IDENTIFICATION OF EXTRACELLULAR MATRIX PROTEINS SUPPORTIVE OF ENDOTHELIAL DIFFERENTIATION

A **THESIS**

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Dedication

This work is dedicated to patients struggling with cardiovascular diseases.

Abstract

Efficient differentiation of pluripotent stem cells into endothelial cells represents one potential means of resolving vascularization issue in the field of tissue engineering. Recently, extracellular matrix proteins (ECM) have been shown potent stimulators of differentiation, including endothelial differentiation. Unclear is how the ECM can exhibit varying signaling stimuli over the course of a 14-day differentiation time scale. Here we test the possibility that an ECM deposited by cells over time could provide the temporal cues needed for endothelial differentiation. Mass spectrometry was employed as a means of measuring changes in deposition of ECM by cells and remodeling with ECM-triggered endothelial differentiation. As a start, the workflow for attaining accurate quantification was optimized. Then temporal dynamics of ECM expression were represented as a heat map and linear graphs of those ECM proteins with unique temporal shifts relative to control cultures. Collagen XVIII, Fibrilin 2, Fibulin 1, Galectin 9, Laminin subunit 5, Nidogen 2 fulfill these criteria and support the possibility that endothelial differentiation spurred by exogenously provided ECM is brought to completion by an evolving ECM composite.

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CHAPTER 1

INTRODUCTION

Successful endothelial differentiation represents one means to promote vascularization, which is the main goal of the tissue engineering field. Vascularization is important for tissue, as it provides cells with oxygen, nutrients and removes waste materials[1]. Overall, development of the circulatory system is critical for growing organism, since it provides developing tissues with oxygen and nutrients as well as eliminates products of metabolism[2]. For any cell to survive, the proximity to blood vessel should not be further than 200 μm[3]. Endothelial cells comprise the primary cell type of the luminal surface of all vessels of the body. One means to garner large populations of endothelial cells is via differentiation of pluripotent stem cells to this mature phenotype.

Pluripotent stem cells are known for their self-renewal ability and capability to differentiate into cells of all 3 germ layers[4]. Since the successful generation of induced pluripotent stem cells (iPSCs)[5,6] reprogramming of somatic cells to the state of pluripotency makes production of patient-specific endothelial cells possible with potential of use in transplantation, disease model and drug screening studies[7]. The ability to differentiate into various cell types makes iPSCs valuable for tissue regeneration and cell therapy studies. In the current study, murine iPSCs were used to investigate cues required for endothelial differentiation.

Differentiation of endothelial cells from human iPSCs has been achieved via temporal stimulation with soluble factors that modify components of canonical Wnt signaling pathways. It is known that newly arising endothelial cells during embryogenesis are

induced from mesoderm[8]. And mesoderm can be derived from pluripotent stem cells via Wnt signal pathway activation[9,10]. The activation of Wnt signaling pathways is mainly achieved via use of soluble factors, such as CHIR or GSK3 inhibitor[11,12]. Therefore, existing endothelial differentiation protocols utilize combination of various soluble factors and growth factors (Fibroblast Growth Factor 2 (FGF2) and Bone Morphogenetic Protein 4 (BMP4)) to induce mesoderm specification[13-15] and growth factors, such as Vascular endothelial growth factor (VEGF) to further induce endothelial lineage commitment[16,17]. However, efficiency of such protocols and homogeneity of derived endothelial cell populations requires significant improvement. Reports of efficiency of various chemically defined endothelial differentiation protocols vary from 50% to 60%[18,19], but in practice is rarely greater than 40%.

Hence, the motivation behind current work is the potential of utilization of insoluble factors, mainly family of ECM proteins, to guide endothelial differentiation. The advantage of the use of insoluble factors is in the possibility of creating a regulated and controllable system for endothelial lineage commitment. Since ECM proteins are not susceptible to diffusion, it allows one to create more controllable and regulated system. Also, it is important to create more reproducible culture conditions for differentiation, that could reveal key interactions required for guided differentiation[20.] It was shown that certain formulations of such insoluble factors lead to efficient cardiac differentiation[11]. Also, use of ECM proteins as signaling molecules is advantageous to structurally support tissue development[21]. Therefore, the current study aims to understand the ways that specific ECM proteins can be used to trigger endothelial differentiation.

Furthermore, motivation behind this project is strengthened by preliminary results from work conducted by PhD student, Mikayla Hall (Fig.1). Immunocytochemistry analysis of 2D endothelial differentiation of miPSCs demonstrated that from 5 tested ECM proteins used as coating for cell growth: Collagen I, Collagen IV, Laminin 111, Laminin 411 and Laminin 511, coating with Laminin 411 resulted in significantly higher number of cells, staining positive for CD31 by Day 14. Whereas, Collagen I showed the lowest number of CD31-positive cells. In other words, the outcome of 2D differentiation study revealed higher efficiency in production of endothelial cells when seeding miPSCs on Laminin 411-coated wells and low endothelial differentiation on Collagen I-coated plates. It should be noted that these studies did not employ soluble factor signaling of any kind and yet still could yield proportion of endothelial cells exceeding 40% of the total cell number.

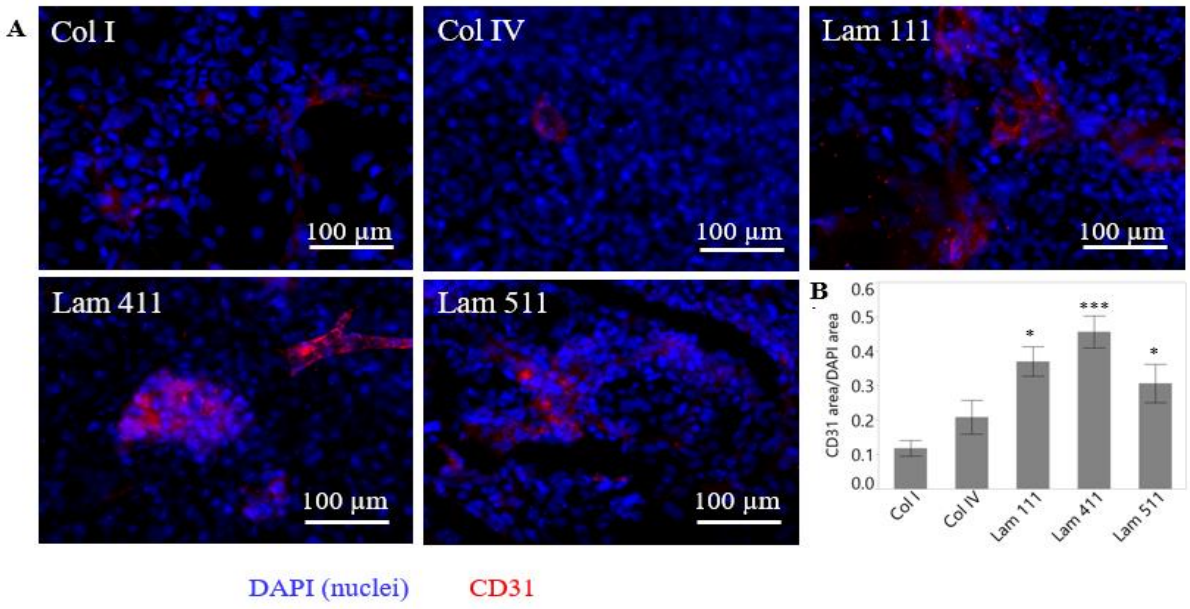

Figure 1. *Unpublished data from study, conducted by PhD student, Mikayla Hall.* ***A.****Images of immunostaining against CD31 from 2D endothelial differentiation protocol on Day 14 with 5 different ECM conditions: Collagen I, Collagen IV, Laminin 111, Laminin 411 and Laminin 511.* ***B.*** *quatitative comparison of CD31 fluorescence intensity, normalized by DAPI.* *P<0.05 **P<0.001

Moreover, these results are further supported by previously conducted work in this field, demonstrating that differentiation of pluripotent stem cells can be induced or inhibited via signaling cues of ECM proteins[22]. And, specifically, induction of endothelial cells with use of ECM proteins was demonstrated in animal models[23].

Based on work conducted by Mikayla Hall, we concentrated on two types of ECM proteins: Laminin 411 and Collagen I. Laminins constitute basement membrane of blood vessels[24]. The network created by laminins provides biological activity to blood vessels[25]. α4 and α5 chains of laminins are expressed in vascular endothelial cells and studies showed them to organize into laminins 411 and 511 by associating with laminin β1 and γ1 chains[26-28]. Laminin 411 was chosen for the current experiment since it is known to be found in basement membrane of various vessel types at distinct stages of their growth, unlike Laminin 511, which is present at later stages of growth of some types of blood vessels[27]. Moreover, there was another recent study demonstrating that Laminin 411 can be used to successfully guide pluripotent stem cells to endothelial commitment in the presence of soluble factors including VEGF[29]. Collagen I was used as a negative control (i.e., ECM engagement without endothelial specification). Type I collagen is known to be present in dense connective tissue types: bone, tendon and skin. It is comprised of two al(l)-chains and one a2(l)-chain[30,31]. Moreover, Type I collagen increases the expression of stem cell-associated markers according to other studies[32]. This knowledge and results from unpublished data (Fig.1B) makes Collagen I an ideal negative control for our study.

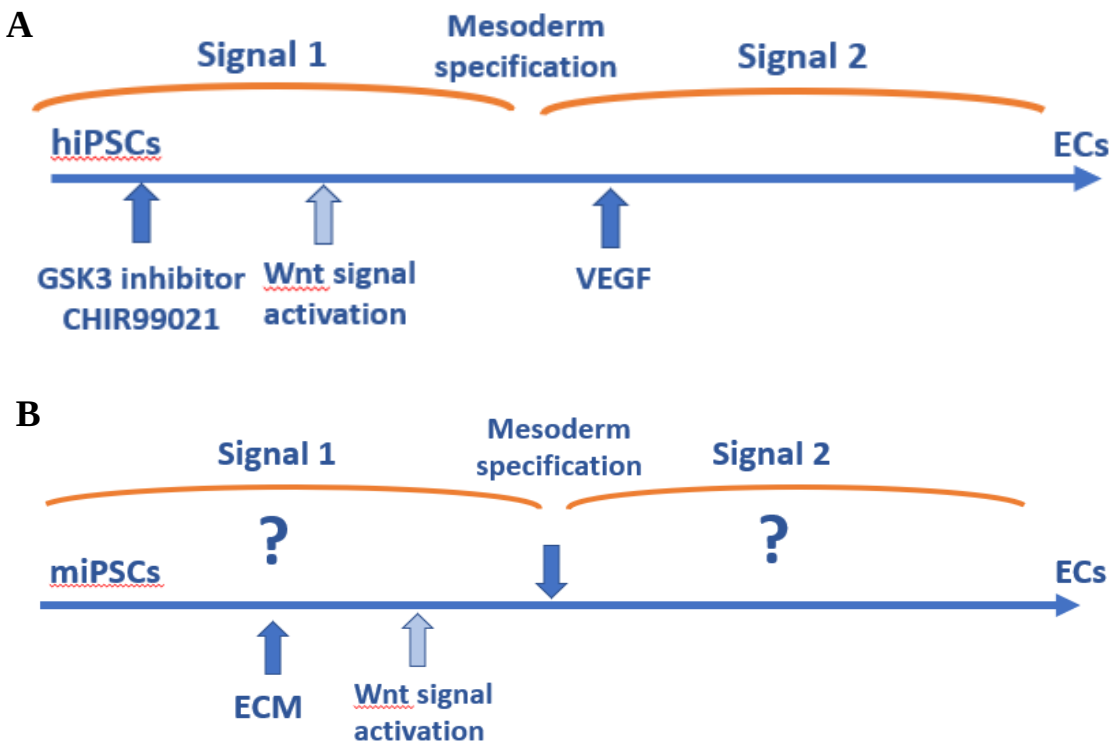

Figure 2**. Endothelial lineage commitment occurs via Wnt signal activation and subsequent mesoderm specification.* ***A. *Differentiation protocol of hPSCs to Endothelial cells via use of soluble factors: GSK3 inhibitor CHIR99021 and VEGF (Lian et al., 2014).* ***B.*** *Differentiation protocol of miPSCs to Endothelial cells via use of insoluble factors (ECM proteins).*

Extracellular matrix proteins provide essential external signals for cell adhesion, proliferation and migration[33]. Moreover, ECM is not just acellular structure surrounding cells of tissue and providing it with physical support, but also continuously remodeling cell microenvironment and signaling[21,34,35]. In this project I aimed to investigate composition and temporal dynamics of ECM remodeling at various stages of endothelial differentiation of miPSCs to elucidate signal specific ECMs, leading to mesoderm switch and further to endothelial cell commitment.

By looking at established protocols of endothelial differentiation that use soluble factors as signaling cues for Mesoderm specification and production of endothelial cells (Fig.2A)

we are interested in elucidation of possible signals that deposited/degraded ECM proteins can provide (Fig.2B) Therefore, we hypothesize that exogenous ECM proteins provided at the initiation of endothelial differentiation spur deposition or degradation of additional ECM proteins over the 14 day differentiation period providing dynamic signaling cues that guide endothelial specification. For this purpose, miPSCs were seeded on Laminin 411 and Collagen I-coated wells, grown and allowed to differentiate over the period of 14 days during which time deposited ECM proteins were analyzed using Mass Spectrometry tool at specified intervals.

CHAPTER 2

METHODS

Current study investigated evolvement of ECM proteins over 14-day ECM-induced differentiation protocol of miPSCs into endothelial cells. The workflow for subsequent experiment was established with use of Fibronectin-coated plates and human umbilical venous endothelial cells (HUVECs). Samples were decellularized at distinct time points and ECM depositions were analyzed with Mass Spectrometry tool. The subsequent experiment was implemented with miPSCs on Laminin 411- and Collagen I- coated plates. Changes in deposited ECM proteins were summarized in constructed heatmaps, linear graphs and pie charts.

Plate coating

In the first experiment, a 96-well plate was coated with Fibronectin (Corning, Human plasma), which had been dissolved in deionized water at a concentration of 0.1 mg/mL. 8 wells were coated, 4 each for a Fibronectin condition and a Fibronectin plus HUVECS condition. The plate was parafilmed and kept at 4ºC overnight. The next day, wells for HUVEC seeding were washed twice with sterile 1X Phosphate Buffered Saline (PBS - Fisher Bioreagents, Thermo Fisher Scientific) before seeding of the cells. HUVECs were trypsinized (0.05%) for detachment and seeded at a density of 1200 cells/well.

In second experiment total of 6 x 96-well plates were coated with 4 wells each of Collagen I and Laminin 411. The setup for each plate is shown in Figure 2. Collagen I was used as a negative control for endothelial differentiation and previous work in the Ogle laboratory has shown Laminin 411 to be important to endothelial differentiation

(unpublished). One plate of samples was decellularized at distinct time points: Day 1, Day 3, Day5, Day 10 and Day 14. These time points were chosen to embrace early,

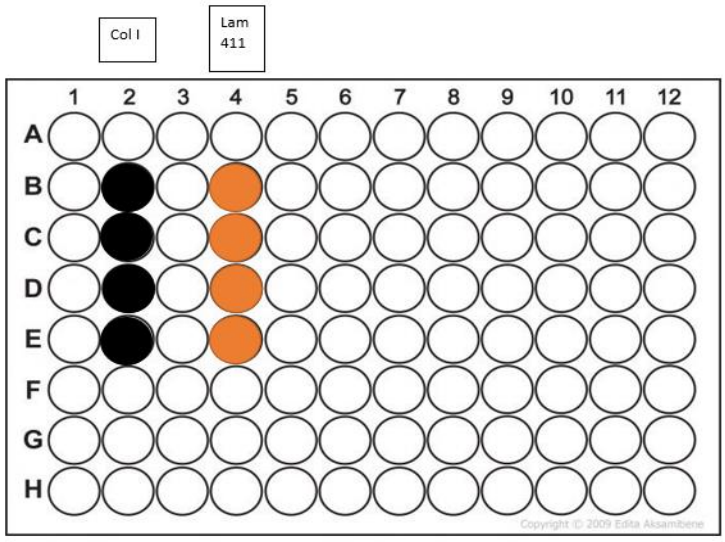

Figure 3*. Experiment plate set-up for Mass Spectrometry analysis and staining. Black wells show Collagen I-coated wells and orange wells show Laminin 411-coated wells.*

middle and later stages of endothelial differentiation. Plate 6 was used for immunostaining against cell-surface protein CD 31.

ECM protein solutions for coating of 48 wells were prepared as follows: 38.4µL of Laminin 411 (BioLamina, human rLaminin-411) at a concentration of 0.1 mg/mL was dissolved in 41. 6mL DPBS. 44.8µL of Collagen I (Corning, rat tail) at a concentration of 3.8mg/mL was dissolved in 47.6mL of 0.1M acetic acid. A total of 6 plates (5 for mass spectrometry and 1 for staining) were coated, parafilmed and kept at 4ºC overnight. The next day the plates were washed twice with 1X PBS before seeding of the cells.

Cell culture

HUVECs were maintained in a T75 cell culture flask and fed with fully supplemented EGM-2 media (Lonza).

miPSCs were acquired from Dr. Deepak Srivastava (Gladstone Institute of Cardiovascular Disease, University of California, San Francisco). They were derived according to the following protocol[36]: the tail tip was acquired from 8-week old male NKX2.5-GFP mice[107], cleaned with ethanol, washed in PBS, chopped into small pieces, and placed in collagenase IV/trypsin 0.25% solution at 37 °C for one hour with 3 breaks for additional rounds of chopping. Obtained tissue was cultured in DMEM/F12 (+glutamine +HEPES +penicillin-streptomycin) with 10% FBS to isolate fibroblasts as described[6]. Modified induction of pluripotency by retrovirus-mediated transfection with four factors (Oct-4, Sox-2, Klf4, c-Myc) was performed[6] as follows: feeder-free gelatinized culture plates at induction and maintenance were used for iPSC growth. Cells were fed with Glasgow Minimum Essential Medium (GMEM) supplemented with glutamine, 0.1 mM MEM non-essential amino acids, sodium pyruvate, 10%(v/v) fetal bovine serum (characterized, Hyclone), a 1:1000 dilution of beta-mercaptoethanol stock solution (0.35% made from Sigma M7522), and 500–1000 units per ml of leukocyte inhibitory factor [Chemicon ESG1107]. Valproic acid (2 mM, Calbiochem) was added from day 2–9 post infection.

After miPSCs were thawed, they were seeded in a 0.1 % Gelatin-coated 6-well plate in modified GMEM minimum medium (Gibco Glasgow's MEM - Thermo Fisher Scientific) supplemented with 10% [v/v] fetal bovine serum (Gibco, Thermo Fisher Scientific), 1% L-glutamate [v/v] (Gibco, Thermo Fisher Scientific), 1% MEM non-essential amino acids [v/v] (Gibco, Thermo Fisher Scientific), 0.7% betamercaptoethanol [v/v] (MP Biomedicals), 0.08% glycine [w/v] (Sigma-Aldrich) and leukocyte inhibitory factor (2 µL per mL of medium). The next day, cells were split and

seeded at a density of 35,000 cells per well in a 6-well gelatinized plate in the same media conditions. They were fed with modified GMEM media 2 days later and split the next day to be used in the differentiation experiment.

For differentiation of miPSCs into endothelial cells, the media was GMEM minimum supplemented with 10% [v/v] fetal bovine serum, 1% L-glutamate [v/v], 1% MEM non-essential amino acids, 1% sodium pyruvate [v/v] and 1% betamercaptoethanol [v/v].

Endothelial differentiation

The protocol for endothelial differentiation was modified based on a previously developed protocol[11]. The endothelial differentiation takes 14 days. 96-well plates were coated with ECM, parafilmed and kept at 4ºC overnight. The next day the plates were washed twice with PBS before seeding the cells. The next day, ECM-coated 96-well plates were seeded with miPSCs at a density of 1200cells/well. Samples were fed every day with GMEM minimum media as described above.

Decellularization method

The method used in this study is modified from a protocol originally developed by Chen et al.[37]. Cell cultures were treated with hypotonic solution of nonionic detergent and washed multiple times with buffers[38]. First, media was removed from cell culture wells and they were rinsed twice with 200µL 1X PBS. Then cells were washed 3 times with 200µL of Wash buffer 1 (100mM Na_2HPO_4, 2mM $MgCl_2$, 2mM EDTA, pH 9.6). Wash buffer 1 contained Na_2HPO_4 known to be an alkaline solution. EDTA, other component of Wash buffer 1 was used to detach cells from ECM. After that, 200µL of Lysis buffer (8mM Na_2HPO_4, 1% Triton, pH 9.6) was applied and samples were incubated at 37ºC for

15 min to increase the rate of reaction. Active component of Lysis buffer, Triton, is a detergent used for cell lysis. After 15 min, fresh Lysis buffer was added and incubated at 37ºC for 1 hour. Cell removal from samples was checked under microscope (Fig.A1, A2). Finally, Lysis buffer was removed, and samples were washed 3 times with 200µL of Wash buffer 2 (10mM Na_2HPO_4, 300mM KCl, pH 7.5) to prevent non-specific binding of proteins. Then the samples were washed 4 times with deionized water to remove residual cell debris and solutions. Plates were allowed to air dry, then parafilmed and frozen at -20ºC.

Preparation of samples for Mass Spectrometry

Protocol for sample preparation was modified from *"In-solution tryptic digest protocol for proteomics"* (website of Department of Chemistry, University of Oxford)[39]. Buffers for trypsinization were prepared freshly on the experiment day: 0.4M Tris, 6M Urea, 200mM DTT (in Tris), 200mM iodoacetamide (in Tris), 1mM $CaCl_2$, Trypsin solution (concentration of 0.2µg/µL).

Plates were taken out of the freezer and left for 20 mins at room temperature (RT). 25µL of 6M urea was added to each well. 1.25µL of 200mM dithiothreitol (DTT) was added to each well. Each well was pipetted to mix solutions. Plates were incubated for 1 hour at RT. 5µL of 200mM iodoacetamide was added to each well and pipetting was performed at each well to mix. Plates were incubated for 1 hour at RT in the dark. 5µL of 200mM DTT was added to each well and pipetted. Plates were incubated for 1 hour at RT in the dark. 113.75 µL of 1mM $CaCl_2$ was added to each well and pipetted for mixing. pH was checked and adjusted to 7.8-8.7 (for trypsin activity) with addition of 2µL increments of 5M NaOH solution until desired pH was accomplished. 5µL of trypsin (at concentration

of 0.2µg/µL) was added to each sample and pipetted for mixing. All plates were incubated overnight at 37ºC. The next day, samples were collected from the plates and transferred to 1.5 mL Eppendorf® LoBind microcentrifuge tubes. The tubes were then frozen and lyophilized.

