets (Supplementary Material). Images were exported and figure panels assembled using Adobe Photoshop (San Jose, CA).

**Experimental Design and Statistical Rationale of the Transcriptome and Proteome Analyses**—The experimental design is summarized in Fig. 1A and supplementary Table S1. A total of 28 HUVEC samples from six umbilical veins were analyzed corresponding to native HUVEC from six different donors, 2 × 5 propagated HUVEC (passage 1) cultivated on Xellulin for 3 and 6 weeks, respectively, and 2 × 6 propagated HUVEC (passage 1) cultivated on plastic with 1 and 5% FCS, respectively. We profiled transcriptomes of all 28 samples corresponding to at least 5 biological replicates per condition using the MACE technology. We profiled proteomes of a subset of 12 samples corresponding to biological triplicates of donor-matched propagated HUVEC on Xellulin for 3 and 6 weeks, and propagated HUVEC on plastic with 1 and 5% FCS using a TMT10plex-based quantification approach. Because of limited sample amounts, we did not include native HUVEC in the proteome study. In addition, we analyzed the proteomes of pooled HUVEC samples from Xellulin and plastic culture (pools of the same samples used for TMT10plex quantification; no replicates) using a label-free intensity based proteomics approach.

**RNA Preparation**—For RNA isolation from native HUVEC, the umbilical vein was washed with DPBS (1x) w/o Ca & Mg and thereafter incubated with 5 to 7 ml RNAprotect cell reagent (Qiagen, Hilden, Germany, 76526) for 2 mins. The liquid was transferred to a centrifuge tube. After another washing step with DPBS (1x) w/o Ca & Mg, HUVEC were isolated by incubation with pre-warmed (37 °C) Cell protease inhibitor for 2 mins. Cells were detached with a cell scraper and transferred into a centrifuge tube. For maximization of the cell yield, residual HUVEC on Xellulin and plastic surface, respectively, were washed with an additional ml of DPBS (1x) w/o Ca & Mg and the liquid was transferred to the centrifuge tube used previously. The detached cells were pelleted.

For extracting RNA from HUVEC passage 1, the cells were washed with DPBS (1x) w/o Ca & Mg and then incubated with 600 μl RNAprotect cell reagent for 2 mins. Cells were detached with a cell scraper and transferred into a centrifuge tube. For maximization of the cell yield, residual HUVEC on Xellulin and plastic surface, respectively, were washed with an additional ml of DPBS (1x) w/o Ca & Mg and the liquid was transferred to the centrifuge tube used previously. The detached cells were pelleted.

RNA was isolated and purified using the RNeasy Mini Kit (Qiagen, 74104) according to the manufacturer’s protocol. The eluates were stored at −20 °C until further processing.

**RNA Quantification and Integrity**—A DNase I digestion of all RNA samples was made with Baseline-Zero™ DNase (Epicerin, Madison, WI; provided by Biozym Scientific GmbH, Hessisch Oldendorf, Germany) in solution to ensure that the samples were completely free of DNA. RNA samples were subsequently purified using RNA Clean & ConcentratorTM-5 Kit (Zymo Research Europe, Freiburg Germany).

Total RNA concentration was measured with Quibit 2.0 Fluorometer and Quibit RNA HS Assay Kit (Life Technologies GmbH, Darmstadt, Germany). RNA quality was estimated in a dilution series with the LabChip GX by PerkinElmer Rodgau, Germany (Software Version 4.2.1745.0). The RNA quality of all samples was estimated using the RNA Integrity Number (RIN) (20) and RIN scores between 6.4 and 10 were obtained.

**Transcriptome Sequencing and Quantification**—The transcriptome of HUVEC samples was sequenced and quantified using the Massive Analysis of cDNA Ends (MACE) technology in combination with the TrueQuant method, eliminates and corrects for PCR introduced copies, thereby enabling a more accurate transcriptome quantification (21).

From each RNA sample a MACE cDNA library was constructed targeting sequences near the cDNA 3’-ends. MACE was conducted by GenXPero GmbH as described (22, 23). Briefly, the polyadenylated mRNA was reverse transcribed and the resulting cDNA was immobilized with biotinylated oligo(dT) primers via streptavidin coated magnetic beads after random fragmentation with directed ultrasound (Biounitor, Diagonede, Belgium). The remaining fragments were discarded, 50–600 bp long fragments before the Poly-A tail starting from the 5’-ends were sequenced (single-read) using the Illumina Next Seq500 platform (Illumina Inc., San Diego, CA), generating 75bp long reads. Illumina’s Control Software was used for sequencing and real-time analysis followed by Illumina Consensus Assessment of Sequence and Variation procedure for base calling and demultiplexing. To prevent PCR-biased sequencing by Illumina sequencing, the described TrueQuant method was applied.

**E-selectin mRNA Expression Assay**—HUVEC in passage 2 were seeded on collagen I-coated plastic or Xellulin discs. After ~2 days, the cells reached confluence and were stimulated with 5 ng/ml human recombinant TNFalpha (hrTNFa, Biomol #50435, Lot#2214–1) for 3 h. Subsequently, cells were washed once in ice-cold HBSS w/Ca²⁺ / Mg²+ and lysed in 300 μl RLT-Lysis Buffer/1% beta-mercaptoethanol (Qiagen) and snap frozen in liquid nitrogen. RNA was isolated according to manufacturer’s instructions (RNeasy MiniKit, Qiagen Cat. No.: 74106). For cDNA synthesis, 100 ng RNA were used according to manufacturer’s protocol (Omniscript RT Kit, Qiagen, Cat. No.: 205113). The PCR reaction was performed using 2.5 μl cDNA using the following primers and conditions: hRPRL32: forward 5′GTTATCATCCGGACACGTAGCA3′, reverse 5′ACGTGCACATGAGCTGCCTA3′; 5 min 95 °C, cycles: 30 s, 95 °C, 60 °C, 45 s, 72 °C, 1 min, 24 cycles, 72 °C 3 min, 389 bp, hE-Selectin: forward 5′TGGCCTGCTGCTGCTGCT4′, reverse 5′TCAGTGAGCGCCCTCCTTG3′; 5 min 95 °C, cycles: 30 s, 95 °C, 55 °C, 45 s, 72 °C, 1 min, 33 cycles, 72 °C 5 min, 474 bp. The PCR products were separated on a 1% agarose gel and documented using the Gel Doc XR+ system and the Quantity One Software, Version 4.6.9 (Bio-Rad). Gray intensities were quantified using Image J Software (Version 1.49a).

**Sample Preparation and Isobaric Labeling for Quantitative Proteome Profiling**—HUVEC were lysed using lysis buffer containing 8 M urea, 40 mM Tris/HCl (pH 7.6), 1X EDTA-free protease inhibitor mixture (Complete Mini, Roche). The protein concentration was determined using the Bradford method (Coomassie Protein Assay Kit, Thermo Scientific). Proteins were reduced and alkylated with 10 mM tris(2-carboxyethyl)phosphin (TCEP) at 37 °C for 1 h followed by 25 mM chloro-acetamide for 45 min at room temperature in the dark. The protein mixture was diluted with 40 mM Tris/HCl to a final urea concentration of 1.6 M. Protein aliquots of 50 μg were digested by adding sequencing-grade trypsin (Promega, Mannheim, Germany; 1:100 enzyme/substrate ratio) and samples were incubated at 37 °C for 4 h. Another 1:100 aliquot of trypsin was added for overnight digestion at 37 °C. Samples were acidified with formic acid to pH 2.
and subsequently cleaned up and concentrated using C18 StageTips essentially as described (24).

To compare and normalize Tandem Mass tag (TMT) reporter intensities between different TMT10-plex LC-MS/MS experiments, identical samples representing a pooled digest of all samples were used as common reference. For the common reference pool, 15 µg of peptide digest per sample were combined.

Peptide aliquots were dried in vacuo, reconstituted in 40 µl 50 mM triethyl ammonium bicarbonate and incubated at room temperature for 15 min. TMT10-plex reagents (Thermo Fisher Scientific, Dreieich) were dissolved in 42 µl of water-free acetonitrile and 10 µl of TMT was added per sample. After incubation at room temperature for 1 h, the labeling reaction was quenched by adding hydroxylamine to a final concentration of 0.2% (v/v).

Labeled peptides were mixed in equal amounts, acidified with formic acid, and desalted using C18 SepPak cartridges according to the manufacturer’s instructions (C18 cartridges, Sep-Pak Vac, 1 cc, 50 mg, Waters Corp., Eschborn, Germany). Eluates were dried down and stored at −80 °C. Samples were further fractionated into 24 fractions using hydrophilic strong anion exchange chromatography (hSAX) as described previously (25), and peptides were desalted and concentrated using C18 StageTips.

Sample Preparation of Label-free Quantitative Proteome Profiling for Global Proteome Composition Analyses—Peptide aliquots of six Xellulin and six plastic samples were pooled separately (see also supplemental Table S1), desalted and concentrated using C18 StageTips and fractionated in StageTips as described (26) using a stepwise gradient of ACN in 25 mM ammonium formiate (flowthrough, 5%, 10%, 15%, 17.5%, 50%) before fraction 1 and 5 and 2 and 6 were pooled.

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)—Nanoflow LC-MS/MS analysis of peptide samples was performed on an UltiMate 3000 RSLCNano System (Thermo Scientific, Dreieich, Germany) coupled to a Q-Exactive HF mass spectrometer (Thermo Scientific, Bremen, Germany). Peptides were delivered to a trap column (100-µm inner diameter × 2 cm, packed with 5-µm C18 resin (Reprosil GOLD, Dr. Maisch, Germany) at a flow rate of 5 µl/min for 10 min in 100% solvent A (0.1% FA in HPLC-grade water). After loading and washing, peptides were transferred to an analytical column (75 µm × 40 cm C18 column; R erosil GOLD, 3 µm, Dr. Maisch, Germany) and separated using a linear gradient of 110 min ramping from 4% to 32% solvent B (0.1% FA, 5% dimethyl sulfoxide in ACN) at a flow rate of 300 nL/min. 5% (v/v) DMSO were used in solvent A and B to boost the nanoelectrospray response (27).