Protein purification

Samples were acidified by adding Formic Acid, 99.5+% (Optima™ LC/MS) until a pH of 4 or less. Purification was performed by STAGE (STop And Go Extraction) TIPS desalting procedure[40]. The stage tips used for purification were assembled as follows. C18 Empore reversed-phase extraction disks (SDB-XC reversed-phase material, 3M product number 2240/2340) were placed on a clean, hard surface and a 17 or 18-gauge, blunt-ended syringe needle was pressed onto it to core out a piece of the filter material. This was done twice to create 2 cores in the syringe needle, as a double layer of filter material added extra loading capacity. After that, the needle was placed into a 200 µL pipette tip and the disks were pushed with PEEK or fushed silica tubing to release them into the pipette tip. Finally, the filter material was gently packed into the end of the pipette tip being careful to leave a gap of several millimeters between the disk and the end of the tip. The estimated binding capacity per core is 2-4 ug.

Stage Tip/Tube constructs were assembled as follows: a cap was removed from a 1.5 mL Eppendorf tube and a hole was made in the center of the cap. This cap was placed onto a new 1.5 mL Eppendorf® LoBind microcentrifuge tube and the assembled pipette tip with Empore disk cores was placed into the hole in the cap with the tip of the pipette approximately 1 cm from the bottom of the tube. 20 assemblies were prepared for all samples.

Solutions for purification were prepared freshly at given ratios: *Solution 1*: Wash solvent: 98:2:0.1%, water:acetonitrile:formic acid (FA); *Solution 2*: Wetting solvent: 80:20:0.1%, acetonitrile:water:formic acid (FA); *Solution 3*: Elution solvent: 60:40:0.1%, acetonitrile:water:formic acid (FA).

Lyophilized samples were reconstituted in 60 µl of wash solvent, vortexed for 45 seconds and centrifuged at 5000 x g for 1 min. pH was checked using pH strips and adjusted to be ≤3 by using 99.5% aqueous FA. Then 60 µl of wetting solvent was pipetted onto the Stage Tip/Tube assemblies and tubes were centrifuged at 5000 x g for 30 seconds. 60 µl of wash solvent was pipetted onto the Stage Tip/Tube assemblies and centrifuged at 5000 x g for 30-45 seconds. Liquids in the bottom of Eppendorf tube were discarded. Next, samples were pipetted into the Stage Tips and centrifuged at 5000 x g for 30-45 seconds. Then, in order to wash trapped peptide samples, 60 µl of wash solvent was pipetted onto the Stage Tip/Tube assemblies and they were centrifuged at 5000 x g for 30-45 seconds. This step was repeated. Finally, cap/Stage Tip assemblies were placed onto new 1.5 mL Eppendorf® LoBind microcentrifuge tubes, labelled respective to samples. Peptides were eluted from C18 material by pipetting 60 µl of elution solvent onto the Stage Tip/Tube assembly. Tubes were centrifuged at 5000 x g for 30-45 seconds. Peptide mixtures were then frozen and lyophilized.

Mass Spectrometry

Samples were analyzed at the Mass Spectrometry facility in the Masonic Cancer Center's Analytical Biochemistry Shared Resource at the University of Minnesota. Samples were run on the Orbitrap Elite™ Hybrid Ion Trap-Orbitrap Mass Spectrometer, which is a

liquid chromatography-mass spectrometry (LC-MS) system. On overall, samples with volume of 10µL were analyzed.

Data analysis

Data was run in the Proteome Discoverer™ Software against the mouse protein database as obtained from UniPprot to reveal the list of cellular, nuclear and ECM proteins in each sample. Since large number of detected peptides is being compared to a large database of proteins, there is some probability of identifying a protein which is not actually present in the sample. Therefore, it is necessary to establish a cutoff for the peptide match to avoid including proteins that were incorrectly identified. There are tools used to evaluate the peptide-spectrum match (PSM) in Mass Spectrum analysis. Statistical significance of the PSM can be measured using either the false-discovery rate (FDR) or the Posterior Error Probability (PEP) scores[41]. It was decided to use the sum PEP scores acquired from Proteome discoverer to eliminate incorrectly identified peptides. The PEP score itself describes the probability that the matched peptide is incorrect, whereas the sum PEP score is the negative logarithm of the corresponding PEP value of the related PSMs[42]. Based on this only samples with a sum PEP score greater than 3.0, corresponding to PEP score of 0.5, were included. ECM proteins were manually identified and listed in separate document.

Next the method developed by Ishihama et al.[43] was used to estimate the absolute protein concentration. In their paper, Ishihama et al.[43] reported that the exponentially modified protein abundance indexes (emPAI) are proportional to the fraction percentages of respectful proteins. Protein abundance index is defined as the ratio of the number of

observed peptides per protein to the number of observable peptides per protein[44] and emPAI is given as follows:

$$emPAI = 10^{PAI} - 1$$

The corresponding protein molar fraction percentages are calculated by multiplying the ratio of emPAI of given protein to total emPAI by 100. These calculations were performed for each given ECM peptide, both with total emPAI values of all acquired proteins, including cellular and nuclear and with total emPAI values of ECM proteins only. Since amount of cellular and nuclear proteins is variable because we remove it, ECM emPAI values were calculated in case the results are infiltrated by total proteins.

Excel spreadsheets for each sample were analyzed to isolate data relevant to ECM proteins only. Furthermore, all the data with sum PEP scores below 3.0 was excluded. Then, overall emPAI ($emPAI_{tot.prot}$) and total emPAI of ECM proteins only ($emPAI_{tot.ECM}$) were calculated for each sample. Two sets of data emPAI/$emPAI_{tot.prot}$ and emPAI/$emPAI_{tot.ECM}$ were created. Both sets of data were multiplied by 100 to obtain molar concentrations[43]. Heatmaps and pie charts were constructed according to obtained molar fraction percentages.

Cell Fixation

Media from Plate 6 was removed, and samples were washed with 1X PBS. Then samples were incubated in 4% Paraformaldehyde (PFA) for 15 minutes at RT. Samples were washed twice with PBS and kept in PBS at 4ºC until staining procedure was applied. PFA is known to be a cross-linking agent that reacts with cell proteins and nucleic acids[45,46]. Fixation method by PFA is preferred because it leads to low shrinkage levels and

preserves cellular structure in good shape[47]. Finally, PFA does not lead to significant structural changes in proteins[48].

Immunocytochemistry

Plate 6 was stained on Day 14 of differentiation. Triton-x 100 (0.2%- prepared by addition of 20 µL to 10mL non-sterile PBS) was added to the samples in plate 6 and incubated at RT for 1 hour. Then, Triton was removed and blocking buffer (0.2% dry milk in Triton 100X) was added for reduction of background interference. After 2 hours of incubation at RT the samples were washed 3 times with PBS. Next the Anti-CD31 primary antibody (Abcam -28364) was added to all samples, except for one which was kept as a secondary antibody only control. Plate 6 was parafilmed and incubated at 4ºC overnight. The next day 0.2% Tween-20 was added and incubated at RT for 5 min. This step was repeated twice. Samples were then washed twice with PBS. The secondary antibody, Goat anti-Rabbit, Alexa Fluor 647 (Invitrogen™), was added and samples were incubated at RT for 1.5 hours. The secondary antibody was removed, and samples were washed with 0.2% Tween-20 twice. Then samples were washed with PBS twice. 2% 4′,6-diamidino-2-phenylindole (DAPI - 20 µL of DAPI dissolved in 1mL DI water) was added to stain for DNA and the plate was incubated at RT for 15 min. Finally, DAPI solution was removed and samples were washed with PBS twice. Samples were imaged using a Leica microscope and acquired images were analyzed using ImageJ software. Cy 5 channel (red) was used to visualize CD 31 staining and DAPI channel – to visualize nuclei staining. Images were taken at adjusted exposure time for DAPI and Cy5 respectively. Fluorescence intensities were calculated as area for DAPI and Cy5 channel images from Collagen I-coated samples and of Laminin 411 samples (Table 1).

Collagen I images	DAPI area	Cy5 area	Cy5/DAPI	Laminin 411 images	DAPI area	Cy5 area	Cy5/DAPI
1	27.215	0.068	0.002499	**1**	38.504	0	0
2	39.274	0.109	0.002775	**2**	43.567	0	0
3	24.661	2.028	0.082235	**3**	25.177	2.273	0.090280812
4	34.217	0.445	0.013005	**4**	30.682	8.556	0.27886057
5	54.093	2.106	0.038933	**5**	34.995	10.034	0.286726675
6	45.784	0.286	0.006247	**6**	40.193	4.551	0.113228672
7	37.806	0.117	0.003095	**7**	40.673	6.886	0.169301502
8	52.464	0.096	0.00183	**8**	36.369	5.064	0.139239462
			Ave: 0.018827	**9**	39.405	22.041	0.559345261
			StdDev.S: 0.02848				Ave: 0.181886995
							StdDev.S: 0.17468493

Table 1. *Areas of DAPI and CD 31 staining (Cy5 channel) for 8 images from Collagen I-coated samples and 9 from Laminin 411-coated samples. Approximation for percentage of CD31-positive cells was calculated as ratio of Cy5 area/DAPI area.* ***Ave.*** *– average of areas of samples; error corresponds to* ***StdDev.S*** *– standard deviation of samples.*

The intensity of CD31 – positive cells was approximated by normalizing area of CD31 to DAPI area (Fig.4).

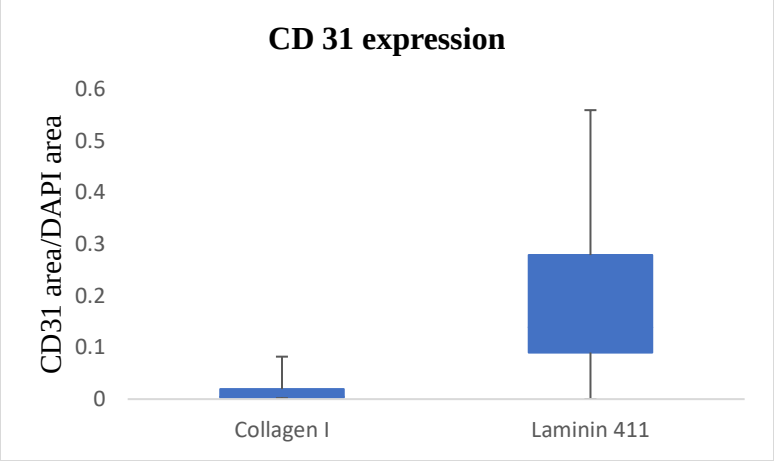

Figure 4*. Approximations for percentage of CD31-positive cells were calculated as ratios of areas of CD 31 staining (Cy5) to DAPI for 8 images from Collagen I-coated samples and 9 – from Laminin 411-coated samples, and presented by a box and whisker plot.*

CHAPTER 3

RESULTS

Workflow establishment

To validate the workflow for extraction of ECM proteins, associated purification, and preparation for Mass Spectrometry analysis, human umbilical vein endothelial cells (HUVECs) were seeded in multiwell plates coated with the ECM protein Fibronectin. HUVECs were chosen as a surrogate for iPSC-derived endothelial cells that is easier to handle and cheaper to expand and Fibronectin was chosen because it promotes robust adhesion and proliferation of cells. The overall experiment set up is shown in Figure 5. 96-well plates were coated with Fibronectin and left overnight. The next day, HUVECs were seeded in half of the wells and the other half were left cell-free as a control for Fibronectin and associated proteins that might have accompanied the "purified" protein from a commercial source. The day after seeding, decellularization was performed on samples with HUVECs. Next, all samples were trypsinized (including those without

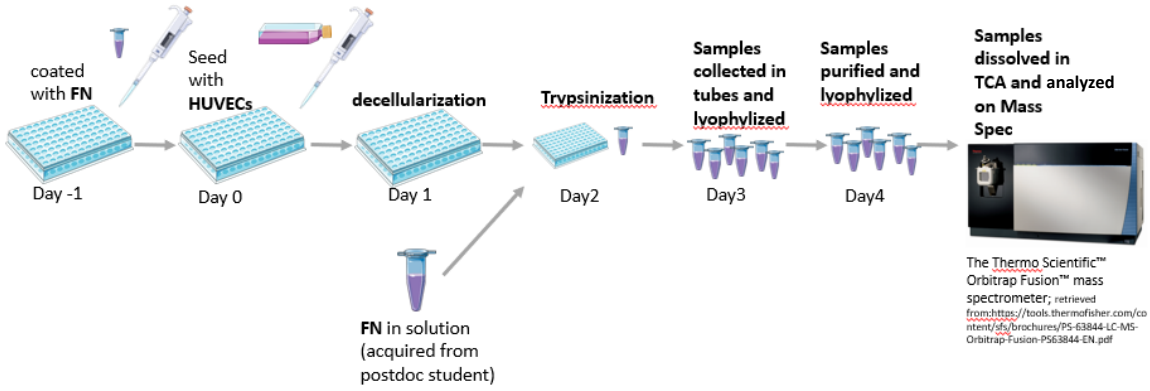

Figure 5. *Experiment 1 set-up. Plates were coated with Fibronectin, HUVECs were seeded the next day. One day later, samples were decellularized, trypsinized, collected, purified and analyzed on Mass Spectrometer.*

cells), ECM collected and run on the Thermo Scientific Orbitrap Fusion Mass Spectrometer at the Analytical Biochemistry facility in Masonic Cancer Center at University of Minnesota.

Raw data obtained from Mass Spectrometry analysis was run against the human Uniprot database in Proteome Discoverer and manually analyzed in Microsoft Excel. Obtained data demonstrated the presence of different cellular, nuclear and ECM proteins. Samples with Fibronectin only (Fibronectin-coated plates and Fibronectin in solution) contained some nuclear and other ECM proteins, revealing that commercially available Fibronectin is not completely pure, likely because it was purified from human plasma. This could explain presence of fibrinogen, serum albumin and immunoglobulins in the sample with Fibronectin only (Table A21). Keratin was also detected in the Fibronectin control samples (Table A21) and could reflect sloughed skin cells during preparation of samples for Mass Spectrometry analysis. Table 2 below summarizes ECM proteins observed in

Fibronectin-coated plates without cells	**Fibronectin in solution without cells**	**Fibronectin + HUVECs**
Fibronectin	Fibronectin	Fibronectin
Vitronectin	Extracellular matrix protein 1	Thrombospondin-1
	von Willebrand factor	Periostin
	Fibulin-1	Collagen alpha-1(IV)
		Collagen alpha-1(I)
		Collagen alpha-2(IV)
		Collagen alpha-1(XVIII)
		Collagen alpha-2(I)
		Collagen alpha-1(VI)

Table 2. *List of ECM proteins observed in samples of Fibronectin-coated plates only, Fibronectin in solution and samples of HUVECs grown on Fibronectin-coated plates.*

Fibronectin-coated, Fibronectin in solution and Fibronectin with HUVECs samples.

Of the two tested samples, one of the Fibronectin-coated wells seeded with HUVECs, revealed ECMs, that were produced endogenously by the cells, since they were not observed in samples with Fibronectin only (Table 2). The replicate sample (Fibronectin + HUVECs) did not show as many endogenously produced proteins but preparation of this sample suffered sample loss at the purification step. Thus, we confirm the workflow for purification of ECM from decellularization matrices demonstrating possibility of detection of endogenously produced ECM.

Identification of ECM proteins evolving over time

Having validated the overall workflow, we sought to evaluate ECM proteins deposited over 14 days of differentiation of miPSCs into endothelial cells. The differentiation protocol used was itself ECM-based. The Ogle lab has shown that ECM proteins provided exogenously and without further augmentation with exogenous growth factor or small molecules can induce differentiation to cardiac cell types including endothelial cells. ECM-driven differentiation to endothelial cells has been shown via the work of Mikayla Hall, graduate student in the Ogle Lab (Fig.1B). Her work identified the ECM stimulus without use of additional soluble factors. In this context it was observed that Laminin 411 was best condition for differentiation of miPSCs into endothelial cells, whereas Collagen I showed the lowest efficiency of endothelial differentiation. Differentiation in this context occurs over the course of 14 days, but the initial ECM stimulus is only provided on the first day. It would be valuable to discern subsequent signals that contribute to ECM-guided differentiation. One possibility is that the initial ECM stimulus triggers endogenous production of ECM

that further enables endothelial differentiation. This is the hypothesis to be tested here. To this end, differentiation conditions wherein exogenous Laminin 411 provides the initial differentiation signal was set as the "high" endothelial differentiation condition and exogenous Collagen I was set as the "low" endothelial differentiation condition, or, in other words, as negative control, since it did not give the desired differentiation response. After seeding of iPSCs on each ECM substrate, ECM composition was assessed at distinct time points during endothelial differentiation (Fig.6). The time points included early (1st and 3rd days), middle (5th day) and later stages (10th and 14th days) of endothelial differentiation. For this, six 96-well plates were used. 4 wells of each plate

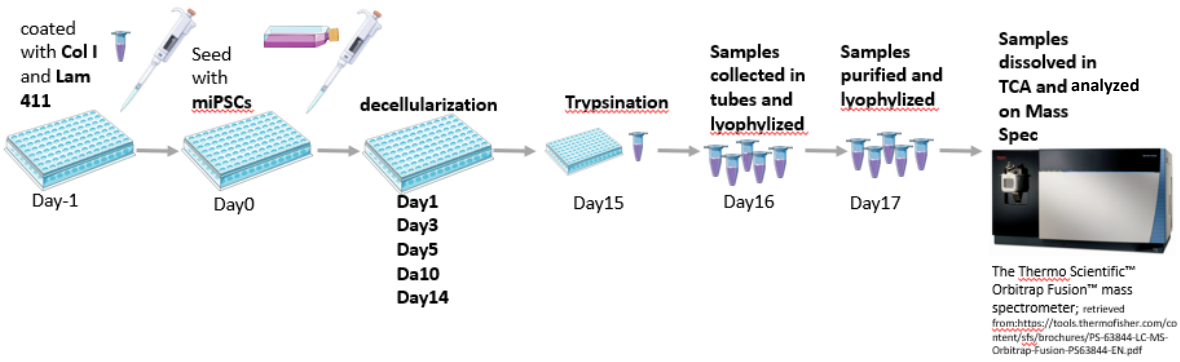

Figure 6. *Experiment 2 set-up. 4 wells of 96-well plates were coated with Collagen I and 4 plates- with Laminin 411. The next day, 5 plates were seeded with mIPSCs at density of 1200 cells/well. Plates were decellularized at following time points: Day1, Day3, Day 5, Day 10 and Day 14; Later, samples were decellularized, trypsinized, collected, purified and analyzed on Mass Spectrometer.*

were coated with Collagen I and 4 other wells with Laminin 411.

They were seeded with miPSCs the next day. There was 1 plate for each given time point: Day 1, Day 3, Day 5, Day 10 and Day 14. A sixth plate was prepared for staining for the endothelial cell-surface marker CD31 on Day 14 of the endothelial differentiation protocol to ensure efficient differentiation in the Laminin 411 condition and limited differentiation in the Collagen I condition (Fig.A1). At each time point 2 samples were

prepared for Mass Spectrometry analysis. Data was analyzed with use of Ishihama's method[42]. Calculated molar fraction percentages (Table A1-A20) were plotted in the form of heatmaps (Fig.7, A3). The heatmap in Figure 7 is constructed based on calculations of molar fraction percentages of ECM proteins only. In this case, since data is not being diluted by presence of all other cellular and nuclear proteins, change trends of relative amounts of ECM proteins can be more readily visualized.

In evaluating the mass spectrometry data, changes not only in ECM differentially produced in the samples exposed to Laminin 411 vs. Collagen I, but also to discern profiles that would seem to be bi or tri-phasic thus perhaps corresponding to various shifts in the differentiation process.