Peptides were ionized using a 2.2-kV electrospray voltage and a capillary temperature of 275 °C. The mass spectrometer was operated in data-dependent acquisition mode, automatically switching between MS and MS2. Full-scan MS spectra (m/z 360–1300) were acquired in the Orbitrap at 75,000 (m/z 200) resolution with an automatic gain control (AGC) target value of 3e6. MS spectra were on the fly re-calibrated using the signal at m/z 401.92339 as lock mass (27). Fragment mass (MS2) spectra were generated for up to 25 precursors with a normalized collision energy of 33% using higher energy collision-induced dissociation (HCD). The fixed first mass was set to 120 m/z for fragment mass spectra and fragment ions were read out in the Orbitrap mass analyzer at a resolving power of 35,000 at m/z 200, which corresponds to a resolving power of ∼50,000 of the TMT10-plex reporter ions. The isolation window was set to 1.3 Th and a MS2 AGC target value of 2e5 (32) was used (28). Sequenced precursor ions were dynamically excluded for 20 s.

Protein Identification and Quantification—Raw MS data were processed by MaxQuant (version 1.5.1.0) for peak detection and quantification (29). MS/MS spectra were searched against the current version of the human Ensembl database (version GRCh 38; 99,436 entries, supplemented with common contaminants) using the Andromeda search engine (30) with the following search parameters: TMT10plex, full tryptic specificity, up to two missed cleavage sites, carboxyamidomethylation of cysteine residues set as a fixed modification, and N-terminal protein acetylation and methionine oxidation as variable modifications. Mass spectra were recalibrated within MaxQuant (first search: 20 ppm precursor tolerance) and subsequently re-searched with a mass tolerance of 6 ppm. Fragment ion mass tolerances were set to 20 ppm. Search results were filtered to a maximum false discovery rate (FDR) of 2.5% for proteins and for 1% peptide spectrum matches and a minimum peptide length of at least 7 amino acids was required. TMT10plex reporter ion intensities were summed per protein group. Only tandem mass spectra with a precursor intensity fraction (PIF) of at least 75% were used for protein quantification.

For label-free proteome quantification, iBAQ values were obtained from MaxQuant and used as proxy for the protein abundance (31). Data Analysis—Data analysis was performed within R statistical computing environment (version 3.2.1) (32) and TIGR MultiExperiment Viewer (version 4.9) (33).

Transcript and protein expression within each MACE and TMT10plex batch were normalized with a linear regression approach. Small batch effects in the MACE dataset were eliminated using a simple gene- and batch-wise normalization of gene expression (manuscript in preparation). Briefly, we assumed that the average expression per gene is constant between different batches and adjusted the expression per gene and batch accordingly by multiplying each gene expression value with a batch- and gene-specific constant. After normalization, only genes with summed expression values >50 across all samples were retained for further analyses to eliminate transcripts of very low expression. The protein intensities between the two TMT10plex experiments were aligned via common references comprised in each TMT10plex experiment.

Principal component analysis (PCA) was performed using the R function “prcomp”. To this end, the data was z-score transformed and proteins with missing values were removed. The first two principal components (PCs) were compared with various experimental factors (sample type, donor, % FCS, and cultivation time) to identify the most influential experimental factors. Transcripts differentially expressed between native HUVEC, HUVEC on Xellulin and HUVEC on plastic, were identified using an ANOVA statistical test resulting in 7165 differentially expressed transcripts with a Benjamini-Hochberg corrected p value < 0.05. Differentially expressed transcripts were further z-score transformed, clustered into distinct clusters using K-means clustering (metric: Pearson correlation) (34), and subsequently curated manually. Single clusters were enriched for functional protein interactions and associations using STRING (35) and the resulting networks were further dissected into highly connected sub-networks using the GLay community cluster algorithm implemented in Cytoscape (version 3.2.1) (36). Functional and pathway information for transcripts comprised in these sub-networks was retrieved through DAVID (37).

Differentially expressed transcripts of HUVEC cultured on Xellulin for 3 or 6 weeks, as well as differentially expressed transcripts and proteins of pair-wise comparisons were identified using a two-sided t test assuming equal variances. The p values were subjected to Benjamini-Hochberg multiple test correction procedure and transcripts/proteins with corrected p values lower than 0.05 were considered differentially expressed.

The integrative analysis of mRNA and protein data was performed using the Consensus PCA approach, which represents a generalization of PCA onto the multiple tables scenario (38). It identifies consensus PCs that represent the most concordant structures between datasets. Similar to PCs, the consensus PCs could be passed to a
clustering algorithm, to identify the joint pattern of two data sets. In this analysis, hierarchical clustering using Euclidean distance and ward linkage was employed to cluster the top two consensus PCs. 

Proteomaps were generated from the transcriptome data using the median expression per transcript and group and from the proteome data using iBAQ values of pooled samples using the Proteomaps tool (39). Only transcripts/proteins were used which could be mapped from Ensembl gene identifiers and gene names to Uniprot accession numbers.

**RESULTS**

**Culture of HUVEC and Other Cell Types on Collagen-coated Xellulin**—As novel support for two-dimensional cell culture, we have developed a BC-based hydrological biomaterial, named Xellulin. Xellulin consists of BC and 99% (w/w) water, demonstrated by weight loss subsequent to drying at 30 °C in a drying cabinet. Scanning electron microscopy revealed an amorphous network of cellulose nanofibers, indicating an elastic, hydrophilic support with a large surface:volume ratio (supplemental Fig. S1A).

We observed that HUVEC (see below) as well as smooth muscle cells, fibroblasts, mesenchymal stem cells and hepatocytes (supplemental Fig. S1B i–iv) can be cultivated on collagen-coated Xellulin for up to several weeks without splitting. Hepatocytes formed typical clusters on Xellulin, whereas the other cell types resulted in cell monolayers.

Before embarking on the molecular profiling experiments, we confirmed the phenotype and purity of HUVEC isolated from six umbilical veins. All cells investigated stained positive for the endothelial cell markers CD31 (also known as PECAM-1) and vWF (supplemental Fig. S2). HUVEC on conventional collagen-coated plasticware formed monolayers 2 days after seeding. In contrast, HUVEC monolayers on Xellulin were observed not until two to 4 days after seeding. The cells grown both on plastic and Xellulin showed cobblestone-like morphology. After reaching confluence, HUVEC on Xellulin revealed a smaller cellular size (supplemental Fig. S3). Cellular monolayers were fed twice per week with standard medium. Notably, we observed that HUVEC cultivated on Xellulin can be readily kept in culture for up to one year without loosing distinct expression patterns of marker proteins like CD31 and von Willebrand Factor (vWF) and the ability to re-initiate proliferation after contact inhibition is lost (supplemental Fig. S4).

**Comparative Transcriptomic and Proteomic Analysis of Native, Freshly Isolated HUVEC and HUVEC on Xellulin and Plastic**—Xellulin allowed the continuous cultivation of quiescent HUVEC monolayers for more than one year without signs of degradation or dedifferentiation. To better understand whether HUVEC on Xellulin can serve as *in vitro* model for *in vivo* endothelial cell biology, and to reveal the underlying molecular differences induced by the native and *in vitro* microenvironments, we set out to analyze the transcriptomic and proteomic phenotype of native and propagated HUVEC on Xellulin and plastic.

A total of 28 HUVEC samples from six umbilical veins were analyzed corresponding to 6 native HUVEC, 2 × 5 propagated HUVEC (passage 1) cultivated on Xellulin for 3 and 6 weeks, respectively, and 2 × 6 propagated HUVEC (passage 1) cultivated on plastic with 1 and 5% FCS, respectively. We profiled transcriptomes of all 28 samples and proteomes of a subset of 12 samples corresponding to biological triplicates of propagated HUVEC on Xellulin for 3 and 6 weeks, and propagated HUVEC on plastic with 1 and 5% FCS (Fig. 1A, supplemental Table S1). Because of limited sample amounts, we did not include native HUVEC in the proteome study.

Quantitative transcriptome profiling was performed using the recently described Massive Analysis of cDNA Ends (MACE) sequencing technology, a digital gene expression method sequencing 50 to 200 bp of either the 3’ or 5’ cDNA of a transcript (21). The 3’ MACE approach resulted in 37,090 to 41,210 quantified transcripts per batch. After stringent filtering of low abundance transcripts (supplemental Fig. S5A), this approach resulted in the quantification of 12,475 genes analyzed in this study (supplemental Table S2).

Quantitative proteome profiling of a subset of 12 samples was performed using an isobaric quantification strategy based on tandem mass tags (TMT) (40) and resulted in the identification of 7831 proteins (protein FDR < 0.01), of which 7417 proteins corresponding to 7257 genes were quantified (supplemental Table S3 and S4).

A comparison of genes revealed 6333 genes exclusively identified on mRNA level and 1115 genes exclusively found on protein level (Fig. 1B). Most transcripts not identified on protein level are of low abundance. Among the proteins not identified on transcript level are numerous important extracellular proteins and major regulators of angiogenesis such as Angiopoietin-2 (antagonist of Angiopoietin-1), Angiopoietin-like 2 and Angiopoietin-like 4, as well as coagulation factors and vasodilatory proteins (such as ACE, DDAH2, and ECE), demonstrating that proteomics contributes information not accessible on transcript level and vice versa.

Unsupervised analysis of all 28 samples based on their transcript expression profiles by principle component analysis (PCA) revealed three distinct clusters corresponding to the three major experimental groups (Fig. 1A and 1D). By far the largest proportion of the experimental variance is captured by principle component (PC) 1 (62%), which separates native from HUVEC cultures and to a much lesser extent by PC2 (17%), which separates Xellulin samples from the other two groups (supplemental Fig. S5B). It is of note that the Xellulin cluster is considerably closer to the native HUVEC cluster on the dominant PC1 axis, indicating a more similar transcriptomic phenotype. Additional experimental factors such as donor, cultivation time on Xellulin, concentration of fetal calf serum (FCS) or sample batch are of minor relevance or irrelevant (see supplemental Fig. S5C and S5D).