As shown in Figure 7, Day 1 Collagen I-coated samples demonstrated highest amounts of Collagen I subunits, Fibronectin, Metalloproteinase inhibitor 3 and Thrombospondin I. On Day 1 Collagen I subunits and Thrombospondin I remained in molar fraction percentages through Day 3 as well. Amounts of Fibronectin remained high through all 5-time points for both conditions, except for Day 3 in Collagen I-coated sample. The majority of ECM proteins seemed to appear on Day 14, with Fibronectin and BMSHSPCP demonstrating the highest molar fraction percentages, as depicted by intensities of colors on the heatmap. Almost the same amount of basement membrane-specific heparan sulfate proteoglycan core protein (BMSHSPCP) was observed in both Collagen I and Laminin 411 conditions from Day 5 to Day 14, although for

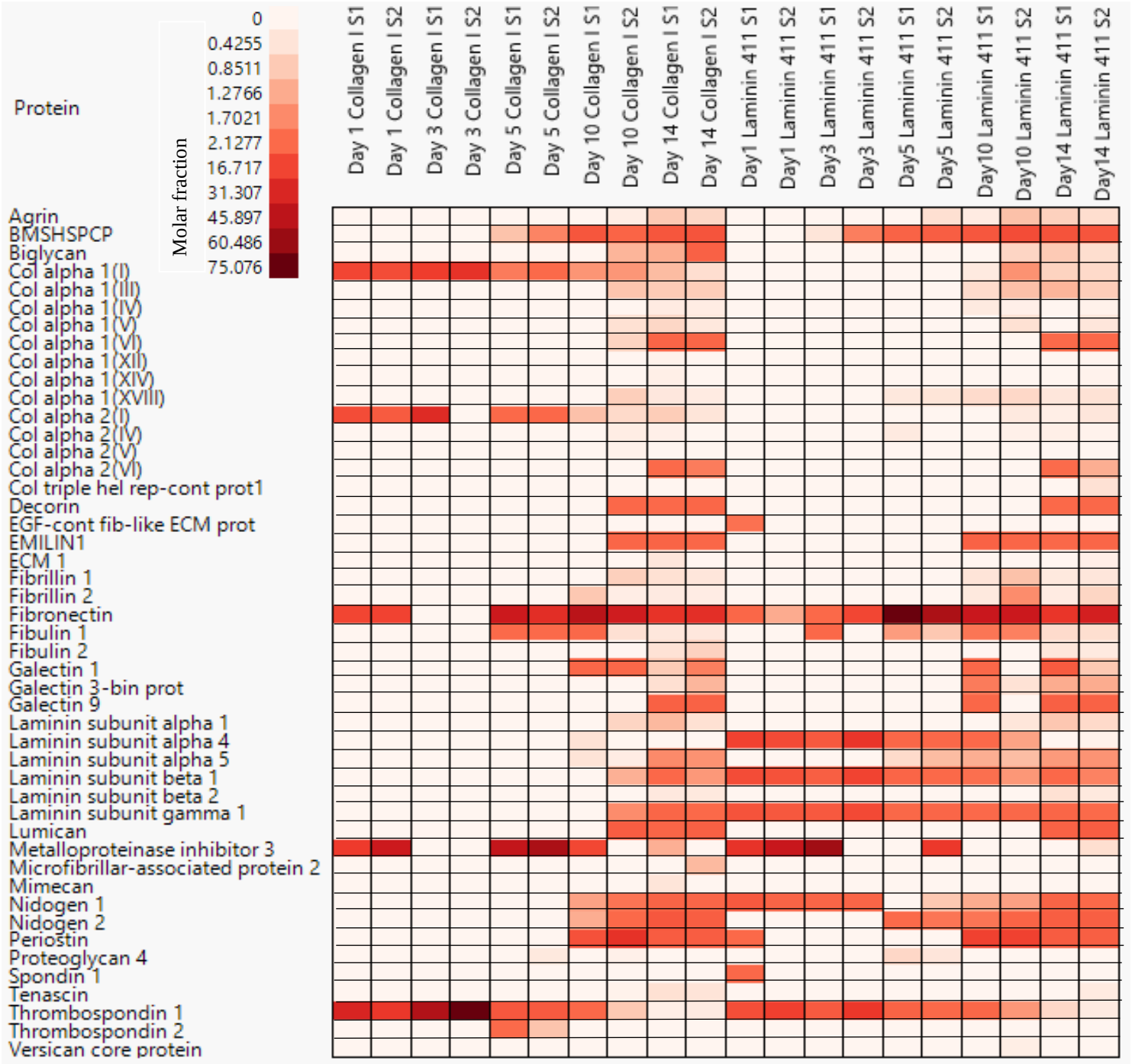

Figure 7. *Molar fraction percentages of ECM proteins, calculated based on ECM protein emPAI values. Data is given for 2 conditions – Laminin 411- and Collagen I-coated wells with duplicate samples for each conditon (S1- Sample 1, S2- Sample 2).*

Laminin 411-coated sample, the first appearance of BMSHSPCP was slightly earlier, on Day 3, as opposed to Day 5 in the Collagen I-coated condition.

The ECM profile for Laminin 411-coated samples was slightly different. Metalloproteinaise inhibitor 3 was present on Days 1, 3 and 5 in one of the duplicates, however absent at later stages – Day 10 and Day 14. In addition to Laminin 411 subunits, Fibronectin and Metalloproteinase inhibitor 3, which were observed in Collagen I-coated samples, significant amounts of Nidogen I were observed on Day 1 and Day 3, slightly less on Day 5 and Day 10 and high on Day 14 again. Some proteins, such as Periostin, Spondin 1, EGF Containing Fibulin Extracellular Matrix Protein 1 (EFEMP1) appeared on Day 1 in one of the duplicate Laminin 411-coated samples. Day 14 demonstrated high amounts of majority of ECM proteins in both conditions with some differences in Tenascin, Microfibrillar-associated protein 2 and Mimecan, which were higher in Collagen I-coated samples. Overall, the heatmap from Figure 7 demonstrated that by Day 14, most of listed ECM proteins were observed at high molar fraction percentages for both of conditions.

Finally, protein subunits of the exogenously added Collagen I and Laminin 411 in respective Collagen I- and Laminin 411-coated samples seemed to decrease in mole fractions from Day 1 to Day 14. This may give us hint of dynamic nature of ECM proteins.

Deposition and degradation of individual ECM proteins over time

Since some ECM proteins demonstrated fluctuations in mole fractions over given time points, linear graphs based on the average molar fraction percentages of duplicate

samples were constructed for the following proteins – Metalloproteinase inhibitor 3, Collagen XVIII alpha subunit 1, Fibrillin 2, Fibulin 1, Galectin 9, Laminin subunit alpha 5 and Nidogen 2 (Fig. 8-14, A4-A10).

Metalloproteinase inhibitor 3, a member of the remodeling family of ECM proteins, demonstrated curious trend of high amounts on Day 1 in both conditions (Fig.8).

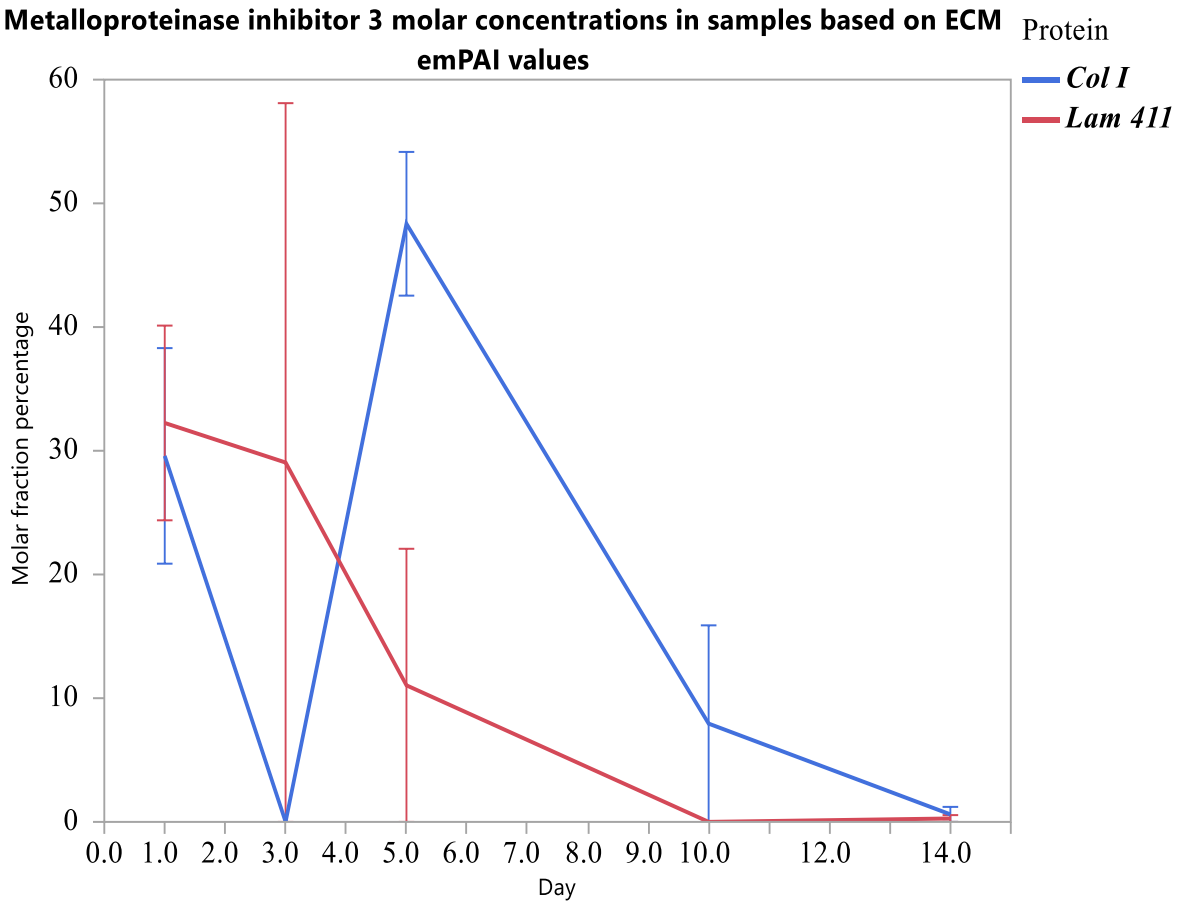

Figure 8. *Molar fraction percentages of Metalloproteinaise inhibitor 3 over 5 time points: Day1, Day3, Day5, Day10 and Day14. Values are calculated based on ECM emPAI values and taken as average of duplicate samples. Error bars are showing standard deviation for samples within duplicates. Data is given for 2 conditions – Laminin 411- and Collagen I-coated wells.*

Levels of Metalloproteinase inhibitor 3 demonstrated decreasing trend after Day 3 and Day 1 for Laminin 411 and Collagen I samples respectively (Fig.A4). The trend was consistently decreasing for Laminin 411, when ECM protein emPAI values only were considered, however unlike Laminin 411 samples, Collagen I samples showed a peak on Day 5 (Fig.8).

The next protein that showed interesting evolvement profiles was the Proteoglycan, Collagen XVIII alpha subunit 1 (Fig.9). It appeared at an earlier time point (Day 3) in Laminin 411-coated sample as opposed to Collagen I-coated condition (Day 5). In both conditions Collagen XVIII alpha subunit 1 reached peak molar concentration on Day 10

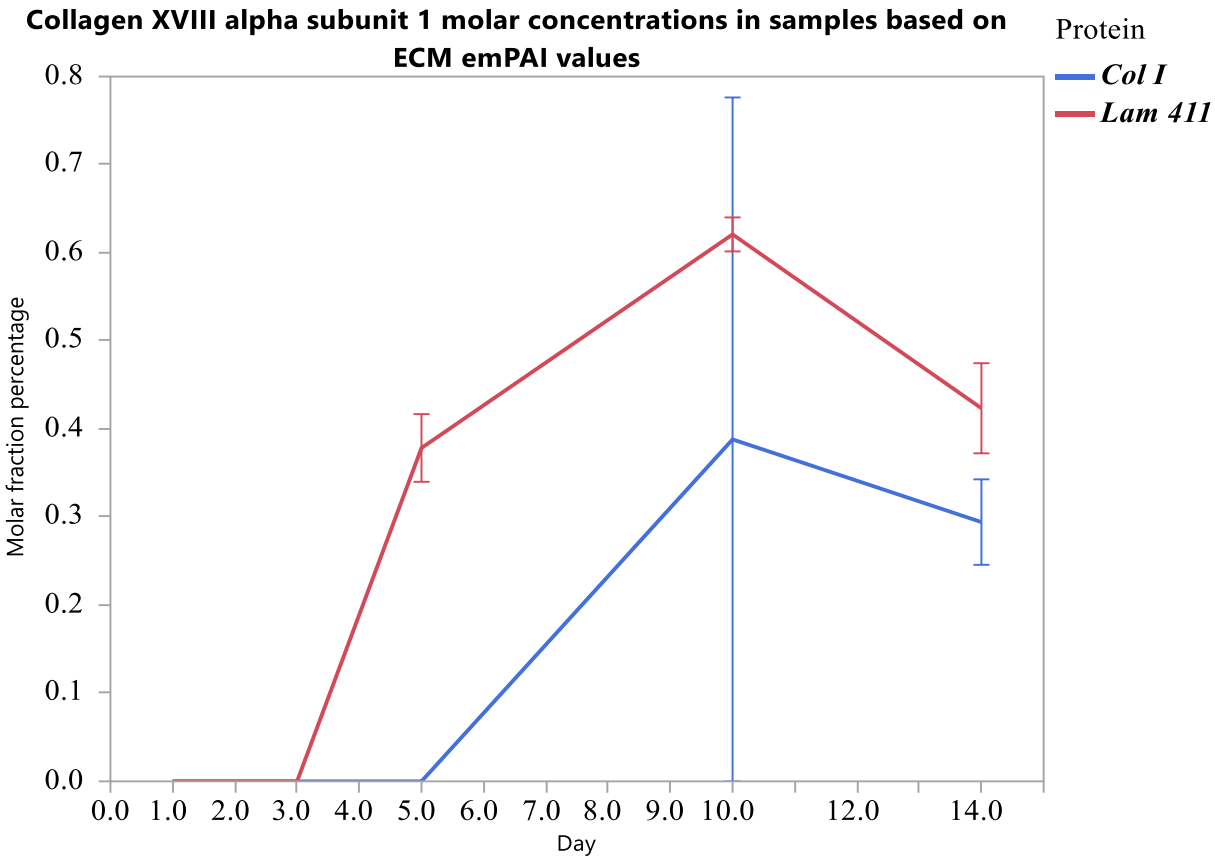

Figure 9. *Molar fraction percentages of Collagen XVIII alpha subunit 1 over 5 time points: Day1, Day3, Day5, Day10 and Day14. Values are calculated based on ECM emPAI values and taken as average of duplicate samples. Error bars are showing standard deviation for samples within duplicates. Data is given for 2 conditions – Laminin 411- and Collagen I-coated wells.*

and decreased by Day 14.

For Microfibrillar ECM protein, Fibrillin 2 (Fig.10), the profile looked similar for both conditions, however deposition in Laminin 411-coated sample was almost twice as high molar fraction as in Collagen I-coated condition.

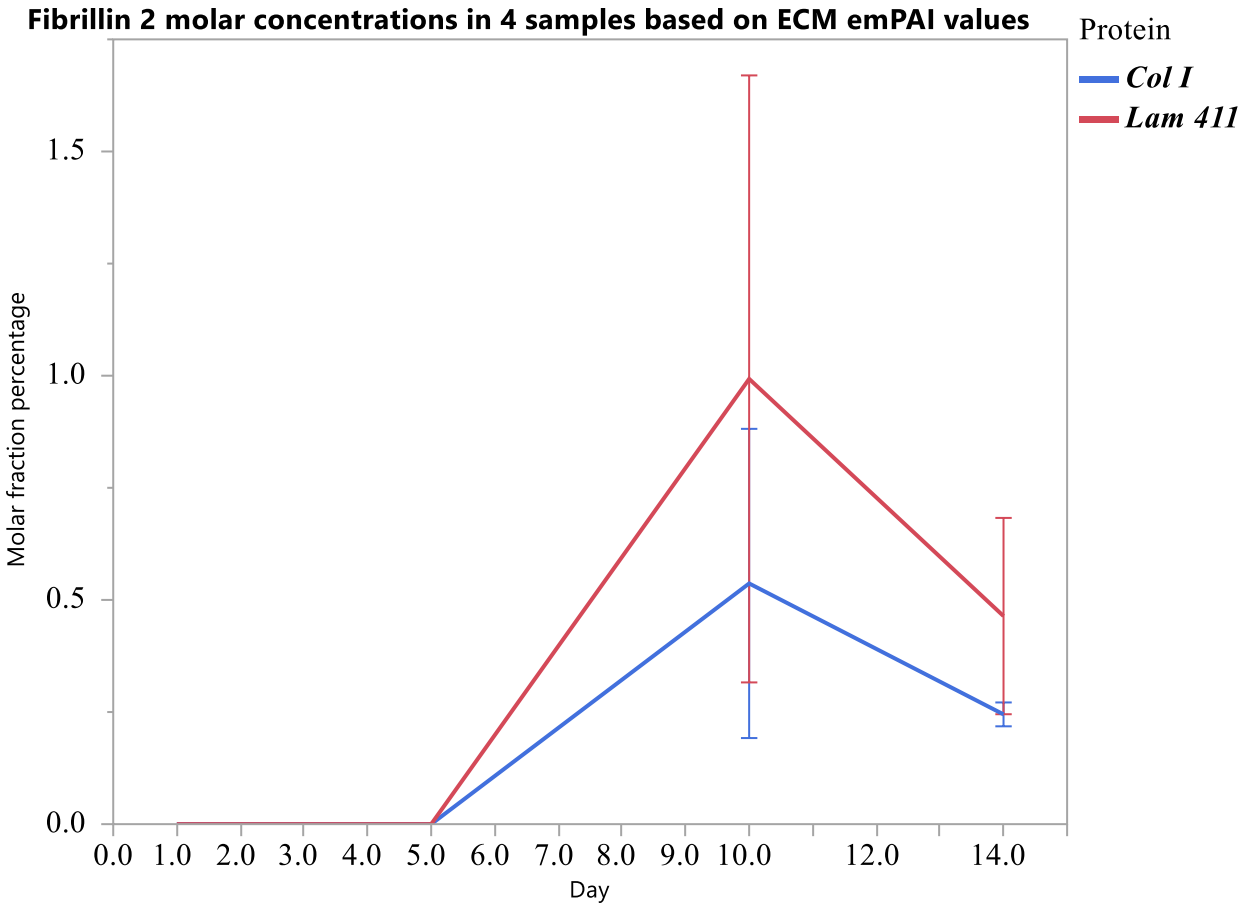

Figure 10. *Molar fraction percentages of Fibrillin 2 over 5 time points: Day1, Day3, Day5, Day10 and Day14. Values are calculated based on ECM emPAI values and taken as average of duplicate samples. Error bars are showing standard deviation for samples within duplicates.. Data is given for 2 conditions – Laminin 411- and Collagen I-coated wells.*

Fibulin 1, a Basement-membrane ECM protein, showed a different deposition profile for the 2 conditions (Fig.11). The Collagen I-coated sample demonstrated a peak amount of Fibulin 1 on Day 5, with the percent decreasing on further days whereas the Laminin 411

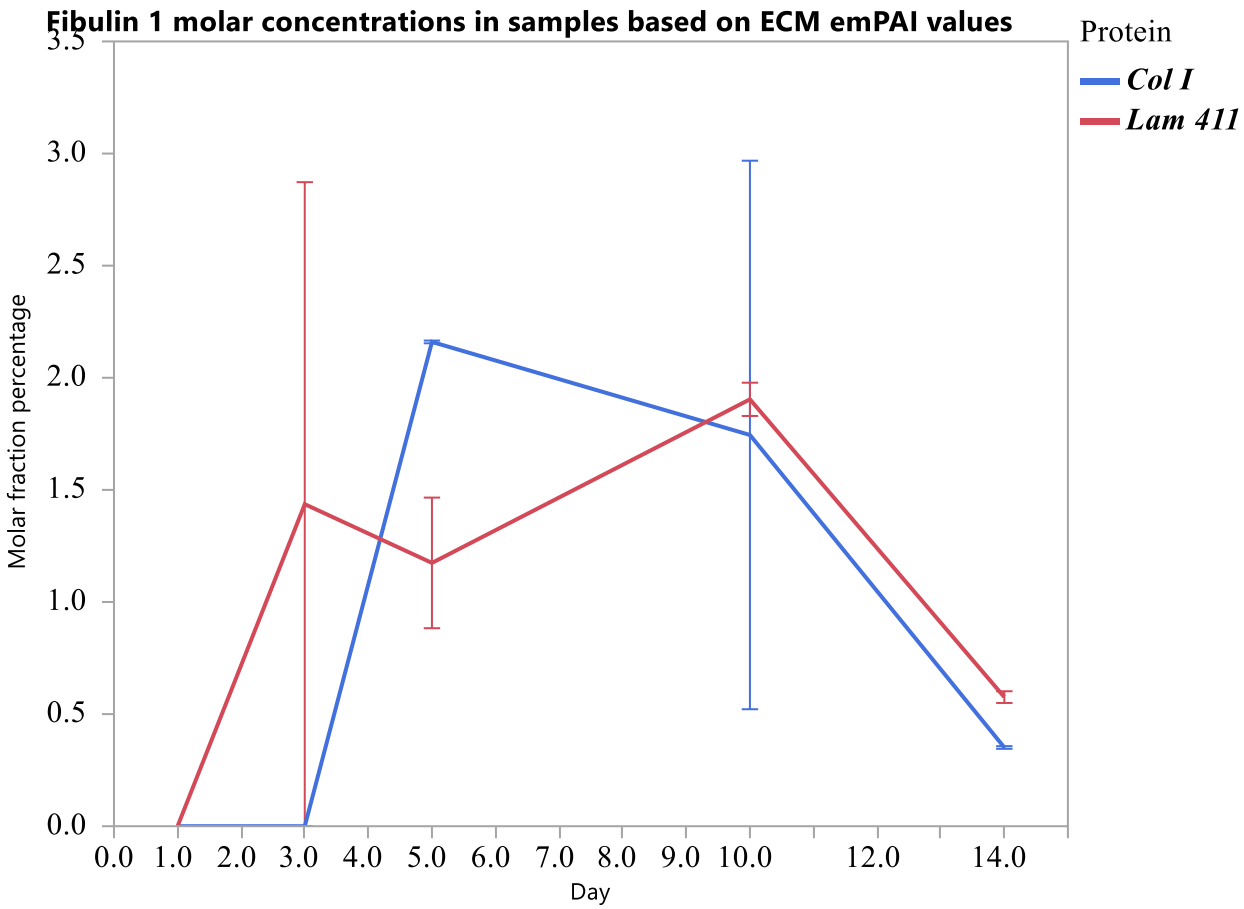

Figure 11. *Molar fraction percentages of Fibulin 1 over 5 time points: Day1, Day3, Day5, Day10 and Day14. Values are calculated based on ECM emPAI values and taken as average of duplicate samples. Error bars are showing standard deviation for samples within duplicates. Data is given for 2 conditions – Laminin 411- and Collagen I-coated wells.*

condition demonstrated a wavy profile – molar fractions reached a peak on Day 3, then decreased sighlty on Day 5, with an increase on later stage- Day 10 and following decrease to day 14.

Unlike the previously described proteins, Galectin 9 (Fig.12) was deposited at later stages in both conditions. However, Collagen I-coated samples demonstrated the first

appearance of Galectin 9 as late as on Day 14, whereas, in Laminin 411-coated samples, Galectin 9 first appeared on Day 10 and increased through Day 14.