To confirm the PCA results, we performed a correlation analysis of all Xellulin (*n* = 10) and plastic (*n* = 12) transcript-
tome samples against all native HUVEC samples \((n = 6)\). The Pearson correlation coefficients are significantly higher \((p < 2.2e-16)\) for the Xellulin samples (median 0.84) compared with plastic samples (0.79) (Fig. 1D), supporting the above observation.

To assess how consistent transcriptome and proteome profiles of six plastic samples and six Xellulin samples are, we followed a consensus PCA approach, which is a generalization of PCA for multiple omics levels and allows the projection of transcriptome and proteome profiles onto the same plane. As depicted in Fig. 1E, the transcriptome and proteome (arrow base and arrow head, respectively) of plastic and Xellulin samples cluster together, whereas the two sample groups are clearly separated along PC1, indicating that transcriptome and proteome represent the same biological variation, and that the biological differences between the two samples groups are much larger than the variation between proteome and transcriptome.

Taken together, these data indicate that native HUVEC and propagated HUVEC on Xellulin and plastic represent distinct transcriptomic and proteomic phenotypes with a significant degree of similarity between native HUVEC and HUVEC on Xellulin.

**Systematic Analysis of the Significantly Regulated Transcriptome**—To further analyze the underlying gene expression differences, we identified significantly regulated transcripts between the three groups using an ANOVA test (threshold FDR = 0.05) resulting in 7165 differentially expressed transcripts \((57\%\) of all quantified transcripts, Fig. 2A), indicating a massive remodeling of gene expression and cellular physiology between native and propagated HUVEC. K-means clustering of all differentially expressed transcripts revealed six...
(very) large clusters of distinct expression profiles, each associated with distinct functions, cellular components and pathways up- or downregulated in HUVEC on Xellulin and plastic (Fig. 2B, supplemental Table S5). Although most genes exhibit, by and large, similar expression levels between native HUVEC and HUVEC passage 1 on Xellulin (5,804 genes, corresponding to cluster 1, 2, 4, and 5), there is one cluster with genes upregulated exclusively in HUVEC on Xellulin...
(cluster 3) and one cluster with genes generally downregulated in propagated HUVEC (cluster 6).

All K-means clusters have been further enriched with protein interactions and associations using STRING and subjected to an additional round of sub-network clustering using the GLay community clustering algorithm (Fig. 2C, 2D and supplemental Fig. S6). A very large proportion of genes highly expressed in HUVEC on plastic material (2832 or 40%, cluster 1 and 2) is tightly linked to proliferation. The sub-networks are depicted in Fig. 2C and supplemental Fig. S6. These clusters comprise cell cycle and mitosis regulators such as aurora kinase, cyclins and cyclin-dependent kinases, and checkpoint kinases 1 and 2 as well as hundreds of other genes required for proliferation (translation, RNA processing/splicing, DNA replication, energy and building block supply, protein transport). Importantly, these two clusters also comprise hundreds of genes related to ROS detoxification, DNA damage repair (including p53) and unfolded protein response, all of which have the potential to trigger cellular senescence (41), and, hence, phenotypic alterations.

Cluster 3 comprises 547 genes highly expressed in HUVEC on Xellulin. Many of these genes (>150) are secreted and glycoproteins as well as lysosomal and endosomal proteins, including collagen type IV, laminin, and fibrillin. These proteins represent essential components of the basal lamina, which separates endothelial cells from connective tissue.

Cluster 4 and 5 comprise 2945 genes (41%) representing cellular functions which are downregulated or completely lost upon sub-cultivation on conventional plasticware and at least partially preserved on Xellulin. Above all, these preserved functions involve signaling and transcription factor networks. To better understand and visualize the underlying differences of the signaling and transcription factor network, we reconstructed these networks based on cluster 4. Fig. 3 exemplifies this reconstruction and shows that gene expression of important receptors and signaling networks are downregulated on plastic, but not on Xellulin support, and eventually also the expression of numerous key transcription factors of endothelial cell biology, such as ELK3 and 4, FOXO1 and 4, MEF2 and EF2C and various NFATs. However, it is of note that the downregulation of transcripts does not necessarily mean that the proteins are absent (e.g. FOXO1 expression remains unchanged on protein level).

Cluster 6 consists of 789 genes linked to ECM organization, response to wounding, cell adhesion and cell-cell recognition.
These genes are downregulated in both Xellulin and plastic HUVEC, and some examples are tightly associated with endothelial cell functions, such as the BMP-binding endothelial regulator protein (BMPER), the platelet endothelial aggregation receptor 1 (PEAR1) and the endothelial kinase Tie2 (TEK).

These results indicate that Xellulin preserves numerous important functions and signaling mechanisms of native HUVEC, but at the same time exhibits noteworthy differences such as the increased expression of secreted and glycoprotein genes and the decreased expression of a broad array of native HUVEC genes.

**Systematic Analysis of the Significantly Regulated Proteome**—A statistical analysis of the quantitative proteomics data comparing HUVEC cultivated on Xellulin and plastic material supports the observations on transcriptome level and likewise reveals massive differences on proteome level (Fig. 4A). Of the 1771 differentially regulated proteins, 1016 are upregulated in plastic samples, and the vast majority (85%) of these proteins is involved in ribosome biogenesis, protein biosynthesis and cell cycle (Fig. 4B). In contrast, the 755 proteins upregulated in HUVEC cultivated on Xellulin originate from distinct biological functions and cellular compartments (Fig. 4C) including a large cluster of lysosomal proteins and numerous groups of proteins involved in protein secretion, glycan, glycolipid, and glycoprotein biosynthesis, extracellular matrix and cell adhesion. Among them we identified proteins which characterize native endothelial cells and support distinct functional properties of these cells upon angiogenic activation (Fibronectin 1, MMP2, Neuropilin 1 and 2, PDGFB, EPHB2 and numerous downstream signaling molecules), coagulation (vWF, thrombin/coagulation factor II and coagulation factors V, IX, X, XII and thrombin receptor), and inflammation (numerous signaling proteins including AKT3, IKKβ, sphingosine kinase 1, phospholipase C, REL, KRAS) (supplemental Table S4; supplemental Fig. S7). The proteomic phenotypes of propagated HUVEC indicate that cells cultured on Xellulin preserve key features of a quiescent, differentiated
phenotype, including a reduced expression of proliferation-associated proteins.

Comparing Transcriptome and Proteome of HUVEC After 3 and 6 Weeks of Cultivation on Xellulin—Having observed massive differences between native and propagated HUVEC cultivated on plastic and Xellulin, we further analyzed whether the cultivation time of HUVEC on Xellulin influences their transcriptome and proteome profiles, and thus their phenotype. A PCA of the transcriptome of HUVEC cultured on Xellulin for three and 6 weeks does not show a clear segregation of samples according to cultivation time (supplemental Fig. S8A). Additionally, there are no significantly regulated transcripts or proteins (t test, Benjamini-Hochberg corrected p value <0.05) (supplemental Table S3 and S4). Nonetheless, when analyzing the transcriptome data for global, but weaker trends (nonconnected p value <0.05; supplemental Fig. S8B), it appears that proliferation-related processes (supplemental Fig. S8C) are increased after 3 weeks on Xellulin, whereas proteins pointing to a quiescent and differentiated phenotype are upregulated on prolonged cultivation (supplemental Fig. S8D). Among these proteins are numerous negative regulators of proliferation (such as FGF13, NOG, cyclin-dependent kinase inhibitor 1A and PPARγ and KLF4), receptors, signaling proteins and chemokines that orchestrate responses of the immune system (such as TLR4, IRAK4, PELI1, IL8, CX3CL, and CXCL3) as well as autophagy related proteins (such as ATG5, MAP1LC3B, and MAP1LC3B).

Comparison of Absolute Abundance Differences on Transcriptome and Proteome Level—One might argue that the signaling competence of cells on conventional plastic is considerably reduced compared with native HUVEC and HUVEC cells on Xellulin because a vast fraction of the cellular transcriptional and translational capacity is allocated to the synthesis of (abundant) proliferation-associated proteins such as those comprised in cluster 1 and 2. To further investigate this hypothesis, we analyzed the transcriptome and proteome composition as a proxy for the transcriptional and translational capacity involved in different cellular functions using Proteomaps (39) (supplemental Fig. S9). The proteome composition was obtained from pooled Xellulin and plastic samples (n = 6 for each group; see also supplemental Table S1) analyzed by label-free LC-MS/MS and quantified using the iBAQ approach (31).

It becomes apparent—mainly on transcript level—that proliferation-associated processes and functions (i.e. the KEGG categories “Genetic Information Processing” representing DNA maintenance, transcription, translation, and protein folding, sorting and degradation and “Metabolism”) are considerably more abundant in cells growing on plastic material than in native HUVEC and Xellulin samples. In contrast, transcripts related to biological functions important for native cells (“Environmental Information Processing” and “Organismal Systems”) are considerably less abundant in plastic samples supporting the above hypothesis. The remarkable differences on transcript level are mirrored on protein level, but to a much smaller extent (this mainly reflects the fact that the most abundant proteins are expressed on a similar level). Only the category “Organismal Systems” (~29%) shows a strong decrease in abundance, which is represented by two frequently used endothelial cell lineage marker proteins, vWF (8.8-fold downregulated) and the cell surface glycoprotein MCAM (CD146; 3.3-fold downregulated).

Assessment of the Endothelial Differentiation of HUVEC Cultured on Xellulin—The sialomucin CD34 is an important marker of differentiated and quiescent endothelial cells. To validate the native character of HUVEC on Xellulin, CD34 expression was monitored by immunostaining 2 days, one and 3 weeks after passaging from conventional collagen-coated plastic support. After 1 week, the expression of CD34 in both Xellulin HUVEC and conventional plastic HUVEC was low. However, after 3 weeks, nearly all HUVEC on Xellulin were CD34 positive, whereas CD34 expression was not upregulated in HUVEC on plastic culture plates (Fig. 5A). The reexpression of CD34 is consistent with the expression levels in the transcriptomic and proteomic data (Fig. 5B).