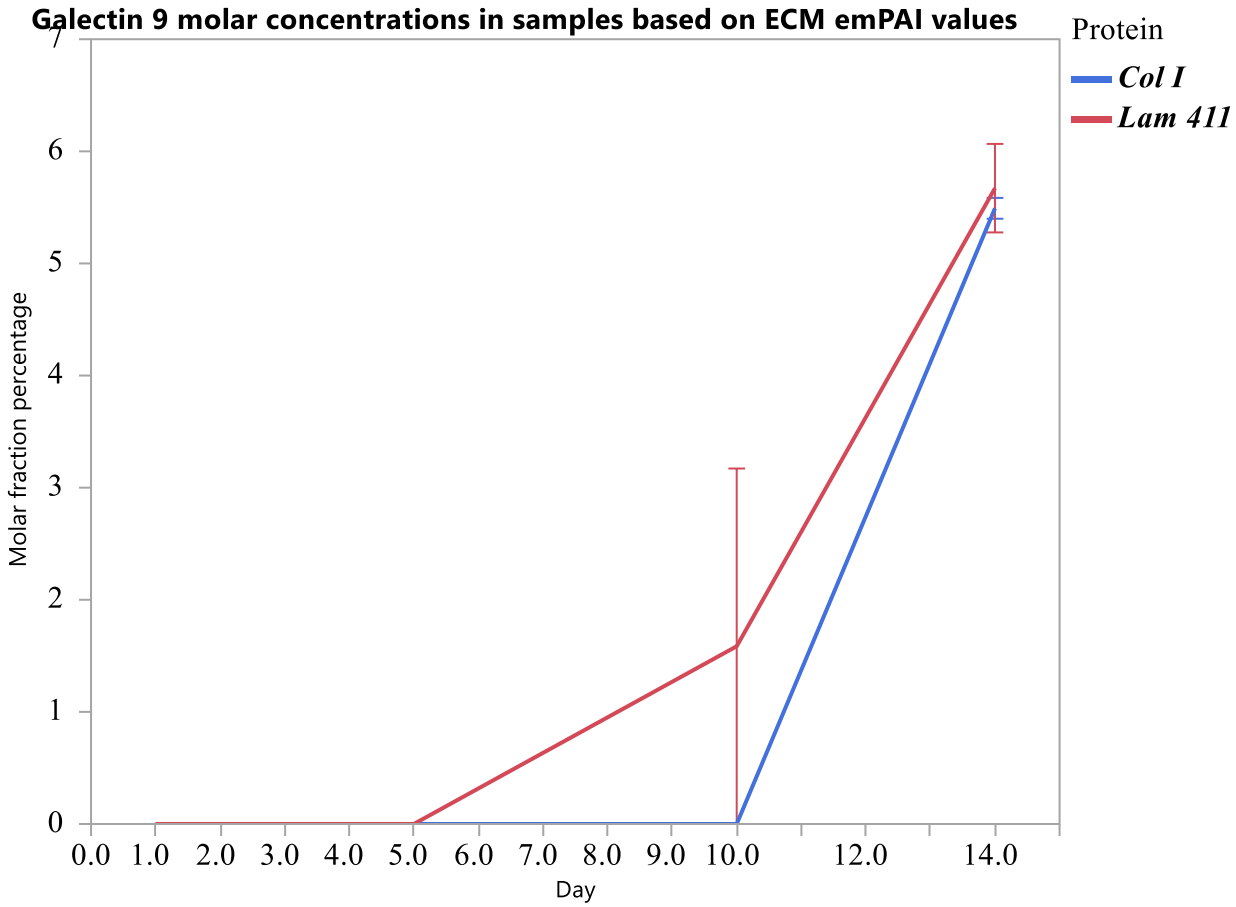

Figure 12. *Molar fraction percentages of Galectin 9 over 5 time points: Day1, Day3, Day5, Day10 and Day14. Values are calculated based on ECM emPAI values and taken as average of duplicate samples. Error bars are showing standard deviation for samples within duplicates. Data is given for 2 conditions – Laminin 411- and Collagen I-coated wells.*

Laminin subunit alpha 5, representative of Basement membrane proteins, was deposited earlier, on Day 3, in Laminin 411-coated sample as opposed to Collagen I, in which the first appearance of Laminin subunit alpha 5 occured later, on Day 5 (Fig.13). Curiously, after the first appearance, Laminin subunit alpha 5 showed increase in amounts for both conditions, although this increase was steeper for Collagen I-coated samples.

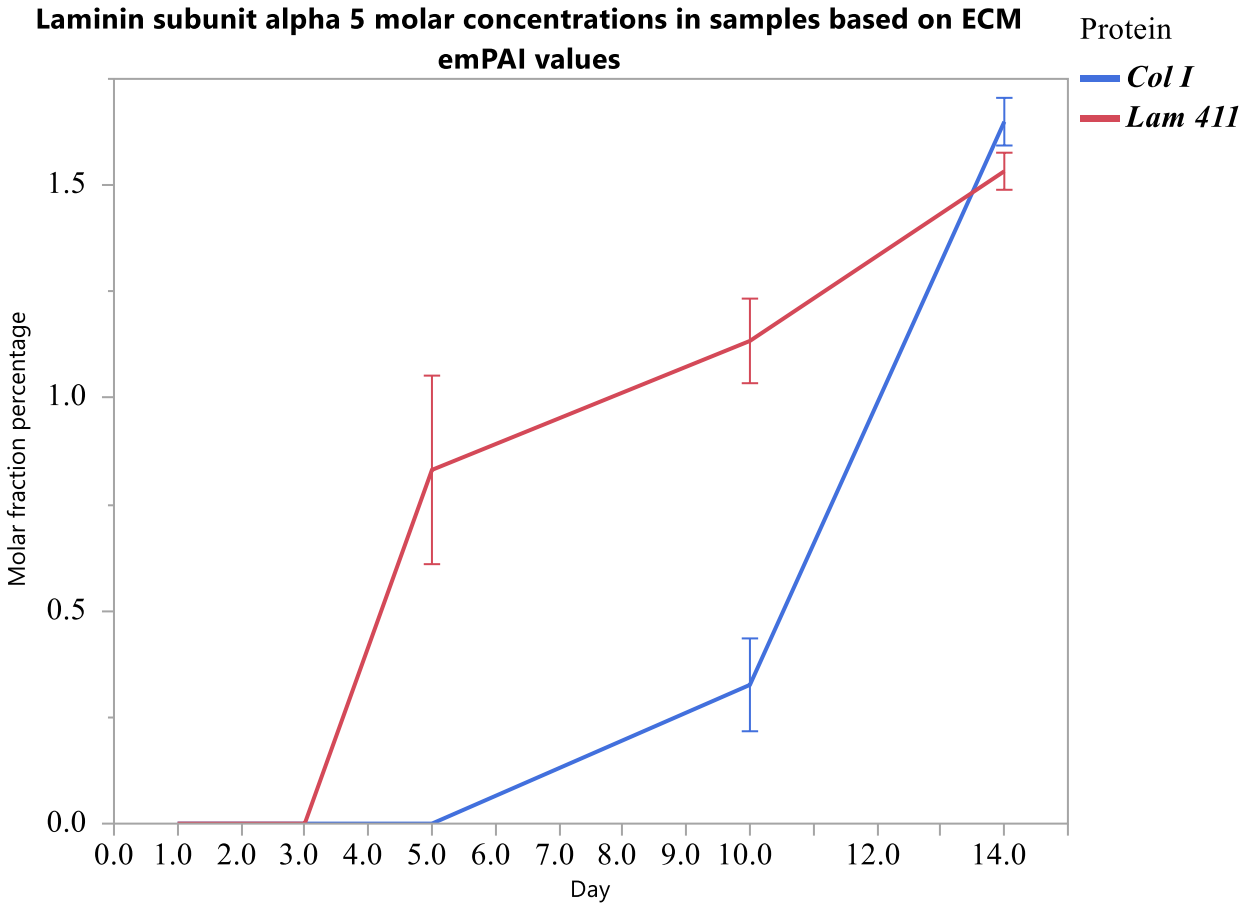

Figure 13. *Molar fraction percentages of Laminin subunit alpha 5 over 5 time points: Day1, Day3, Day5, Day10 and Day14. Values are calculated based on ECM emPAI values and taken as average of duplicate samples. Error bars are showing standard deviation for samples within duplicates. Data is given for 2 conditions – Laminin 411- and Collagen I-coated wells.*

Finally, another representative of Basement-membrane proteins, Nidogen 2, showed a profile resembling that of Laminin subunit alpha 5 (Fig.14). However, the amounts of Nidogen 2 by Day14 in both conditions were almost 6 times higher than those for Laminin subunit alpha 5.

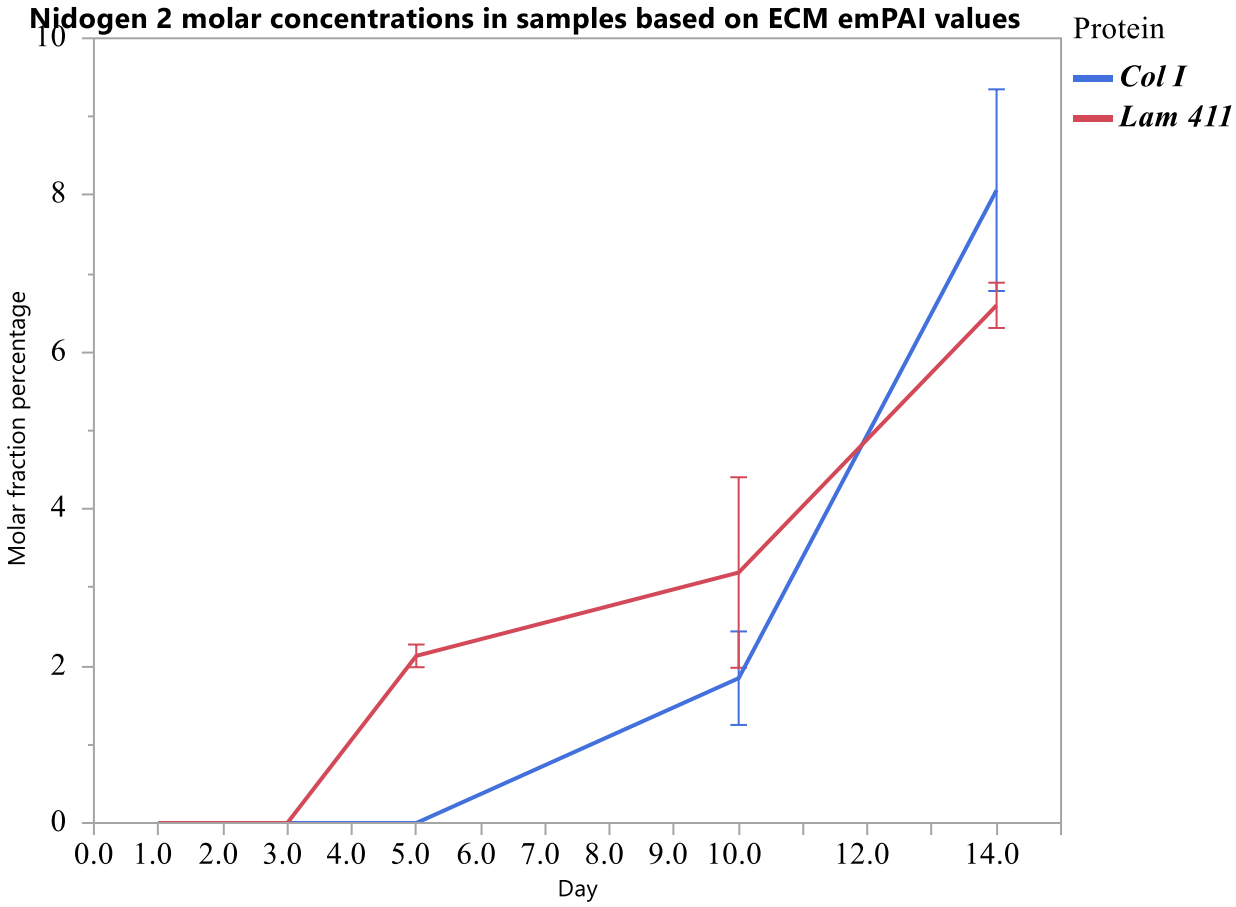

Figure 14 *Molar fraction percentages of Nidogen 2 over 5 time points: Day1, Day3, Day5, Day10 and Day14. Values are calculated based on ECM emPAI values and taken as average of duplicate samples Error bars are showing standard deviation for samples within duplicates. Data is given for 2 conditions – Laminin 411- and Collagen I-coated wells.*

Relative contributions of distinct ECM protein families

To further understand which family of ECM proteins most contributed to the ECM present at distinct time points, the list of ECM proteins was organized into 9 families[21,49-71,79] as shown in Table A22: Basement-membrane, Basement-membrane associated, Fibrillar, Fibril-associated, Matricellular, Microfibrillar, Microfibril-associated, Proteoglycan and Remodelling. Pie charts with percentages of respective ECM protein

families were constructed (Fig.15, A11, A12). Figure 15 demonstrates data for molar fraction percentages calculated by including ECM proteins only.

From Figure 15, it can be seen that at early stages of the differentiation protocol, Collagen I-coated samples were comprised of Fibrillar, Matricellular and Remodeling ECM proteins. Since the Fibrillar family was mostly represented by Collagen I, which is known to be exogeneously added, and Fibronectin, the incline in its amounts was visible in heatmap (Fig.7). As it was seen from heatmap (Fig.7), the Matricellular family was mostly represented by Thrombospondin I and the Remodeling family - by the presence of Metalloproteinaise inhibitor 3. An interesting trend to observe in Collagen I-coated samples was the increasing proportion of Remodeling protein family towards Day 5. However, Day 10 and 14 brought significant changes, wherein the proportion of Fibrillar proteins seemed to go down from 49% and 40% on Day 10 to 28% and 29% on Day 14, which could either indicate changes in relative proportions of protein families or degradation over time.

The former seems to be more plausible, since deposition of new ECM proteins could have changed the overall percentage contributions over time. Another observation was related to the appearance of Basement membrane ECM proteins on Day 10 at more than two times increase in amount towards Day 14. The proportion of Remodeling ECM proteins, constituting 43% and 54% on Day 5, reduced significantly towards Day 14. Day 14 also demonstrated the appearance of Basement membrane-associated proteins, Proteoglycans and Microfibril-assocciated proteins.

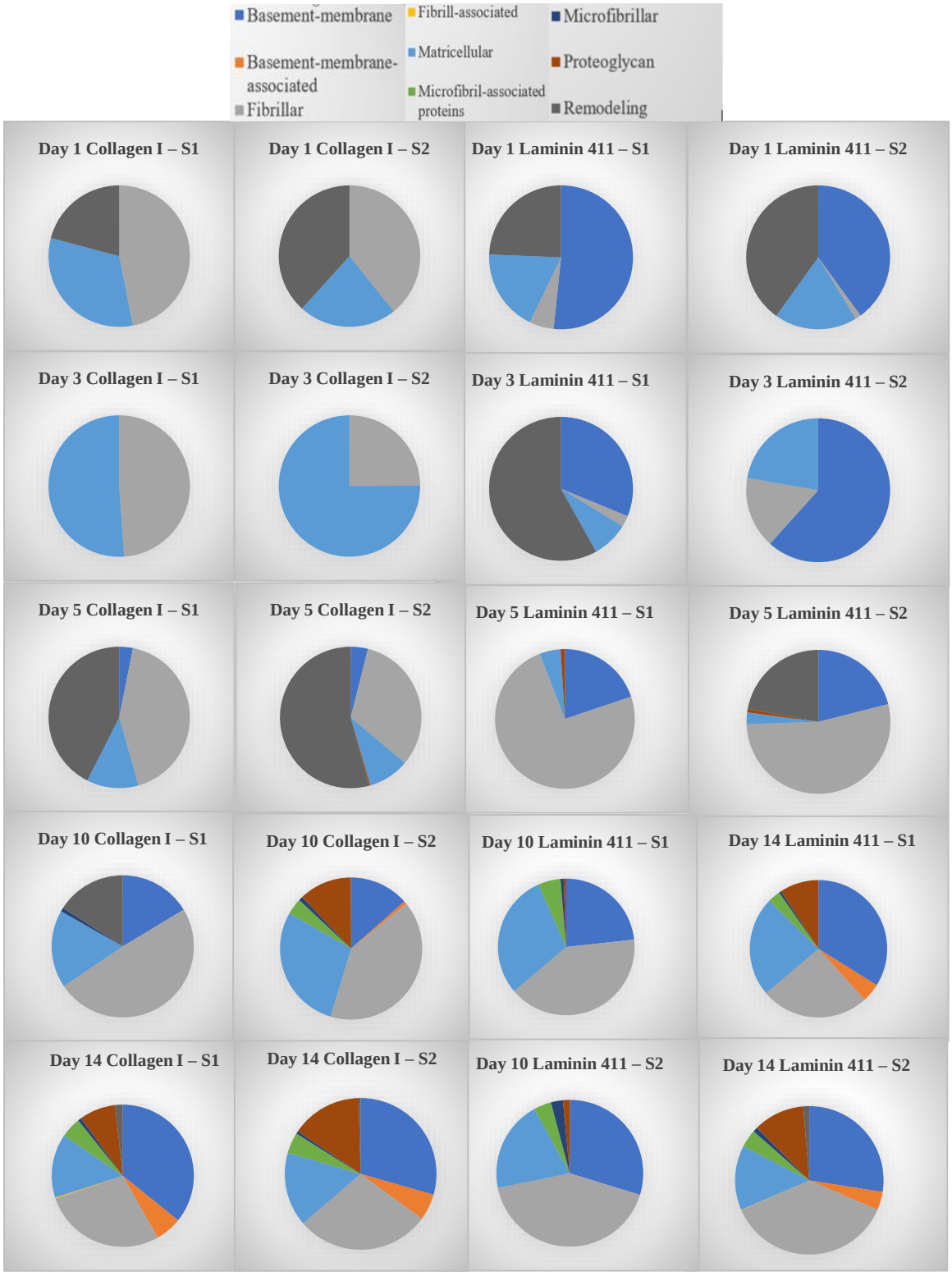

Figure 15. *Pie charts of ECM families, based on fraction percentages of ECM proteins, calculated via emPAI values of ECM proeins only. Data is given for Collagen I- and Laminin 411-coated wells at 5 distinct time points, with duplicate samples for each time point (S1-*

Laminin 411-coated samples from Figure 15 showed a slightly different profile of ECM families. Since these plates were coated with Laminin 411, there was a high percentage of Basement Membrane proteins, which at day 1 was largely comprised of exogeneously added ECM protein. Early stages of differentiation again demonstrated that Matricellular and Remodeling proteins contributed the most to ECM profile. However, after Day 1 it was hard to draw any conclusions about Remodeling proteins, since this family was observed in just one of the duplicates both on Day 3 and Day 5. Whether the percentage of Remodeling proteins increased on Day 3 and then reduced on Day 5 should be further investigated. Just like in the previous condition (Collagen I-coated samples), Laminin 411-coated samples also showed an increase in the proportion of Fibrillar proteins, which was mostly due to increasing molar fractions of Fibronectin.

Another observation was that the proportion of Basement- membrane proteins reduced from 52% and 40% on Day 1 to 20% and 21% on Day 5, which could be indicative of either changing relative proportions of ECM proteins or their degradation over time. On Day 10, the Laminin 411-coated condition showed almost the same relative proportions of Matricellular, Basement membrane and Fibrillar proteins. By Day 14 the relative proportion of observed proteins in Laminin 411-coated condition resembled that of Collagen I-coated samples. This was quite unexpected, since the initial starting conditions were different in terms of ECM proteins used for coating.

CHAPTER 4

DISCUSSION

The current study aimed to determine how the composition of the ECM changes in the course of endothelial differentiation of miPSCs triggered by exogenously provided ECM (namely Laminin 411). Mass Spectrometry analysis of the ECM of mouse iPSCs committed to endothelial lineage was performed in order to reveal the spectrum of ECM proteins appearing at distinct time points over the 14-day endothelial differentiation protocol. The analyses of data from performed experiments were conducted using a method described by Ishihama et al.[42]. Absolute molar concentrations of ECM were calculated based on exponentially modified Protein Abundancy Index (emPAI) values corresponding to proteins of interest. Heatmaps of all ECM proteins and linear graphs of individual ECM proteins were constructed based on the collected data. The proteins were organized into families and the differences in relative proportions of those families over the differentiation protocol were observed.

In establishing the workflow, a few interesting facts about Fibronectin were identified. Since Fibronectin-coated samples without cells demonstrated the presence of other cellular and ECM proteins, it could be concluded that commercially available Fibronectin is not completely pure, but does contain immunoglobulins, albumin and fibrinogen. This could be explained by the source of Fibronectin, which was human plasma. Moreover, the presence of proteins as Fibulin 1, Extracellular matrix protein 1, Vitronectin and von Willebrand factor, implied that other ECM proteins can also contribute to the impurity of Fibronectin. Overall, it was confirmed that cells seeded on ECM-coated plates can be

hypotonically decellularized, exogenously added ECM and endogenously produced protein can be detached and run on Mass Spectrometry for further analysis.

With respect to the evolution of proteins at distinct time points during ECM-induced differentiation of miPSCs into endothelial cells, it should be noted that current work is based on results from two technical replicates. Therefore, the experiment should be reproduced in order to obtain statistically significant data. However, trends in ECM evolution were observed and can be interpreted.

The constructed heatmap of the data collected revealed considerable amounts of BMSHSPCP in both Laminin 411- and Collagen I-coated samples, although the protein arises more rapidly and with greater proportion in Laminin 411 samples. BMSHSPCP, also known as Perlecan or heparan sulfate proteoglycan 2, is one of the largest ECM proteins and significant constituent of basement membrane of endothelial cells. It also plays a role in cell adhesion, development, proliferation, differentiation, interaction with growth factors and stabilization of vasculature[72-74]. Thus, the appearance of this ECM protein at later stages is quite promising in the context of endothelial differentiation.

The presence of a high molar fraction of Thrombospondin I in both samples on Day 1 could possibly be explained as an impurity of the ECM proteins used for coating – Collagen I and Laminin 411. Since it demonstrated almost the same proportions on Day 1, it possibly could be present as impurity in Collagen I and Laminin 411 ECM protein solutions used for coating of plate wells.

One of the ECM proteins with an interesting profile was Metalloproteinaise inhibitor 3 or TIMP3. TIMP3 is a representative of the family of proteins responsible for inhibition of matrix metalloproteinases (MMPs), such as gelatinases, collagenases and others[75]. TIMPS play a role in turnover of ECM, cellular behavior and remodeling of tissue[76]. However, TIMP3, unlike other members of the MMP family, can bind to the ECM and was shown to have an inhibitory action on angiogenesis which is mediated by vascular endothelial growth factor (VEGF)[77]. The results (Fig.8) demonstrated high molar fractions of TIMP3 on Day 1 in both samples which could be explained either by ECM remodeling or by an impurity of Collagen I and Laminin 411 ECM protein solutions used for coating of plate wells. Curiously, both samples, showed decrease in proportion of TIMP3 over the time, although Collagen I samples demonstrated a slight increase in molar fraction towards Day 5. This could be explained by the presence of large amounts of Collagen I in the coating. Since a decrease in proportion of Collagen I is observed at later stages, it could be explained by degradation. If this was the case, cells could have produced high amounts of collagenases, and in order to maintain the balance, the proportion of TIMP3 could have increased in response to possibly higher proportion of collagenases[75]. Other possible explanation for elevated proportion of TIMP3 in samples could be apoptosis-related processes going in the samples at later stages of differentiation[78].