To further assess as to whether HUVEC cultured on Xellulin may acquire an activated and migratory phenotype, a scratch assay was performed (Fig. 6A). The artificial wounds created on HUVEC monolayers grown on Xell-Disc or plastic surfaces were comparably closed in both experimental setups within two and 4 days on collagen-coated plastic and on collagen-coated Xellulin, respectively.

In addition, the functional responsiveness of HUVEC grown on Xellulin or plastic to a prototypic pro-inflammatory stimulus was compared. The result of the corresponding experimental approach showed that the TNFα-dependent E-Selectin mRNA expression was significantly pronounced in HUVEC on Xellulin (Fig. 6B).

We also observed spontaneous tube formation in a small number of HUVEC monolayers cultured on Xellulin, indicating that HUVEC on Xellulin are capable of leveraging their angiogenic potential. The tubes were located underneath the HUVEC monolayers. In an immunocytochemical and immunohistological analysis (supplemental Figs. S10–S12) we compared the expression of endothelial-specific proteins in spontaneously formed tubes on Xellulin, with endothelial cells in umbilical veins, HUVEC on plastic, and HUVEC on Xellulin. This demonstrated that the tube forming endothelial cells were clearly positive for angiopoietin-2, CD34, and VE-Cadherin, in addition to PECAM-1 (CD31), and ZO-1.

DISCUSSION

Our transcriptomic and proteomic analysis of HUVEC shows that the phenotypic differences of native and propagated HUVEC on collagen-coated BC or conventional collagen-coated polystyrene material involve significant changes of 57% of the transcriptome and 24% of the proteome.
We investigated the transcriptome of both native and propagated HUVEC to a depth of 12,475 transcripts and the proteome of propagated HUVEC to a depth of 7831 proteins, which represents the most in depth endothelial proteome generated so far. The comprehensiveness of the quantitative transcriptomic and proteomic approach allowed us to shed light on the multitude of phenotypic and physiological differences between native and propagated HUVEC on BC and plastic material.

By and large, our results are consistent with and extend recently published transcriptome and proteome studies of cultured endothelial cells and their composition of the extracellular matrix showing that the properties of the extracellular matrix and the level of confluence have an important impact on their physiology (42–44).

The endothelial cell phenotype is controlled by a variety of factors, such as growth factors, inflammatory cytokines or hemodynamic flow-induced mechanical forces (45, 46). In this context, the analysis of key transcript and protein data related to signaling, cell cycle and endothelial markers suggests that collagen-coated Xellulin but not conventional collagen-coated plastic promotes a stable, quiescent, mature endothelial cell state. This is for instance reflected by the observation that VE-cadherin is significantly upregulated on Xellulin on protein level (compared with plastic), whereas YAP1, a transcriptional co-activator of proliferation associated genes, is consistently downregulated on transcript level on Xellulin and plastic, together contributing to a quiescent state. In vivo, cell-cell contacts composed of adherens and tight junctions play important roles in maintaining the quiescence of an mature endothelial cell monolayer. Vascular endothelial cadherin (VE-cadherin or CD144, encoded by the gene CDH5) is the major adherens junction protein of endothelial cells and stabilizes the vasculature through interactions with growth factor receptors (such as VEGFR2 and TGFBR), thereby activating pro-quiescent signaling mechanisms. As recently discovered, contact inhibition of endothelial cells is mediated by Hippo-Yes-associated protein (YAP) signaling (47), a key regulator of cell growth and organ size restriction (48) via the PI3K-AKT axis.

In addition to molecules organizing cell-cell contacts, integrins, receptor tyrosine kinases and GPCRs have been shown to facilitate sensing of biophysical cues such as substrate stiffness, nanotopography, and mechanical force (49, 50),
by modulating the phenotype of endothelial cells. Adhesion to extracellular matrix proteins is mainly mediated through collagen receptors, such as integrins, discoidin domain receptors (DDR1 and DDR2), LAIR1 (leukocyte-associated immunoglobulin-like receptor 1), mannose receptors such as MRC1, MRC2 and the secretory phospholipase A2 receptor PLA2R. Almost all of the proteins above show similar transcript expression levels in native HUVEC and propagated HUVEC on Xellulin which is contrasted by a considerably reduced expression on plastic support.

Further evidence that Xellulin in fact promotes the development of a mature endothelium as it usually predominates in vivo may be deduced from the analyses of endothelial differentiation markers. For instance, both transcriptome and proteome data revealed higher levels of the endothelial markers PECAM-1 (CD31) and vWF in HUVEC cultured on Xellulin indicating their quiescent state. In fact, vWF expression is detectable in most types of vascular endothelia in vivo (51, 52). However, upon propagation under standard culture conditions, expression and deposition of vWF is gradually decreased (53). Moreover, deficiency of vWF is associated with angiogenic activation of endothelial cells (54).