Collagen XVIII was demonstrated to have properties of both proteoglycans and collagens[79] and to be a constituent of Basal lamina along with laminin and perlecan. It is found in endothelial basement membranes of blood vessels, but Collagen XVIII is also present in basal laminae of the peripheral nervous system and kidney[80,81]. Being a

heparan sulfate proteoglycan, collagen XVIII was proposed to have a function in the interaction between the basal lamina and endothelial cells[79]. The results of this study (Fig.9) demonstrated an increase in the proportion of Collagen XVIII over the time, with Laminin samples having slightly higher molar fractions by Day 14, which could be due to the Laminin 411 coating or some behavior of the cells caused by this coating. Also increase in molar fraction of Collagen XVIII could be explained by degradation of other ECM proteins.

Another ECM protein with an interesting evolution profile was Fibrillin 2, which is structurally known to be a glycoprotein[82]. Fibrillin 2 and Fibrillin 1 were demonstrated to participate in the formation of a scaffold for assembly of elastic fibers[83]. Specifically, Fibrillin 2 was shown to be present within matrices rich with elastic fibers in the fetal aorta and cartilaginous tissue[84]. Although our Mass Spectrometry data did not reveal the presence of elastin, the presence of Fibrillin 2 could be explained by the fact that Fibrillin 2 is deposited more than Fibrillin 1 during early stages of embryogenesis[85]. Additionally, as could be seen from the results (Fig.10), the molar fraction of Fibrillin 2 from the Laminin 411 samples was almost twice that of the Collagen I samples, implying that the presence of former endogenous ECM protein could have effect on the proportion of Fibrillin 2 deposited, or of the deposition and degradation of other ECM proteins, that changed the overall distribution of the relative proportions of ECM proteins present in the sample.

Curiously, immunohistochemical data and *in vitro* binding data from previous studies demonstrated that Fibrillin 1 interacts with Fibulin 2 in several tissues, including the

blood vessel intima[86]. However, our results did not demonstrate any visible changes in the percentage of these fibrillin and fibulin types.

Fibulin 1, the next interesting and related ECM protein, is known to interact with Nidogens, Fibronectin, Collagen IV, Collagen VI, and perlecan[87]. Moreover, the association of Fibulin 1 and basement membrane and elastic fibers was demonstrated by previous studies[88,89]. Other work revealed the suppressive effect of Fibulin 1 on Fibronectin's motility inducing activity[90]. This study could possibly explain why in our results (Fig.11), Fibulin 1 molar proportion increased towards Day 5 and showed a decreasing trend towards Day 14. As it can be seen from heatmap (Fig.7), Fibronectin also increases towards Day 5 and slightly decreases towards Day 14. However, it is unclear why Laminin 411 samples showed a drop in molar fraction on Day 5. The experiment should be repeated before solid conclusions can be drawn.

The next ECM protein of interest, Galectin 9, a representative of glycan-binding proteins family[91], was demonstrated to possess a possible role in inflammation, coagulation and angiogenesis balance during wound healing[92]. Endothelial cells were shown to express Galectin 9 in healthy[93] and inflamed tissues[94]. Moreover, another study showed deposition of Galectin 9 in tumor vasculature[95]. Our results (Fig.12) demonstrated an increase in molar fraction of Galectin 9 in both samples at later stages, however Laminin 411 sample showed an earlier appearance of this ECM protein. The increase in molar fraction of Galectin 9 could be related to the fact, that miPSCs were grown *in vitro*, which is far from natural conditions required for growth of cells, could cause such an increase in the proportion of Galectin 9. However, further analysis of the absolute values

of Galectin 9 is required, because an increase in molar fraction could also be caused by degradation of other ECM proteins.

Another protein of interest was Laminin subunit alpha 5. In general, laminins are known to provide cell adhesion via the amino acid sequence IKVAV (Isoleucine-Lysine-Valine-Alanine-Valine)[96]. The presence of Laminin subunit alpha 5 could imply the presence of Laminin 511 which was shown to provide *in vivo* endothelial cells with shear stress mechanotransduction[97]. These results (Fig.13) demonstrate an increase of molar fraction over time in both samples, with Laminin 411 samples showing an earlier appearance compared to Collagen I samples. The presence of endogenous laminin could induce deposition of the Laminin alpha 5 subunit. Also, it was demonstrated that Laminin 511 appears at later stages of blood vessel growth[27]. Again, this explanation should be further tested, since an increase in molar fraction could be due to degradation of other proteins in the sample rather than an increase in the amount of Laminin alpha 5 subunit present.

Finally, Nidogen 2 showed an increase in molar fraction, resembling the trend observed in Laminin subunit alpha 5. Nidogens in general were demonstrated to cross-link Collagen IV and laminin[98], whereas Nidogen 2 specifically was found in the basal lamina of endothelial cells[99]. Although both samples demonstrated increases in molar fractions of Nidogen 2, the appearance of Nidogen 2 in Laminin 411 was at an earlier stage. This could be explained by a previous study demonstrating that the complex of nidogen with laminin has a modulatory role in angiogenesis[100]. Also, by Day 14 both samples reached a higher molar fraction of Nidogen 2, as compared to values of Laminin subunit alpha 5.

Overall, these results demonstrated the appearance of some ECMs with time, some, such as Thrombospondin and TIMP3, at earlier stages and others, such as certain

Proteoglycans and Nidogens, at later stages. The presence of Nidogen at later stages in Collagen I-coated samples could be related to the fact that Nidogens are mostly associated with Laminins, and that is why Laminin 411-coated samples demonstrated early appearance of Nidogen I as opposed to Collagen I-coated samples[99].

Thrombospondin 1, amounts of which were robust, is known to be associated with Collagens I and V, Fibronectin and Laminins[101]. However, although high amounts of Thrombospondin 1 in both conditions could be related to the early appearance of Fibronectin, it should be emphasized that some of the ECM proteins observed at early time points could be contributed by impurities of Laminin 411 and Collagen I protein solutions used for coating. Another interesting observation from the heatmap in Figure 7 is that protein subunits of exogenously added Collagen I and Laminin 411 in respective Collagen I- and Laminin 411-coated samples seem to decrease in amounts from Day 1 to Day 14. This may provide a hint at the dynamic nature of ECM proteins and may imply that a balance between formation of ECM and degradation is taking place.

However, according to numerous studies the effect of Thrombospondin 1 is anti-angiogenic[102]. Its presence in tumor cells, for example, was demonstrated to be associated with reduced vascularization[103]. At the same time, Thrombospondin 1 is known to promote cell motility and migration[104]. This could have explained why Thrombospondin 1 is seen up to Day 10, but no later. However, further experiments are required to draw more accurate conclusions. The experiment should be repeated, this time concentrating on early time points, during which it is known that mesoderm induction is occurring. Also, it could be useful to perform immunostaining of ECM proteins with the highest molar concentrations at various time points to confirm their presence in samples.

It should also be noted that the samples used in current experiment showed slightly diminished level of differentiation in Laminin 411 samples (Fig.4) as compared to Mikayla Hall's preliminary data (Fig.1B). This could be related to the fact that experiment was performed only once and was not reproduced to choose for samples with higher differentiation to CD31 expressing cells. So, for more accurate results, it is important to limit future experiments to samples that demonstrate at least 40% of differentiation to CD31 expressing cells.

Another point to be improved is the development of more a robust decellularization method that would filter out cellular and nuclear proteins but preserve the ECM proteins. ECM proteins are known to possess bulky structures, and it makes protein purification and sample runs on the Mass Spectrometer complicated. Therefore, more accurate quantification of ECM would be possible with more robust protocol of ECM isolation, that would not interfere with Mass Spectrometry analysis but would produce pure samples of solubilized ECM proteins[105].

There are certain points in this study, where protein loss was encountered. For instance, low number of ECM proteins observed in Day 3 for Collagen I-coated samples could be related to possible loss of proteins during the purification step and/or the lyophilization steps. Although further replications of this experiment are required, by looking at certain ECM proteins on Day 3, such as Fibronectin for example, it could be confirmed that loss of the sample did take place. Therefore, protein purification and lyophilization are critical steps in sample preparation. In order to eliminate peptide loss during protein purification, it is important to optimize the steps of centrifugation, handle samples accurately during transfers from tubes, and use low protein binding collection Eppendorf tubes.

Lyophilization is another important step in the workflow, during which peptide loss can occur. Loss in the Collagen I-coated sample for Day 3 likely occurred during this step. It is very important to make sure that samples are properly frozen (visually assessed) before placing them into lyophilizer (Methods-Preparation of samples for Mass Spectrometry).

Future directions of our project include further optimization of the procedures for decellularization, trypsinization, and protein purification. The studies should be reproduced and immunostaining against ECM proteins robustly encountered at given time points should be performed. In order to confirm the presence of such ECM proteins, more accurate quantitative Mass Spectrometry analysis can be performed. For instance, stable isotope labeling could provide absolute and relative protein amounts[106].

Another important stage of the study was data analysis. For example, depending on FDR and p values used, outcomes vary widely. It could be useful to analyze data using at least 2 distinct software programs, such as Proteome Discoverer and Scaffold for example, and 2 methodologies for data analysis, such as emPAI and average total ion current values. This step might require collaboration with Proteomic analysis specialists for creating a custom workflow in order to perform analysis of ECM proteins.

Next, it could be useful to look into the effect of combinations of ECM proteins that can be used as microenvironments for guiding endothelial differentiation and investigating the ECM protein evolvement profile generated from such samples. The overall aim is to elucidate the effect of the presence of exogenously provided ECM on signal pathways leading to the mesoderm switch. According to these results, this could be not only the presence, but also the degradation, of certain proteins through time, which could also potentially be causing signal changes in differentiating cells. Further work is required for

elucidation and confirmation of ECM proteins having the most efficient guiding effect on endothelial differentiation. This includes, knocking-down of some of the key ECM proteins and investigating its effect on differentiation process, that could potentially be measured by levels of CD31 expression. Knocking-down of specific ECM proteins could demonstrate whether particular proteins have signaling role in differentiation process or are deposited in response to other factors or proteins. In this way, the project could investigate relative effect of these proteins and their amounts and find the ECM formulation most supportive of endothelial lineage commitment. The efficient ECM formulation could be used in tissue engineering assays to generate endothelial cells for potential vascularization of tissue constructs.

The current study was implemented to reveal evolution of ECM proteins over time in ECM-induced differentiation of miPSCs into endothelial cells. This study is important for future studies implementing identification of ECM formulations for efficient endothelial differentiation. Robust endothelial differentiation protocol could potentially be used for successful vascularization of tissue constructs.

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APPENDIX

Tables A1-20: emPAI values were acquired from Proteome discoverer after analysis of Mass Spectrometry data. emPAI fractions of total proteins, molar concentrations (%), ECM emPAI fractions, ECM molar concentrations (%) were calculated according to the method described under *Data analysis* section of METHODS.

Table A1 Day 1 Collagen I-coated samples

Sample 1

ECM protein	emPAI	emPAI fraction of tot proteins	mol conc (%)	ECM emPAI fraction	ECM mol conc (%)
Collagen alpha-1(I) chain OS=Mus musculus OX=10090 GN=Col1a1 PE=1 SV=4	0.334	0.00520809	0.520808969	0.164047151	16.40471513
Collagen alpha-2(I) chain OS=Mus musculus OX=10090 GN=Col1a2 PE=1 SV=2	0.284	0.004428436	0.442843555	0.139489194	13.94891945
Thrombospondin-1 OS=Mus musculus OX=10090 GN=Thbs1 PE=1 SV=1	0.656	0.010229062	1.022906239	0.322200393	32.22003929
Fibronectin OS=Mus musculus OX=10090 GN=Fn1 PE=1 SV=4	0.337	0.005254869	0.525486894	0.165520629	16.55206287

Metalloproteinase inhibitor 3 OS=Mus musculus OX=10090 GN=Timp3 PE=1 SV=1	0.425	0.00662706	0.662706024	0.208742633	20.87426326
	Tot.64.131			Tot. 2.036	

Table A2 Day 1 Collagen I-coated samples

Sample 2

ECM protein	emPAI	emPAI fraction of tot proteins	mol conc (%)	ECM emPAI fraction	ECM mol conc (%)
Fibronectin OS=Mus musculus OX=10090 GN=Fn1 PE=1 SV=4	0.473	0.00550557	0.550556959	0.175705795	17.57058
Collagen alpha-1(I) chain OS=Mus musculus OX=10090 GN=Col1a1 PE=1 SV=4	0.369	0.004295043	0.429504266	0.137072808	13.70728
Thrombospondin-1 OS=Mus musculus OX=10090 GN=Thbs1 PE=1 SV=1	0.605	0.007042008	0.704200761	0.22473997	22.474
Collagen alpha-2(I) chain OS=Mus musculus OX=10090 GN=Col1a2 PE=1 SV=2	0.214	0.002490892	0.249089195	0.079494799	7.94948
Metalloproteinase inhibitor 3 OS=Mus musculus OX=10090 GN=Timp3 PE=1 SV=1	1.031	0.012000512	1.200051215	0.382986627	38.29866
	Tot. 85.913			Tot. 2.692	

Table A3 Day 1 Laminin 411-coated samples

Sample 1

ECM protein	emPAI	emPAI fraction of tot proteins	mol conc (%)	ECM emPAI fraction	ECM mol conc (%)
Laminin subunit alpha-4 OS=Mus musculus OX=10090 GN=Lama4 PE=1 SV=2	0.76	0.005286442	0.528644167	0.179711516	17.97115157
Laminin subunit beta-1 OS=Mus musculus OX=10090 GN=Lamb1 PE=1 SV=3	0.585	0.004069169	0.406916892	0.138330575	13.83305746
Laminin subunit gamma-1 OS=Mus musculus OX=10090 GN=Lamc1 PE=1 SV=2	0.474	0.00329707	0.32970702	0.112083235	11.20832348
Thrombospondin-1 OS=Mus musculus OX=10090 GN=Thbs1 PE=1 SV=1	0.555	0.003860494	0.386049359	0.131236699	13.1236699
Nidogen-1 OS=Mus musculus OX=10090 GN=Nid1 PE=1 SV=2	0.366	0.002545839	0.254583901	0.086545283	8.654528257
Fibronectin OS=Mus musculus OX=10090 GN=Fn1 PE=1 SV=4	0.145	0.001008597	0.100859742	0.034287066	3.42870655
Metalloproteinase	1.031	0.007171475	0.717147547	0.243792859	24.37928588

inhibitor 3 OS=Mus musculus OX=10090 GN=Timp3 PE=1 SV=1					
Periostin OS=Mus musculus OX=10090 GN=Postn PE=1 SV=2	0.108	0.000751231	0.075123118	0.025537952	2.553795223
Spondin-1 OS=Mus musculus OX=10090 GN=Spon1 PE=1 SV=1	0.119	0.000827745	0.082774547	0.02813904	2.813903996
EGF-containing fibulin-like extracellular matrix protein 1 OS=Mus musculus OX=10090 GN=Efemp1 PE=1 SV=1	0.086	0.000598203	0.059820261	0.020335777	2.033577678
	Tot. 143.764			Tot. 4.229	

Table A4 Day 1 Laminin 411-coated samples

Sample 2

ECM protein	emPAI	emPAI fraction of tot proteins	mol conc (%)	ECM emPAI fraction	ECM mol conc (%)
Laminin subunit alpha-4 OS=Mus musculus OX=10090 GN=Lama4 PE=1 SV=2	0.688	0.000673943	0.067394298	0.145731836	14.57318365
Thrombospondin-1 OS=Mus musculus OX=10090 GN=Thbs1 PE=1 SV=1	0.879	0.000861041	0.086104052	0.186189367	18.61893667
Laminin subunit gamma-1 OS=Mus musculus OX=10090 GN=Lamc1 PE=1 SV=2	0.436	0.000427092	0.042709177	0.092353315	9.235331498
Laminin subunit beta-1 OS=Mus musculus OX=10090 GN=Lamb1 PE=1 SV=3	0.549	0.000537783	0.053778299	0.116288922	11.62889218
Metalloproteinase inhibitor 3 OS=Mus musculus OX=10090 GN=Timp3 PE=1 SV=1	1.894	0.001855302	0.185530233	0.401186189	40.11861894
Nidogen-1 OS=Mus musculus OX=10090 GN=Nid1 PE=1 SV=2	0.215	0.000210607	0.021060718	0.045541199	4.55411989

Fibronectin OS=Mus musculus OX=10090 GN=Fn1 PE=1 SV=4	0.06	5.87741E-05	0.00587741	0.012709172	1.270917179
	Tot. 119.819			Tot.4.721	

Table A5 Day 3 Collagen I-coated samples

Sample 1

ECM protein	emPAI	emPAI fraction of tot proteins	mol conc (%)	ECM emPAI fraction	ECM mol conc (%)
Thrombospondin-1 OS=Mus musculus OX=10090 GN=Thbs1 PE=1 SV=1	0.208	0.003362921	0.336292057	0.511056511	51.10565111
Collagen alpha-2(I) chain OS=Mus musculus OX=10090 GN=Col1a2 PE=1 SV=2	0.117	0.001891643	0.189164282	0.287469287	28.74692875
Collagen alpha-1(I) chain OS=Mus musculus OX=10090 GN=Col1a1 PE=1 SV=4	0.082	0.001325767	0.132576676	0.201474201	20.14742015
	Tot.61.851			Tot.0.407	

Table A6 Day 3 Collagen I-coated samples

Sample 2

ECM protein	emPAI	emPAI fraction of tot proteins	mol conc (%)	ECM emPAI fraction	ECM mol conc (%)
Thrombospondin-1 OS=Mus musculus OX=10090 GN=Thbs1 PE=1 SV=1	0.247	0.008843537	0.884354	0.750759878	75.07598784
Collagen alpha-2(I) chain OS=Mus musculus OX=10090 GN=Col1a2 PE=1 SV=2	0.082	0.002935911	0.293591	0.249240122	24.92401216
	Tot.27.93			Tot.0.329	

Table A7 Day 3 Laminin 411-coated samples

Sample 1

ECM protein	emPAI	emPAI fraction of tot proteins	mol conc (%)	ECM emPAI fraction	ECM mol conc (%)
Laminin subunit alpha-4 OS=Mus musculus OX=10090 GN=Lama4 PE=1 SV=2	0.585	0.002909174	0.290917409	0.086615339	8.661533906
Laminin subunit gamma-1 OS=Mus musculus OX=10090 GN=Lamc1 PE=1 SV=2	0.593	0.002948958	0.294895767	0.087799822	8.779982233
Laminin subunit beta-1 OS=Mus musculus OX=10090 GN=Lamb1 PE=1 SV=3	0.445	0.002212961	0.221296149	0.065886882	6.588688185
Thrombospondin-1 OS=Mus musculus OX=10090 GN=Thbs1 PE=1 SV=1	0.555	0.002759986	0.275998568	0.082173527	8.21735268
Fibronectin OS=Mus musculus OX=10090 GN=Fn1 PE=1 SV=4	0.167	0.000830482	0.083048218	0.024726088	2.472608824
Metalloproteinase inhibitor 3 OS=Mus musculus OX=10090	3.924	0.019513845	1.951384468	0.580989044	58.09890435

GN=Timp3 PE=1 SV=1					
Nidogen-1 OS=Mus musculus OX=10090 GN=Nid1 PE=1 SV=2	0.264	0.001312858	0.131285805	0.039087948	3.908794788
Fibulin-1 OS=Mus musculus OX=10090 GN=Fbln1 PE=1 SV=2	0.194	0.000964752	0.096475175	0.028723719	2.872371928
Basement membrane-specific heparan sulfate proteoglycan core protein OS=Mus musculus OX=10090 GN=Hspg2 PE=1 SV=1	0.027	0.00013427	0.013426957	0.003997631	0.399763103
	Tot. 201.088			Tot. 6.754	

Table A8 Day 3 Laminin 411-coated samples

Sample 2

ECM protein	emPAI	emPAI fraction of tot proteins	mol conc (%)	ECM emPAI fraction	ECM mol conc (%)
Laminin subunit alpha-4 OS=Mus musculus OX=10090 GN=Lama4 PE=1 SV=2	0.688	0.003425885	0.342588535	0.233536999	23.35369993
Fibronectin OS=Mus musculus OX=10090 GN=Fn1 PE=1 SV=4	0.473	0.002355296	0.235529618	0.160556687	16.0556687
Thrombospondin-1 OS=Mus musculus OX=10090 GN=Thbs1 PE=1 SV=1	0.656	0.003266542	0.326654185	0.222674813	22.26748133
Laminin subunit beta-1 OS=Mus musculus OX=10090 GN=Lamb1 PE=1 SV=3	0.514	0.002559455	0.255945505	0.174473863	17.44738629
Laminin subunit gamma-1 OS=Mus musculus OX=10090 GN=Lamc1 PE=1 SV=2	0.436	0.002171055	0.217105525	0.147997284	14.79972845
Basement membrane-specific heparan sulfate proteoglycan core protein OS=Mus musculus	0.055	0.000273872	0.027387165	0.018669382	1.866938221

OX=10090 GN=Hspg2 PE=1 SV=1					
Nidogen-1 OS=Mus musculus OX=10090 GN=Nid1 PE=1 SV=2	0.124	0.000617456	0.061745608	0.042090971	4.209097081
	Tot. 200.824			Tot. 2.946	

Table A9 Day 5 Collagen I-coated samples

Sample 1

ECM protein	emPAI	emPAI fraction of tot proteins	mol conc (%)	ECM emPAI fraction	ECM mol conc (%)
Fibronectin OS=Mus musculus OX=10090 GN=Fn1 PE=1 SV=4	2.193	6.90051E-06	0.000690051	0.380003466	38.00034656
Thrombospondin-1 OS=Mus musculus OX=10090 GN=Thbs1 PE=1 SV=1	0.555	1.74637E-06	0.000174637	0.096170508	9.617050771
Collagen alpha-1(I) chain OS=Mus musculus OX=10090 GN=Col1a1 PE=1 SV=4	0.11	3.46127E-07	3.46127E-05	0.019060821	1.906082135
Collagen alpha-2(I) chain OS=Mus musculus OX=10090 GN=Col1a2 PE=1 SV=2	0.149	4.68845E-07	4.68845E-05	0.025818749	2.581874892
Metalloproteinase inhibitor 3 OS=Mus musculus OX=10090 GN=Timp3 PE=1 SV=1	2.455	7.72492E-06	0.000772492	0.425402876	42.54028765
Basement membrane-specific heparan sulfate proteoglycan core	0.055	1.73063E-07	1.73063E-05	0.009530411	0.953041067

protein OS=Mus musculus OX=10090 GN=Hspg2 PE=1 SV=1					
Thrombospondin-2 OS=Mus musculus OX=10090 GN=Thbs2 PE=1 SV=2	0.129	4.05912E-07	4.05912E-05	0.022353145	2.235314504
Fibulin-1 OS=Mus musculus OX=10090 GN=Fbln1 PE=1 SV=2	0.125	3.93326E-07	3.93326E-05	0.021660024	2.166002426
	Tot. 317802.5			Tot.5.771	