In line with these observations, transcripts and/or proteins involved in cell cycle progression such as cyclin-dependent kinase 1 (CDK1) or CCN family members (CCNA2, CCNB1, CCND1, CCNE1) were upregulated in endothelial cells propagated on plastic as compared with Xellulin or native environment. As endothelial cell proliferation may still be considered as one of the most reliable indicator of endothelial cell (e.g. angiogenic) activity (55), these observations underline once more the capacity of Xellulin to maintain a quiescent endothelial cell phenotype.

In contrast, HUVEC on collagen-coated plastic show signs of dedifferentiation and endothelial-mesenchymal transition (EndMT). EndMT of endothelial cells is characterized by the loss of endothelial markers (as observed for VE-cadherin, PECAM-1, vWF or CD34 on transcript and/or protein level), loss of cell-cell contacts (such as integrins, Fig. 3), acquisition of invasive and migratory properties (as indicated by increased vimentin transcript expression, supplemental Table S5), and gain of mesenchymal markers, such as transgelin, a smooth muscle differentiation marker (also known as SM22-alpha) (56, 57).

The observations on transcriptome and proteome level are further corroborated by the elevated level of CD34 detected in HUVEC on Xellulin (Fig. 5). Although CD34 is a well-known marker of hematopoietic cells and detectable on endothelial cells in vivo (58), its expression is rapidly lost upon propagation of cultured endothelial cells or their activation (58–61). Experimental settings allowing re-expression of this molecule in vitro mimic environmental conditions promoting the quiescent endothelial cell phenotype (62, 63). In fact, allowing cultured endothelial cells to acquire quiescence appears to be the prerequisite for enhanced and subtle in vivo-like responses to specific stimuli such as the determinants of angiogenesis (64).

Recently, Yang et al. (65) utilized HUVEC cultured on Xellulin to investigate the mechanism by which local delivery of

Fig. 6. Functional cellular assays. A, Scratched HUVEC monolayers. Artificial surface wounds are covered by HUVEC with the same rate both on plastic plates (upper row) and Xellulin (lower row). Scale bars: 400 μm. B, Responsiveness to TNFα stimulation. HUVEC were grown on plastic plates or on Xellulin discs both coated with rat collagen I. The confluent cell monolayer was stimulated with hrTNFα (5 ng/ml) for 3 h and processed for PCR-analysis of E-Selectin expression (*p < 0.05 versus plastic control, **p < 0.01 versus Xellulin control, ##p < 0.01 versus plastic hrTNFα, n = 3, n.d. = not detectable). All data are displayed as mean ± S.D.
Semaphorin-3C suppresses retinal angiogenesis in newborn mice. Semaphorin-3C administration strongly inhibited the formation of new vascular sprouts and disrupted HUVEC monolayer formation on plastic but did not affect fully developed vascular networks in vivo or quiescent HUVEC monolayers developed on Xellulin. These findings suggest that Xellulin supports the development of a quiescent, mature in vivo-like endothelial cell phenotype. HUVEC cultured on Xellulin may, however, switch their phenotype to become activated and migrate as was evidenced by the scratch assay.

We expect that the observations made for HUVEC, smooth muscle cells, fibroblasts, mesenchymal stem cells, and hepatocytes (supplemental Fig. S1B) also hold true for many other (primary) cell types and commonly used immortal cell lines such as HeLa, K562, or HEK 293, rendering Xellulin a promising cell biology tool. Establishing Xellulin-based cell cultures is straightforward, but may require minor modifications to established cell culture protocols such as the use of alternative enzymes (e.g. collagenases instead of trypsin) to detach cells or slight adaptations to staining procedures in case of water-soluble dyes, which may result in an increased staining background. Xellulin also provides new opportunities for pharmacological and toxicological research in vitro, as platform for serial and repeated testing, and eventually as viable alternative to animal-based research approaches.

Our study shows that applying modern transcriptomic and proteomic technologies to established and novel in vitro models is a powerful approach to characterize molecular phenotypic differences and functional associations in endothelial and other cell types and to better understand the effects of the mechanical and regulatory properties of the cellular microenvironment. Our results provide a comprehensive baseline transcriptome and proteome of native and propagated HUVEC cultivated on conventional plastic support as well as on Xellulin, which complements recent transcriptome, proteome and phosphoproteome studies (42–44, 66, 67–72). The present study may form the basis for further exciting insights on Xellulin, which complements recent transcriptome, proteome and phosphoproteome studies (42–44, 66, 67–72). The present study may form the basis for further exciting insights.

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DATA AVAILABILITY

Transcriptome sequencing and quantification results (MACE data) are available through the ArrayExpress database; accession number E-MTAB-4747). The raw mass spectrometric data generated in this study and the MaxQuant analysis files are available via ProteomeXchange (accession number: PXD003975).

* G.F., J.S., J.B., N.K., E.S., and I.H. are employees of Xellutec GmbH. L.J. is an employee and H.R. is an employee and shareholder of GenXPro GmbH. H.H. is managing director of OmicScouts GmbH. H.H. and B.K. are co-founders and shareholders of OmicScouts GmbH. H.H. is managing director of OmicScouts GmbH. L.J. is an employee and R.H. is an employee and shareholder of GenXPro GmbH.

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