Table A10 Day 5 Collagen I-coated samples

Sample 2

ECM protein	emPAI	emPAI fraction of tot proteins	mol conc (%)	ECM emPAI fraction	ECM mol conc (%)
Fibronectin OS=Mus musculus OX=10090 GN=Fn1 PE=1 SV=4	2.45	3.51309E-05	0.003513093	0.272010658	27.20106584
Thrombospondin-1 OS=Mus musculus OX=10090 GN=Thbs1 PE=1 SV=1	0.764	1.09551E-05	0.001095512	0.084822916	8.482291551
Collagen alpha-2(I) chain OS=Mus musculus OX=10090 GN=Col1a2 PE=1 SV=2	0.248	3.55611E-06	0.000355611	0.02753414	2.753414011
Basement membrane-specific heparan sulfate proteoglycan core protein OS=Mus musculus OX=10090 GN=Hspg2 PE=1 SV=1	0.16	2.29427E-06	0.000229427	0.017763961	1.776396136
Collagen alpha-1(I) chain OS=Mus musculus OX=10090 GN=Col1a1 PE=1 SV=4	0.201	2.88217E-06	0.000288217	0.022315976	2.231597646
Metalloproteinase inhibitor 3	4.878	6.99464E-05	0.00699464	0.541578772	54.15787721

OS=Mus musculus OX=10090 GN=Timp3 PE=1 SV=1					
Fibulin-1 OS=Mus musculus OX=10090 GN=Fbln1 PE=1 SV=2	0.194	2.7818E-06	0.00027818	0.021538803	2.153880315
Thrombospondin-2 OS=Mus musculus OX=10090 GN=Thbs2 PE=1 SV=2	0.084	1.20449E-06	0.000120449	0.00932608	0.932607972
Proteoglycan 4 OS=Mus musculus OX=10090 GN=Prg4 PE=1 SV=2	0.028	4.01496E-07	4.01496E-05	0.003108693	0.310869324
	Tot. 69739.11			Tot. 9.007	

Table A11 Day 5 Laminin 411-coated samples

Sample 1

ECM protein	emPAI	emPAI fraction of tot proteins	mol conc (%)	ECM emPAI fraction	ECM mol conc (%)
Fibronectin OS=Mus musculus OX=10090 GN=Fn1 PE=1 SV=4	6.337	9.26927E-06	0.000926927	0.742994489	74.29944894
Basement membrane-specific heparan sulfate proteoglycan core protein OS=Mus musculus OX=10090 GN=Hspg2 PE=1 SV=1	0.4	5.85089E-07	5.85089E-05	0.046898816	4.68988158
Laminin subunit alpha-4 OS=Mus musculus OX=10090 GN=Lama4 PE=1 SV=2	0.286	4.18339E-07	4.18339E-05	0.033532653	3.35326533
Thrombospondin-1 OS=Mus musculus OX=10090 GN=Thbs1 PE=1 SV=1	0.415	6.0703E-07	6.0703E-05	0.048657521	4.86575214
Laminin subunit gamma-1 OS=Mus musculus OX=10090 GN=Lamc1 PE=1 SV=2	0.262	3.83233E-07	3.83233E-05	0.030718724	3.071872435
Laminin subunit beta-1 OS=Mus musculus OX=10090	0.349	5.1049E-07	5.1049E-05	0.040919217	4.091921679

GN=Lamb1 PE=1 SV=3					
Nidogen-2 OS=Mus musculus OX=10090 GN=Nid2 PE=1 SV=2	0.194	2.83768E-07	2.83768E-05	0.022745926	2.274592567
Laminin subunit alpha-5 OS=Mus musculus OX=10090 GN=Lama5 PE=1 SV=4	0.052	7.60616E-08	7.60616E-06	0.006096846	0.609684605
Fibulin-1 OS=Mus musculus OX=10090 GN=Fbln1 PE=1 SV=2	0.125	1.8284E-07	1.8284E-05	0.01465588	1.465587994
Collagen alpha-2(IV) chain OS=Mus musculus OX=10090 GN=Col4a2 PE=1 SV=4	0.024	3.51053E-08	3.51053E-06	0.002813929	0.281392895
Proteoglycan 4 OS=Mus musculus OX=10090 GN=Prg4 PE=1 SV=2	0.056	8.19125E-08	8.19125E-06	0.006565834	0.656583421
Collagen alpha-1(XVIII) chain OS=Mus musculus OX=10090 GN=Col18a1 PE=1 SV=4	0.029	4.2419E-08	4.2419E-06	0.003400164	0.340016415
	Tot. 683656.7			Tot. 8.529	

Table A12 Day 5 Laminin 411-coated samples

Sample 2

ECM protein	emPAI	emPAI fraction of tot proteins	mol conc (%)	ECM emPAI fraction	ECM mol conc (%)
Fibronectin OS=Mus musculus OX=10090 GN=Fn1 PE=1 SV=4	7.566	1.62167E-05	0.001621666	0.534548538	53.45485375
Basement membrane-specific heparan sulfate proteoglycan core protein OS=Mus musculus OX=10090 GN=Hspg2 PE=1 SV=1	0.934	2.0019E-06	0.00020019	0.065988413	6.598841317
Laminin subunit alpha-4 OS=Mus musculus OX=10090 GN=Lama4 PE=1 SV=2	0.427	9.15214E-07	9.15214E-05	0.03016815	3.016815035
Laminin subunit gamma-1 OS=Mus musculus OX=10090 GN=Lamc1 PE=1 SV=2	0.513	1.09954E-06	0.000109954	0.036244171	3.624417126
Laminin subunit alpha-5 OS=Mus musculus OX=10090 GN=Lama5 PE=1 SV=4	0.149	3.19361E-07	3.19361E-05	0.010527059	1.052705949
Thrombospondin-1 OS=Mus musculus OX=10090	0.371	7.95186E-07	7.95186E-05	0.026211672	2.621167161

GN=Thbs1 PE=1 SV=1					
Laminin subunit beta-1 OS=Mus musculus OX=10090 GN=Lamb1 PE=1 SV=3	0.349	7.48032E-07	7.48032E-05	0.024657341	2.465734068
Metalloproteinase inhibitor 3 OS=Mus musculus OX=10090 GN=Timp3 PE=1 SV=1	3.125	6.698E-06	0.0006698	0.220785644	22.07856436
Nidogen-2 OS=Mus musculus OX=10090 GN=Nid2 PE=1 SV=2	0.281	6.02284E-07	6.02284E-05	0.019853045	1.985304508
Nidogen-1 OS=Mus musculus OX=10090 GN=Nid1 PE=1 SV=2	0.124	2.65777E-07	2.65777E-05	0.008760774	0.876077434
Agrin OS=Mus musculus OX=10090 GN=Agrn PE=1 SV=1	0.075	1.60752E-07	1.60752E-05	0.005298855	0.529885545
Fibulin-1 OS=Mus musculus OX=10090 GN=Fbln1 PE=1 SV=2	0.125	2.6792E-07	2.6792E-05	0.008831426	0.883142575
Proteoglycan 4 OS=Mus musculus OX=10090 GN=Prg4 PE=1 SV=2	0.056	1.20028E-07	1.20028E-05	0.003956479	0.395647873
Collagen alpha-1(XVIII) chain	0.059	1.26458E-07	1.26458E-05	0.004168433	0.416843295

OS=Mus musculus OX=10090 GN=Col18a1 PE=1 SV=4					
	Tot. 466557.3			Tot. 14.154	

Table A13 Day 10 Collagen I-coated samples

Sample 1

ECM protein	emPAI	emPAI fraction of tot proteins	mol conc (%)	ECM emPAI fraction	ECM mol conc (%)
Fibronectin OS=Mus musculus OX=10090 GN=Fn1 PE=1 SV=4	4.179	0.000271659	0.027165943	0.466302165	46.63021647
Basement membrane-specific heparan sulfate proteoglycan core protein OS=Mus musculus OX=10090 GN=Hspg2 PE=1 SV=1	0.883	5.74002E-05	0.005740016	0.098527114	9.852711448
Periostin OS=Mus musculus OX=10090 GN=Postn PE=1 SV=2	1.047	6.80611E-05	0.006806112	0.116826601	11.68266012
Thrombospondin-1 OS=Mus musculus OX=10090 GN=Thbs1 PE=1 SV=1	0.247	1.60564E-05	0.001605644	0.027560812	2.756081232
Collagen alpha-1(I) chain OS=Mus musculus OX=10090 GN=Col1a1 PE=1 SV=4	0.14	9.10082E-06	0.000910082	0.015621513	1.562151306
Metalloproteinase inhibitor 3 OS=Mus musculus	1.424	9.25683E-05	0.009256832	0.158893104	15.88931042

OX=10090 GN=Timp3 PE=1 SV=1					
Collagen alpha-2(I) chain OS=Mus musculus OX=10090 GN=Col1a2 PE=1 SV=2	0.087	5.65551E-06	0.000565551	0.009707655	0.970765454
Fibrillin-2 OS=Mus musculus OX=10090 GN=Fbn2 PE=1 SV=2	0.079	5.13546E-06	0.000513546	0.008814997	0.881499665
Laminin subunit alpha-5 OS=Mus musculus OX=10090 GN=Lama5 PE=1 SV=4	0.039	2.53523E-06	0.000253523	0.004351707	0.435170721
Fibulin-1 OS=Mus musculus OX=10090 GN=Fbln1 PE=1 SV=2	0.266	1.72916E-05	0.001729155	0.029680875	2.96808748
Laminin subunit alpha-4 OS=Mus musculus OX=10090 GN=Lama4 PE=1 SV=2	0.043	2.79525E-06	0.000279525	0.004798036	0.479803615
Nidogen-2 OS=Mus musculus OX=10090 GN=Nid2 PE=1 SV=2	0.112	7.28065E-06	0.000728065	0.01249721	1.249721044
Nidogen-1 OS=Mus musculus OX=10090 GN=Nid1 PE=1 SV=2	0.124	8.06072E-06	0.000806072	0.013836197	1.383619728

Galectin-1 OS=Mus musculus OX=10090 GN=Lgals1 PE=1 SV=3	0.292	1.89817E-05	0.001898171	0.032582013	3.258201294
	Tot. 15383.232			Tot. 8.962	

Table A14 Day 10 Collagen I-coated samples

Sample 2

ECM protein	emPAI	emPAI fraction of tot proteins	mol conc (%)	ECM emPAI fraction	ECM mol conc (%)
Fibronectin OS=Mus musculus OX=10090 GN=Fn1 PE=1 SV=4	8.808	4.04964E-05	0.004049637	0.367612688	36.76126878
Basement membrane-specific heparan sulfate proteoglycan core protein OS=Mus musculus OX=10090 GN=Hspg2 PE=1 SV=1	1.041	4.78618E-06	0.000478618	0.043447412	4.344741235
Periostin OS=Mus musculus OX=10090 GN=Postn PE=1 SV=2	5.989	2.75355E-05	0.002753551	0.249958264	24.99582638
EMILIN-1 OS=Mus musculus OX=10090 GN=Emilin1 PE=1 SV=1	0.874	4.01837E-06	0.000401837	0.036477462	3.647746244
Laminin subunit gamma-1 OS=Mus musculus OX=10090 GN=Lamc1 PE=1 SV=2	0.4	1.83907E-06	0.000183907	0.016694491	1.669449082
Collagen alpha-1(I) chain OS=Mus	0.369	1.69654E-06	0.000169654	0.015400668	1.540066778

musculus OX=10090 GN=Col1a1 PE=1 SV=4					
Nidogen-1 OS=Mus musculus OX=10090 GN=Nid1 PE=1 SV=2	0.477	2.19309E-06	0.000219309	0.01990818	1.99081803
Nidogen-2 OS=Mus musculus OX=10090 GN=Nid2 PE=1 SV=2	0.585	2.68964E-06	0.000268964	0.024415693	2.441569282
Laminin subunit beta-1 OS=Mus musculus OX=10090 GN=Lamb1 PE=1 SV=3	0.288	1.32413E-06	0.000132413	0.012020033	1.202003339
Fibrillin-1 OS=Mus musculus OX=10090 GN=Fbn1 PE=1 SV=2	0.181	8.3218E-07	8.3218E-05	0.007554257	0.75542571
Laminin subunit alpha-1 OS=Mus musculus OX=10090 GN=Lama1 PE=1 SV=1	0.167	7.67813E-07	7.67813E-05	0.00696995	0.696994992
Collagen alpha-1(XVIII) chain OS=Mus musculus OX=10090 GN=Col18a1 PE=1 SV=4	0.186	8.55168E-07	8.55168E-05	0.007762938	0.776293823
Collagen alpha-2(I) chain OS=Mus musculus OX=10090	0.149	6.85054E-07	6.85054E-05	0.006218698	0.621869783

GN=Col1a2 PE=1 SV=2					
Decorin OS=Mus musculus OX=10090 GN=Dcn PE=1 SV=1	0.65	2.98849E-06	0.000298849	0.027128548	2.712854758
Thrombospondin-1 OS=Mus musculus OX=10090 GN=Thbs1 PE=1 SV=1	0.208	9.56317E-07	9.56317E-05	0.008681135	0.868113523
Collagen alpha-1(III) chain OS=Mus musculus OX=10090 GN=Col3a1 PE=1 SV=4	0.22	1.01149E-06	0.000101149	0.00918197	0.918196995
Laminin subunit alpha-5 OS=Mus musculus OX=10090 GN=Lama5 PE=1 SV=4	0.052	2.39079E-07	2.39079E-05	0.002170284	0.217028381
Collagen alpha-1(V) chain OS=Mus musculus OX=10090 GN=Col5a1 PE=1 SV=2	0.123	5.65515E-07	5.65515E-05	0.005133556	0.513355593
Lumican OS=Mus musculus OX=10090 GN=Lum PE=1 SV=2	1.738	7.99077E-06	0.000799077	0.072537563	7.25375626
Collagen alpha-1(VI) chain OS=Mus musculus OX=10090 GN=Col6a1 PE=1 SV=1	0.169	7.77008E-07	7.77008E-05	0.007053422	0.705342237

Agrin OS=Mus musculus OX=10090 GN=Agrn PE=1 SV=1	0.049	2.25286E-07	2.25286E-05	0.002045075	0.204507513
Collagen alpha-2(IV) chain OS=Mus musculus OX=10090 GN=Col4a2 PE=1 SV=4	0.049	2.25286E-07	2.25286E-05	0.002045075	0.204507513
Fibulin-1 OS=Mus musculus OX=10090 GN=Fbln1 PE=1 SV=2	0.125	5.7471E-07	5.7471E-05	0.005217028	0.521702838
Biglycan OS=Mus musculus OX=10090 GN=Bgn PE=1 SV=1	0.274	1.25976E-06	0.000125976	0.011435726	1.143572621
Fibrillin-2 OS=Mus musculus OX=10090 GN=Fbn2 PE=1 SV=2	0.046	2.11493E-07	2.11493E-05	0.001919866	0.191986644
Tenascin OS=Mus musculus OX=10090 GN=Tnc PE=1 SV=1	0.021	9.65513E-08	9.65513E-06	0.000876461	0.087646077
Galectin-1 OS=Mus musculus OX=10090 GN=Lgals1 PE=1 SV=3	0.668	3.07125E-06	0.000307125	0.0278798	2.787979967
Proteoglycan 4 OS=Mus musculus OX=10090	0.028	1.28735E-07	1.28735E-05	0.001168614	0.116861436

GN=Prg4 PE=1 SV=2					
Collagen alpha-2(V) chain OS=Mus musculus OX=10090 GN=Col5a2 PE=1 SV=1	0.026	1.1954E-07	1.1954E-05	0.001085142	0.10851419
	Tot. 217501			Tot.23.96	

Table A15 Day 10 Laminin 411-coated samples

Sample 1

ECM protein	emPAI	emPAI fraction of tot proteins	mol conc (%)	ECM emPAI fraction	ECM mol conc (%)
Fibronectin OS=Mus musculus OX=10090 GN=Fn1 PE=1 SV=4	3.887	0.000731903	0.073190328	0.396309135	39.63091354
Basement membrane-specific heparan sulfate proteoglycan core protein OS=Mus musculus OX=10090 GN=Hspg2 PE=1 SV=1	0.934	0.000175868	0.017586768	0.095228385	9.522838499
Periostin OS=Mus musculus OX=10090 GN=Postn PE=1 SV=2	1.783	0.00033573	0.033573027	0.181790375	18.17903752
EMILIN-1 OS=Mus musculus OX=10090 GN=Emilin1 PE=1 SV=1	0.52	9.79135E-05	0.009791348	0.053017945	5.301794454
Laminin subunit gamma-1 OS=Mus musculus OX=10090 GN=Lamc1 PE=1 SV=2	0.23	4.33079E-05	0.004330789	0.023450245	2.34502447
Thrombospondin-1 OS=Mus musculus OX=10090	0.328	6.17608E-05	0.006176081	0.033442088	3.344208809

GN=Thbs1 PE=1 SV=1					
Laminin subunit alpha-4 OS=Mus musculus OX=10090 GN=Lama4 PE=1 SV=2	0.233	4.38728E-05	0.004387277	0.023756117	2.375611746
Laminin subunit alpha-5 OS=Mus musculus OX=10090 GN=Lama5 PE=1 SV=4	0.121	2.27837E-05	0.002278371	0.012336868	1.233686786
Laminin subunit beta-1 OS=Mus musculus OX=10090 GN=Lamb1 PE=1 SV=3	0.202	3.80356E-05	0.003803562	0.020595432	2.05954323
Nidogen-2 OS=Mus musculus OX=10090 GN=Nid2 PE=1 SV=2	0.194	3.65293E-05	0.003652926	0.019779772	1.977977162
Fibulin-1 OS=Mus musculus OX=10090 GN=Fbln1 PE=1 SV=2	0.194	3.65293E-05	0.003652926	0.019779772	1.977977162
Galectin-9 OS=Mus musculus OX=10090 GN=Lgals9 PE=1 SV=1	0.311	5.85598E-05	0.005855979	0.031708809	3.170880914
Nidogen-1 OS=Mus musculus OX=10090 GN=Nid1 PE=1 SV=2	0.124	2.33486E-05	0.00233486	0.012642741	1.264274062
Collagen alpha-1(I) chain	0.027	5.08397E-06	0.000508397	0.002752855	0.275285481

OS=Mus musculus OX=10090 GN=Col1a1 PE=1 SV=4					
Galectin-3-binding protein OS=Mus musculus OX=10090 GN=Lgals3bp PE=1 SV=1	0.186	3.50229E-05	0.00350229	0.018964111	1.896411093
Collagen alpha-1(IV) chain OS=Mus musculus OX=10090 GN=Col4a1 PE=1 SV=4	0.027	5.08397E-06	0.000508397	0.002752855	0.275285481
Fibrillin-1 OS=Mus musculus OX=10090 GN=Fbn1 PE=1 SV=2	0.042	7.9084E-06	0.00079084	0.004282219	0.42822186
Collagen alpha-1(XVIII) chain OS=Mus musculus OX=10090 GN=Col18a1 PE=1 SV=4	0.059	1.11094E-05	0.001110941	0.006015498	0.601549755
Agrin OS=Mus musculus OX=10090 GN=Agrn PE=1 SV=1	0.024	4.51908E-06	0.000451908	0.002446982	0.244698206
Collagen alpha-1(III) chain OS=Mus musculus OX=10090 GN=Col3a1 PE=1 SV=4	0.059	1.11094E-05	0.001110941	0.006015498	0.601549755
Galectin-1 OS=Mus	0.292	5.49822E-05	0.005498219	0.029771615	2.977161501

musculus OX=10090 GN=Lgals1 PE=1 SV=3					
Fibrillin-2 OS=Mus musculus OX=10090 GN=Fbn2 PE=1 SV=2	0.031	5.83715E-06	0.000583715	0.003160685	0.316068515
	Tot. 5310.811			Tot. 9.808	

Table A16 Day 10 Laminin 411-coated samples

Sample 2

ECM protein	emPAI	emPAI fraction of tot proteins	mol conc (%)	ECM emPAI fraction	ECM mol conc (%)
Fibronectin OS=Mus musculus OX=10090 GN=Fn1 PE=1 SV=4	7.241	2.27285E-05	0.002272852	0.386269071	38.62690707
Basement membrane-specific heparan sulfate proteoglycan core protein OS=Mus musculus OX=10090 GN=Hspg2 PE=1 SV=1	2.546	7.99155E-06	0.000799155	0.135815641	13.58156407
Periostin OS=Mus musculus OX=10090 GN=Postn PE=1 SV=2	3.41	1.07035E-05	0.001070353	0.181905473	18.19054732
EMILIN-1 OS=Mus musculus OX=10090 GN=Emilin1 PE=1 SV=1	0.723	2.2694E-06	0.00022694	0.038568228	3.856822789
Nidogen-2 OS=Mus musculus OX=10090 GN=Nid2 PE=1 SV=2	0.826	2.5927E-06	0.00025927	0.044062733	4.406273338
Laminin subunit gamma-1 OS=Mus musculus OX=10090	0.552	1.73265E-06	0.000173265	0.029446282	2.944628187

GN=Lamc1 PE=1 SV=2					
Fibrillin-2 OS=Mus musculus OX=10090 GN=Fbn2 PE=1 SV=2	0.313	9.82465E-07	9.82465E-05	0.016696895	1.669689534
Fibrillin-1 OS=Mus musculus OX=10090 GN=Fbn1 PE=1 SV=2	0.181	5.68135E-07	5.68135E-05	0.009655393	0.965539315
Laminin subunit alpha-5 OS=Mus musculus OX=10090 GN=Lama5 PE=1 SV=4	0.194	6.0894E-07	6.0894E-05	0.010348874	1.034887443
Laminin subunit alpha-4 OS=Mus musculus OX=10090 GN=Lama4 PE=1 SV=2	0.259	8.12966E-07	8.12966E-05	0.013816281	1.381628081
Laminin subunit beta-1 OS=Mus musculus OX=10090 GN=Lamb1 PE=1 SV=3	0.288	9.03993E-07	9.03993E-05	0.015363277	1.53632775
Collagen alpha-1(I) chain OS=Mus musculus OX=10090 GN=Col1a1 PE=1 SV=4	0.299	9.38521E-07	9.38521E-05	0.015950069	1.595006935
Thrombospondin-1 OS=Mus musculus OX=10090 GN=Thbs1 PE=1 SV=1	0.287	9.00854E-07	9.00854E-05	0.015309933	1.530993279
Nidogen-1	0.264	8.2866E-07	8.2866E-05	0.014083004	1.408300437

OS=Mus musculus OX=10090 GN=Nid1 PE=1 SV=2					
Agrin OS=Mus musculus OX=10090 GN=Agrn PE=1 SV=1	0.183	5.74412E-07	5.74412E-05	0.009762083	0.976208258
Collagen alpha-1(XVIII) chain OS=Mus musculus OX=10090 GN=Col18a1 PE=1 SV=4	0.12	3.76664E-07	3.76664E-05	0.006401366	0.640136562
Collagen alpha-1(III) chain OS=Mus musculus OX=10090 GN=Col3a1 PE=1 SV=4	0.186	5.83829E-07	5.83829E-05	0.009922117	0.992211672
Collagen alpha-1(V) chain OS=Mus musculus OX=10090 GN=Col5a1 PE=1 SV=2	0.097	3.0447E-07	3.0447E-05	0.005174437	0.517443721
Laminin subunit alpha-1 OS=Mus musculus OX=10090 GN=Lama1 PE=1 SV=1	0.073	2.29137E-07	2.29137E-05	0.003894164	0.389416409
Fibulin-1 OS=Mus musculus OX=10090 GN=Fbln1 PE=1 SV=2	0.343	1.07663E-06	0.000107663	0.018297237	1.829723674
Versican core protein OS=Mus musculus	0.035	1.0986E-07	1.0986E-05	0.001867065	0.186706497

OX=10090 GN=Vcan PE=1 SV=2					
Collagen alpha-1(IV) chain OS=Mus musculus OX=10090 GN=Col4a1 PE=1 SV=4	0.027	8.47494E-08	8.47494E-06	0.001440307	0.144030727
Collagen alpha-2(I) chain OS=Mus musculus OX=10090 GN=Col1a2 PE=1 SV=2	0.057	1.78915E-07	1.78915E-05	0.003040649	0.304064867
Galectin-3-binding protein OS=Mus musculus OX=10090 GN=Lgals3bp PE=1 SV=1	0.089	2.79359E-07	2.79359E-05	0.00474768	0.47476795
Biglycan OS=Mus musculus OX=10090 GN=Bgn PE=1 SV=1	0.129	4.04914E-07	4.04914E-05	0.006881468	0.688146805
Collagen alpha-2(IV) chain OS=Mus musculus OX=10090 GN=Col4a2 PE=1 SV=4	0.024	7.53328E-08	7.53328E-06	0.001280273	0.128027312
	Tot. 318586.5			Tot. 18.746	

Table A17 Day 14 Collagen I-coated samples

Sample 1

ECM protein	emPAI	emPAI fraction of tot proteins	mol conc (%)	ECM emPAI fraction	ECM mol conc (%)
Fibronectin OS=Mus musculus OX=10090 GN=Fn1 PE=1 SV=4	8.62	0.014384575	1.438457546	0.246243501	24.62435011
Basement membrane-specific heparan sulfate proteoglycan core protein OS=Mus musculus OX=10090 GN=Hspg2 PE=1 SV=1	3.518	0.005870642	0.587064228	0.100497058	10.04970576
Laminin subunit alpha-5 OS=Mus musculus OX=10090 GN=Lama5 PE=1 SV=4	0.597	0.00099624	0.099624032	0.017054219	1.705421928
Nidogen-2 OS=Mus musculus OX=10090 GN=Nid2 PE=1 SV=2	3.273	0.0054618	0.546179994	0.093498257	9.349825744
Laminin subunit gamma-1 OS=Mus musculus OX=10090 GN=Lamc1 PE=1 SV=2	1.288	0.002149343	0.21493426	0.036793693	3.679369251
EMILIN-1 OS=Mus musculus OX=10090	1.619	0.002701697	0.270169695	0.046249214	4.624921442

GN=Emilin1 PE=1 SV=1					
Laminin subunit beta-1 OS=Mus musculus OX=10090 GN=Lamb1 PE=1 SV=3	0.95	0.001585307	0.158530704	0.027138205	2.713820488
Periostin OS=Mus musculus OX=10090 GN=Postn PE=1 SV=2	2.594	0.004328723	0.432872259	0.074101583	7.410158259
Nidogen-1 OS=Mus musculus OX=10090 GN=Nid1 PE=1 SV=2	1.551	0.002588222	0.258822234	0.04430669	4.430669028
Collagen alpha-1(VI) chain OS=Mus musculus OX=10090 GN=Col6a1 PE=1 SV=1	1.36	0.002269492	0.226949218	0.038850483	3.885048277
Laminin subunit alpha-1 OS=Mus musculus OX=10090 GN=Lama1 PE=1 SV=1	0.381	0.000635792	0.063579156	0.010883848	1.088384848
Fibrillin-1 OS=Mus musculus OX=10090 GN=Fbn1 PE=1 SV=2	0.181	0.000302043	0.030204271	0.005170542	0.517054219
Collagen alpha-2(VI) chain OS=Mus musculus OX=10090 GN=Col6a2 PE=1 SV=3	0.746	0.001244883	0.124488321	0.021310632	2.131063246

Agrin OS=Mus musculus OX=10090 GN=Agrn PE=1 SV=1	0.302	0.000503961	0.050396076	0.008627092	0.86270925
Collagen alpha-2(I) chain OS=Mus musculus OX=10090 GN=Col1a2 PE=1 SV=2	0.284	0.000473923	0.047392337	0.008112895	0.811289493
Collagen alpha-1(I) chain OS=Mus musculus OX=10090 GN=Col1a1 PE=1 SV=4	0.369	0.000615767	0.061576663	0.01054105	1.054105011
Galectin-9 OS=Mus musculus OX=10090 GN=Lgals9 PE=1 SV=1	1.955	0.003262395	0.326239502	0.055847569	5.584756899
Fibrillin-2 OS=Mus musculus OX=10090 GN=Fbn2 PE=1 SV=2	0.095	0.000158531	0.01585307	0.00271382	0.271382049
Tenascin OS=Mus musculus OX=10090 GN=Tnc PE=1 SV=1	0.181	0.000302043	0.030204271	0.005170542	0.517054219
Collagen alpha-1(V) chain OS=Mus musculus OX=10090 GN=Col5a1 PE=1 SV=2	0.233	0.000388817	0.038881741	0.006656002	0.665600183
Collagen alpha-1(III) chain	0.292	0.000487273	0.048727332	0.008341427	0.834142718

OS=Mus musculus OX=10090 GN=Col3a1 PE=1 SV=4					
Lumican OS=Mus musculus OX=10090 GN=Lum PE=1 SV=2	1.371	0.002287848	0.228784837	0.039164715	3.916471462
Decorin OS=Mus musculus OX=10090 GN=Dcn PE=1 SV=1	0.823	0.001373377	0.137337652	0.023510255	2.351025538
Fibulin-2 OS=Mus musculus OX=10090 GN=Fbln2 PE=1 SV=2	0.169	0.000282018	0.028201778	0.004827744	0.482774382
Laminin subunit beta-2 OS=Mus musculus OX=10090 GN=Lamb2 PE=1 SV=2	0.114	0.000190237	0.019023684	0.003256585	0.325658459
Collagen alpha-1(IV) chain OS=Mus musculus OX=10090 GN=Col4a1 PE=1 SV=4	0.085	0.000141843	0.014184326	0.002428155	0.242815517
Collagen alpha-1(XVIII) chain OS=Mus musculus OX=10090 GN=Col18a1 PE=1 SV=4	0.12	0.000200249	0.020024931	0.003427984	0.342798377
Fibulin-1 OS=Mus musculus OX=10090	0.125	0.000208593	0.020859303	0.003570816	0.357081643

GN=Fbln1 PE=1 SV=2					
Thrombospondin-1 OS=Mus musculus OX=10090 GN=Thbs1 PE=1 SV=1	0.065	0.000108468	0.010846838	0.001856825	0.185682454
Collagen alpha-1(XIV) chain OS=Mus musculus OX=10090 GN=Col14a1 PE=1 SV=2	0.053	8.84434E-05	0.008844345	0.001514026	0.151402617
Biglycan OS=Mus musculus OX=10090 GN=Bgn PE=1 SV=1	0.438	0.00073091	0.073090998	0.012512141	1.251214078
Metalloproteinase inhibitor 3 OS=Mus musculus OX=10090 GN=Timp3 PE=1 SV=1	0.425	0.000709216	0.070921631	0.012140776	1.214077587
Galectin-1 OS=Mus musculus OX=10090 GN=Lgals1 PE=1 SV=3	0.292	0.000487273	0.048727332	0.008341427	0.834142718
Galectin-3-binding protein OS=Mus musculus OX=10090 GN=Lgals3bp PE=1 SV=1	0.186	0.000310386	0.031038643	0.005313375	0.531337485
Extracellular matrix protein 1 OS=Mus musculus OX=10090	0.129	0.000215268	0.021526801	0.003685083	0.368508256

GN=Ecm1 PE=1 SV=2					
Mimecan OS=Mus musculus OX=10090 GN=Ogn PE=1 SV=1	0.145	0.000241968	0.024196792	0.004142147	0.414214706
Collagen alpha-2(IV) chain OS=Mus musculus OX=10090 GN=Col4a2 PE=1 SV=4	0.049	8.17685E-05	0.008176847	0.00139976	0.139976004
Collagen alpha-1(XII) chain OS=Mus musculus OX=10090 GN=Col12a1 PE=2 SV=3	0.028	4.67248E-05	0.004672484	0.000799863	0.079986288
	Tot. 599.253			Tot.35.006	

Table A18 Day 14 Collagen I-coated samples

Sample 2

ECM protein	emPAI	emPAI fraction of tot proteins	mol conc (%)	ECM emPAI fraction	ECM mol conc (%)
Fibronectin OS=Mus musculus OX=10090 GN=Fn1 PE=1 SV=4	9.598	0.009712206	0.971220583	0.265057579	26.50575792
Basement membrane-specific heparan sulfate proteoglycan core protein OS=Mus musculus OX=10090 GN=Hspg2 PE=1 SV=1	3.833	0.003878609	0.387860856	0.105851813	10.5851813
Laminin subunit alpha-5 OS=Mus musculus OX=10090 GN=Lama5 PE=1 SV=4	0.577	0.000583866	0.058386568	0.015934385	1.593438458
Nidogen-2 OS=Mus musculus OX=10090 GN=Nid2 PE=1 SV=2	2.455	0.002484212	0.248421185	0.067797078	6.779707824
Periostin OS=Mus musculus OX=10090 GN=Postn PE=1 SV=2	2.594	0.002624866	0.26248658	0.071635691	7.163569081
EMILIN-1 OS=Mus musculus OX=10090 GN=Emilin1	1.215	0.001229457	0.122945719	0.03355334	3.355334015

PE=1 SV=1					
Laminin subunit gamma-1 OS=Mus musculus OX=10090 GN=Lamc1 PE=1 SV=2	0.959	0.000970411	0.097041106	0.026483665	2.648366518
Collagen alpha-1(VI) chain OS=Mus musculus OX=10090 GN=Col6a1 PE=1 SV=1	1.36	0.001376183	0.137618253	0.037557648	3.755764823
Nidogen-1 OS=Mus musculus OX=10090 GN=Nid1 PE=1 SV=2	1.27	0.001285112	0.128511163	0.035072216	3.507221563
Collagen alpha-2(VI) chain OS=Mus musculus OX=10090 GN=Col6a2 PE=1 SV=3	0.682	0.000690115	0.069011506	0.018834056	1.883405595
Laminin subunit beta-1 OS=Mus musculus OX=10090 GN=Lamb1 PE=1 SV=3	0.549	0.000555533	0.055553251	0.015161139	1.516113888
Agrin OS=Mus musculus OX=10090 GN=Agrn PE=1 SV=1	0.241	0.000243868	0.024386764	0.006655436	0.665543619
Fibrillin-1 OS=Mus musculus OX=10090 GN=Fbn1 PE=1 SV=2	0.133	0.000134583	0.013458256	0.003672917	0.367291707
Laminin subunit	0.184	0.000186189	0.01861894	0.005081329	0.508132888

alpha-1 OS=Mus musculus OX=10090 GN=Lama1 PE=1 SV=1					
Biglycan OS=Mus musculus OX=10090 GN=Bgn PE=1 SV=1	1.976	0.001999512	0.199951226	0.054569054	5.45690536
Fibulin-2 OS=Mus musculus OX=10090 GN=Fbln2 PE=1 SV=2	0.264	0.000267141	0.026714131	0.007290602	0.72906023
Collagen alpha-1(I) chain OS=Mus musculus OX=10090 GN=Col1a1 PE=1 SV=4	0.201	0.000203392	0.020339168	0.005550799	0.555079948
Lumican OS=Mus musculus OX=10090 GN=Lum PE=1 SV=2	2.162	0.002187725	0.218772546	0.059705614	5.970561432
Collagen alpha-1(III) chain OS=Mus musculus OX=10090 GN=Col3a1 PE=1 SV=4	0.292	0.000295474	0.029547448	0.008063848	0.8063848
Decorin OS=Mus musculus OX=10090 GN=Dcn PE=1 SV=1	1.228	0.001242612	0.124261187	0.033912347	3.391234708
Galectin-9 OS=Mus musculus OX=10090	1.955	0.001978262	0.197826239	0.053989119	5.398911933

GN=Lgals9 PE=1 SV=1					
Collagen alpha-1(V) chain OS=Mus musculus OX=10090 GN=Col5a1 PE=1 SV=2	0.123	0.000124464	0.012446357	0.003396758	0.339675789
Fibrillin-2 OS=Mus musculus OX=10090 GN=Fbn2 PE=1 SV=2	0.079	7.994E-05	0.007994001	0.002181658	0.218165751
Tenascin OS=Mus musculus OX=10090 GN=Tnc PE=1 SV=1	0.156	0.000157856	0.015785623	0.004308083	0.430808318
Collagen alpha-2(I) chain OS=Mus musculus OX=10090 GN=Col1a2 PE=1 SV=2	0.149	0.000150773	0.015077294	0.004114772	0.411477175
Laminin subunit beta-2 OS=Mus musculus OX=10090 GN=Lamb2 PE=1 SV=2	0.114	0.000115356	0.011535648	0.003148215	0.314821463
Galectin-3-binding protein OS=Mus musculus OX=10090 GN=Lgals3bp PE=1 SV=1	0.407	0.000411843	0.041184286	0.011239679	1.123967855
Galectin-1 OS=Mus musculus OX=10090 GN=Lgals1 PE=1	0.668	0.000675948	0.067594848	0.018447433	1.84474331

SV=3					
Thrombospondin-1 OS=Mus musculus OX=10090 GN=Thbs1 PE=1 SV=1	0.099	0.000100178	0.010017799	0.002733976	0.273397586
Fibulin-1 OS=Mus musculus OX=10090 GN=Fbln1 PE=1 SV=2	0.125	0.000126487	0.012648736	0.00345199	0.345198973
Collagen alpha-1(IV) chain OS=Mus musculus OX=10090 GN=Col4a1 PE=1 SV=4	0.085	8.60114E-05	0.008601141	0.002347353	0.234735301
Collagen alpha-1(XVIII) chain OS=Mus musculus OX=10090 GN=Col18a1 PE=1 SV=4	0.089	9.0059E-05	0.0090059	0.002457817	0.245781669
Microfibrillar-associated protein 2 OS=Mus musculus OX=10090 GN=Mfap2 PE=2 SV=1	0.389	0.000393629	0.039362868	0.010742592	1.074259203
	Tot. 988.241			Tot.36.211	

Table A19 Day 14 Laminin 411-coated samples

Sample 1

ECM protein	emPAI	emPAI fraction of tot proteins	mol conc (%)	ECM emPAI fraction	ECM mol conc (%)
Fibronectin OS=Mus musculus OX=10090 GN=Fn1 PE=1 SV=4	7.566	0.01003008	1.003007963	0.234764801	23.47648008
Basement membrane-specific heparan sulfate proteoglycan core protein OS=Mus musculus OX=10090 GN=Hspg2 PE=1 SV=1	3.58	0.004745927	0.474592719	0.11108353	11.10835298
Nidogen-2 OS=Mus musculus OX=10090 GN=Nid2 PE=1 SV=2	2.219	0.002941679	0.294167945	0.068853171	6.885317116
Laminin subunit alpha-5 OS=Mus musculus OX=10090 GN=Lama5 PE=1 SV=4	0.48	0.000636325	0.063632543	0.014893881	1.48938811
Laminin subunit gamma-1 OS=Mus musculus OX=10090 GN=Lamc1 PE=1 SV=2	1.23	0.001630584	0.163058392	0.03816557	3.816557031
Laminin subunit beta-1 OS=Mus musculus OX=10090 GN=Lamb1	0.905	0.001199739	0.119973858	0.028081172	2.808117165

PE=1 SV=3					
Periostin OS=Mus musculus OX=10090 GN=Postn PE=1 SV=2	2.415	0.003201512	0.320151233	0.074934839	7.493483927
Nidogen-1 OS=Mus musculus OX=10090 GN=Nid1 PE=1 SV=2	1.454	0.001927536	0.192753579	0.045116048	4.511604816
EMILIN-1 OS=Mus musculus OX=10090 GN=Emilin1 PE=1 SV=1	0.954	0.001264697	0.12646968	0.029601589	2.960158868
Laminin subunit alpha-1 OS=Mus musculus OX=10090 GN=Lama1 PE=1 SV=1	0.288	0.000381795	0.038179526	0.008936329	0.893632866
Collagen alpha-1(VI) chain OS=Mus musculus OX=10090 GN=Col6a1 PE=1 SV=1	0.727	0.000963768	0.096376789	0.022558024	2.255802408
Collagen alpha-2(VI) chain OS=Mus musculus OX=10090 GN=Col6a2 PE=1 SV=3	0.682	0.000904112	0.090411239	0.021161723	2.116172273
Collagen alpha-1(I) chain OS=Mus musculus OX=10090 GN=Col1a1 PE=1 SV=4	0.233	0.000308883	0.030888297	0.007229738	0.722973812

Collagen alpha-1(III) chain OS=Mus musculus OX=10090 GN=Col3a1 PE=1 SV=4	0.367	0.000486524	0.048652382	0.011387613	1.138761326
Fibrillin-1 OS=Mus musculus OX=10090 GN=Fbn1 PE=1 SV=2	0.102	0.000135219	0.013521915	0.00316495	0.316494973
Agrin OS=Mus musculus OX=10090 GN=Agrn PE=1 SV=1	0.241	0.000319488	0.031948839	0.007477969	0.747796947
Galectin-9 OS=Mus musculus OX=10090 GN=Lgals9 PE=1 SV=1	1.955	0.0025917	0.259170046	0.060661537	6.066153655
Thrombospondin-1 OS=Mus musculus OX=10090 GN=Thbs1 PE=1 SV=1	0.208	0.000275741	0.027574102	0.006454015	0.645401514
Collagen alpha-1(XVIII) chain OS=Mus musculus OX=10090 GN=Col18a1 PE=1 SV=4	0.12	0.000159081	0.015908136	0.00372347	0.372347027
Laminin subunit beta-2 OS=Mus musculus OX=10090 GN=Lamb2 PE=1 SV=2	0.163	0.000216086	0.021608551	0.005057714	0.505771379
Decorin OS=Mus musculus OX=10090	0.823	0.001091033	0.109103298	0.0255368	2.55368003

GN=Dcn PE=1 SV=1					
Lumican OS=Mus musculus OX=10090 GN=Lum PE=1 SV=2	1.738	0.002304028	0.230402834	0.053928261	5.392826114
Fibrillin-2 OS=Mus musculus OX=10090 GN=Fbn2 PE=1 SV=2	0.079	0.000104729	0.010472856	0.002451285	0.24512846
Galectin-1 OS=Mus musculus OX=10090 GN=Lgals1 PE=1 SV=3	2.594	0.003438809	0.343880869	0.080489016	8.048901576
Fibulin-2 OS=Mus musculus OX=10090 GN=Fbln2 PE=1 SV=2	0.124	0.000164384	0.016438407	0.003847586	0.384758595
Galectin-3-binding protein OS=Mus musculus OX=10090 GN=Lgals3bp PE=1 SV=1	0.407	0.000539551	0.053955094	0.01262877	1.262877001
Fibulin-1 OS=Mus musculus OX=10090 GN=Fbln1 PE=1 SV=2	0.194	0.000257182	0.025718153	0.00601961	0.601961028
Biglycan OS=Mus musculus OX=10090 GN=Bgn PE=1 SV=1	0.274	0.000363236	0.036323577	0.008501924	0.850192379
Collagen alpha-	0.049	6.49582E-05	0.006495822	0.001520417	0.152041703

2(IV) chain OS=Mus musculus OX=10090 GN=Col4a2 PE=1 SV=4					
Collagen alpha-2(I) chain OS=Mus musculus OX=10090 GN=Col1a2 PE=1 SV=2	0.057	7.55636E-05	0.007556365	0.001768648	0.176864838
	Tot. 754.331			Tot.32.228	

Table A20 Day 14 Laminin 411-coated samples

Sample 2

ECM protein	emPAI	emPAI fraction of tot proteins	mol conc (%)	ECM emPAI fraction	ECM mol conc (%)
Fibronectin OS=Mus musculus OX=10090 GN=Fn1 PE=1 SV=4	26.885	0.011699894	1.169989434	0.347701818	34.77018184
Basement membrane-specific heparan sulfate proteoglycan core protein OS=Mus musculus OX=10090 GN=Hspg2 PE=1 SV=1	7.979	0.003472325	0.347232497	0.103191847	10.31918471
Laminin subunit alpha-5 OS=Mus musculus OX=10090 GN=Lama5 PE=1 SV=4	1.219	0.000530488	0.053048805	0.015765241	1.576524146
Nidogen-2 OS=Mus musculus OX=10090 GN=Nid2 PE=1 SV=2	4.878	0.002122823	0.212282256	0.063086832	6.308683169
EMILIN-1 OS=Mus musculus OX=10090 GN=Emilin1 PE=1 SV=1	2.981	0.00129728	0.129728045	0.038553064	3.855306381
Laminin subunit gamma-1 OS=Mus musculus OX=10090 GN=Lamc1	1.673	0.000728061	0.072806112	0.021636792	2.16367916

PE=1 SV=2					
Periostin OS=Mus musculus OX=10090 GN=Postn PE=1 SV=2	4.995	0.002173739	0.217373897	0.064599984	6.459998448
Laminin subunit beta-1 OS=Mus musculus OX=10090 GN=Lamb1 PE=1 SV=3	1.399	0.000608821	0.060882098	0.018093169	1.809316883
Collagen alpha-1(VI) chain OS=Mus musculus OX=10090 GN=Col6a1 PE=1 SV=1	2.101	0.000914319	0.091431943	0.027172086	2.717208556
Fibrillin-2 OS=Mus musculus OX=10090 GN=Fbn2 PE=1 SV=2	0.528	0.000229777	0.022977661	0.006828587	0.682858695
Nidogen-1 OS=Mus musculus OX=10090 GN=Nid1 PE=1 SV=2	1.982	0.000862533	0.086253266	0.025633067	2.563306691
Laminin subunit alpha-1 OS=Mus musculus OX=10090 GN=Lama1 PE=1 SV=1	0.482	0.000209758	0.020975819	0.006233672	0.623367218
Fibrillin-1 OS=Mus musculus OX=10090 GN=Fbn1 PE=1 SV=2	0.284	0.000123592	0.012359197	0.003672952	0.367295207
Collagen alpha-2(VI) chain	0.951	0.000413859	0.041385901	0.012299216	1.229921626

OS=Mus musculus OX=10090 GN=Col6a2 PE=1 SV=3					
Agrin OS=Mus musculus OX=10090 GN=Agrn PE=1 SV=1	0.433	0.000188434	0.018843423	0.005599959	0.559995861
Collagen alpha-1(I) chain OS=Mus musculus OX=10090 GN=Col1a1 PE=1 SV=4	0.481	0.000209323	0.020932301	0.006220739	0.622073925
Collagen alpha-1(III) chain OS=Mus musculus OX=10090 GN=Col3a1 PE=1 SV=4	0.621	0.000270249	0.027024863	0.008031349	0.803134942
Galectin-9 OS=Mus musculus OX=10090 GN=Lgals9 PE=1 SV=1	4.08	0.001775547	0.177554655	0.052766354	5.276635369
Collagen alpha-1(V) chain OS=Mus musculus OX=10090 GN=Col5a1 PE=1 SV=2	0.292	0.000127073	0.012707343	0.003776416	0.377641551
Decorin OS=Mus musculus OX=10090 GN=Dcn PE=1 SV=1	2.325	0.0010118	0.101180042	0.030069062	3.006906185
Lumican OS=Mus musculus OX=10090	5.494	0.002390895	0.239089528	0.071053516	7.105351646

GN=Lum PE=1 SV=2					
Galectin-3-binding protein OS=Mus musculus OX=10090 GN=Lgals3bp PE=1 SV=1	0.978	0.000425609	0.042560895	0.012648405	1.264840537
Tenascin OS=Mus musculus OX=10090 GN=Tnc PE=1 SV=1	0.205	8.92125E-05	0.008921251	0.002651251	0.265125061
Collagen alpha-1(XVIII) chain OS=Mus musculus OX=10090 GN=Col18a1 PE=1 SV=4	0.367	0.000159712	0.015971215	0.004746385	0.474638525
Laminin subunit beta-2 OS=Mus musculus OX=10090 GN=Lamb2 PE=1 SV=2	0.24	0.000104444	0.010444391	0.003103903	0.310390316
Fibulin-2 OS=Mus musculus OX=10090 GN=Fbln2 PE=1 SV=2	0.215	9.35643E-05	0.009356434	0.00278058	0.278057991
Collagen alpha-2(I) chain OS=Mus musculus OX=10090 GN=Col1a2 PE=1 SV=2	0.284	0.000123592	0.012359197	0.003672952	0.367295207
Thrombospondin-1 OS=Mus musculus OX=10090 GN=Thbs1 PE=1	0.208	9.05181E-05	0.009051806	0.002690049	0.26900494

SV=1					
Fibulin-1 OS=Mus musculus OX=10090 GN=Fbln1 PE=1 SV=2	0.425	0.000184953	0.018495277	0.005496495	0.549649518
Biglycan OS=Mus musculus OX=10090 GN=Bgn PE=1 SV=1	0.438	0.00019061	0.019061014	0.005664623	0.566462326
Collagen alpha-2(IV) chain OS=Mus musculus OX=10090 GN=Col4a2 PE=1 SV=4	0.049	2.1324E-05	0.002132397	0.000633714	0.063371356
Laminin subunit alpha-4 OS=Mus musculus OX=10090 GN=Lama4 PE=1 SV=2	0.087	3.78609E-05	0.003786092	0.001125165	0.112516489
Collagen alpha-1(XII) chain OS=Mus musculus OX=10090 GN=Col12a1 PE=2 SV=3	0.014	6.09256E-06	0.000609256	0.000181061	0.018106102
Galectin-1 OS=Mus musculus OX=10090 GN=Lgals1 PE=1 SV=3	0.668	0.000290702	0.029070223	0.008639197	0.863919712
Collagen alpha-1(IV) chain OS=Mus musculus OX=10090 GN=Col4a1 PE=1 SV=4	0.085	3.69906E-05	0.003699055	0.001099299	0.109929904

Extracellular matrix protein 1 OS=Mus musculus OX=10090 GN=Ecm1 PE=1 SV=2	0.129	5.61386E-05	0.00561386	0.001668348	0.166834795
Collagen alpha-1(XIV) chain OS=Mus musculus OX=10090 GN=Col14a1 PE=1 SV=2	0.053	2.30647E-05	0.00230647	0.000685445	0.068544528
Collagen triple helix repeat-containing protein 1 OS=Mus musculus OX=10090 GN=Cthrc1 PE=2 SV=2	0.389	0.000169286	0.016928618	0.00503091	0.50309097
Metalloproteinase inhibitor 3 OS=Mus musculus OX=10090 GN=Timp3 PE=1 SV=1	0.425	0.000184953	0.018495277	0.005496495	0.549649518
	Tot. 2297.884			Tot.77.322	

Table A21 List of proteins other than ECM observed in samples from Experiment 1

Fibronectin	Fibronectin + HUVECs
Fibrinogen alpha chain	Vimentin
Fibrinogen beta chain	Myosin-9
Fibrinogen gamma chain	Elongation factor 1-alpha 1
Serum albumin	Actin, cytoplasmic 1
Immunoglobulin gamma-1 heavy chain	Histone H4
Immunoglobulin lambda-1 light chain	Annexin A2
Immunoglobulin kappa constant	Histone H2B type 1
Keratin, type I cytoskeletal	Histone H2A type 1-B/E
Keratin, type II cytoskeletal 1	40S ribosomal protein S3

Table A22 ECM protein family distribution[21,49-71,79]

Family	Proteins
Basement membrane	Agrin
	BMSHSPCP
	Col alpha 1(IV)
	Col alpha 2(IV)
	ECM 1
	Fibulin 1
	Fibulin 2
	Laminin subunit alpha 1
	Laminin subunit alpha 4
	Laminin subunit alpha 5
	Laminin subunit beta 1
	Laminin subunit beta 2
	Laminin subunit gamma 1
	Nidogen 1
	Nidogen 2
Basement-membrane-associated	Col alpha 1(VI)
	Col alpha 2(VI)
Fibrillar	Col alpha 1(I)
	Col alpha 1(III)
	Col alpha 1(V)
	Col alpha 2(I)
	Col alpha 2(V)
	EGF-cont fib-like ECM prot
	Fibronectin
Fibrill-associated	Col alpha 1(XII)
	Col alpha 1(XIV)
Matricellular	Galectin 1
	Galectin 3-bin prot
	Galectin 9

	Periostin
	Spondin 1
	Thrombospondin 1
	Thrombospondin 2
Microfibril-associated proteins	EMILIN1
	Microfibrillar-associated protein 2
Microfibrillar	Fibrillin 1
	Fibrillin 2
Proteoglycan	Biglycan
	Col alpha 1(XVIII)
	Decorin
	Lumican
	Mimecan
	Proteoglycan 4
	Versican core protein
Remodeling	Col triple hel rep-cont prot1
	Metalloproteinase inhibitor 3
	Tenascin

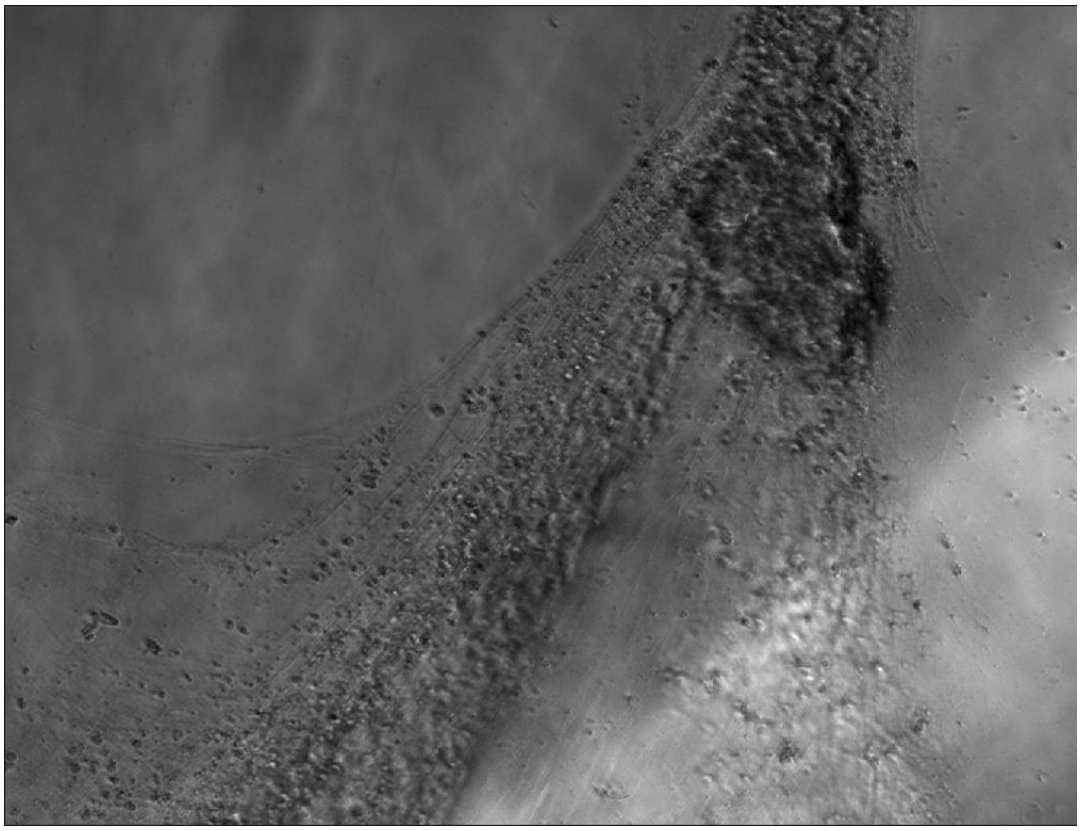


Figure A1. *Colagen I-coated plate well 1, seeded with miPSCs and decellularized 5 days post seeding (10x).*

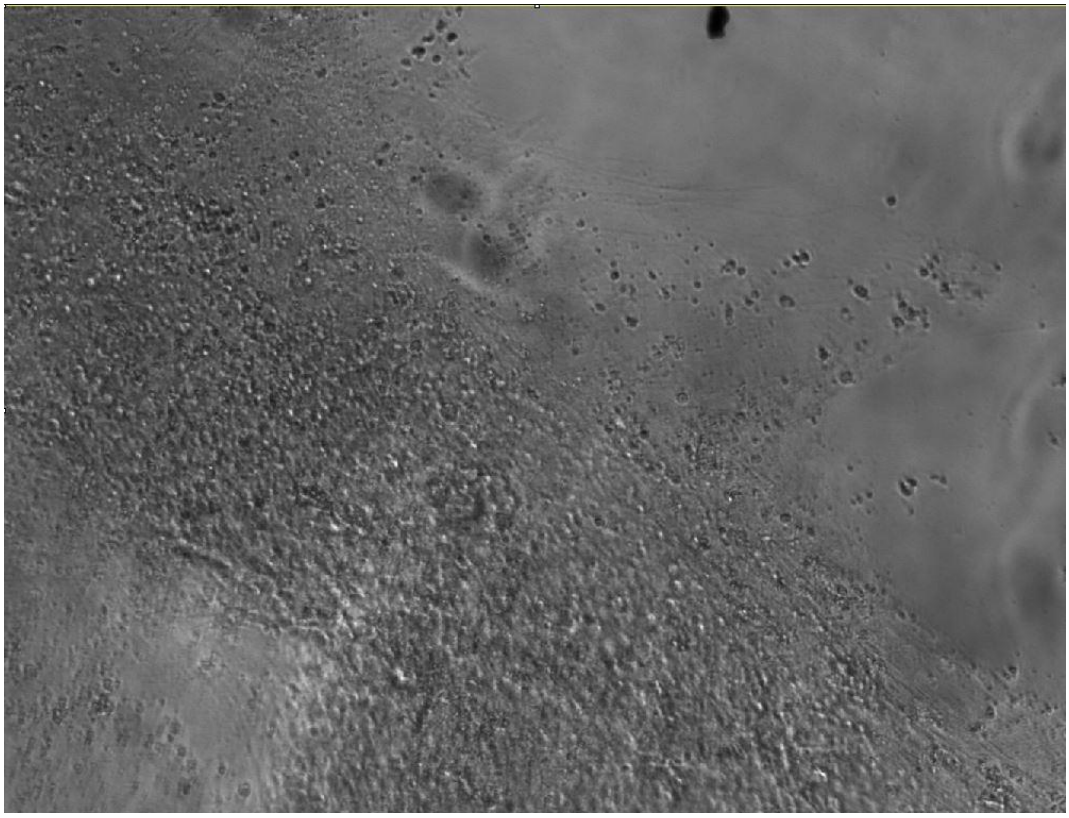


Figure A2. *Laminin 411-coated plate well 1, seeded with miPSCs and decellularized 5 days post seeding (10x).*

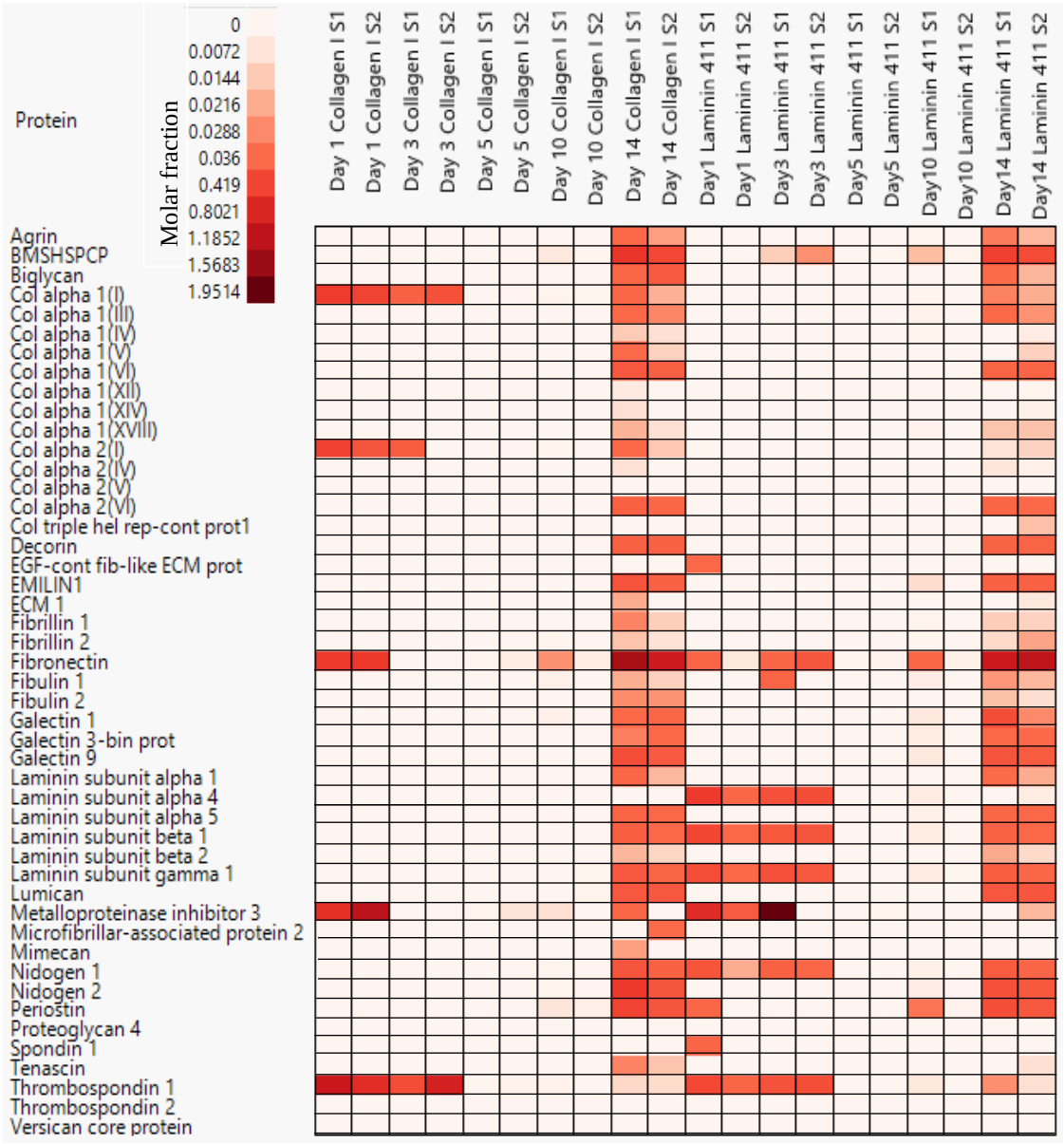


Figure A3. *Molar fraction percentages of ECM proteins, calculated based on total protein emPAI values. Data is given for 2 conditions – Laminin 411- and Collagen I-coated wells with duplicate samples for each conditon (S1-Sample 1, S2- Sample 2).*

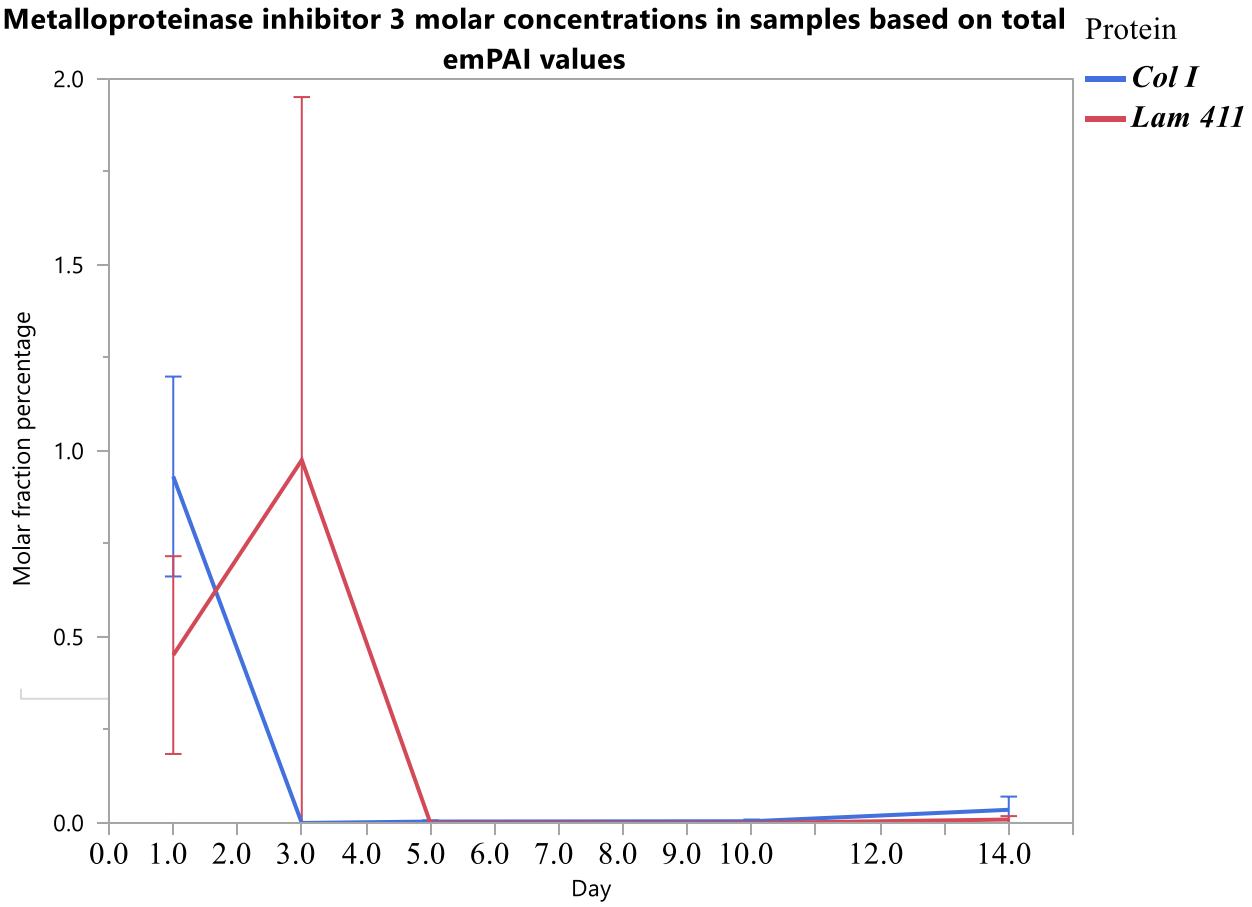


Figure A4. *Molar fraction percentages of Metalloproteinase inhibitor 3 over 5 time points: Day1, Day3, Day5, Day10 and Day14. Values are calculated based on total emPAI values and taken as average of duplicate samples. Error bars are showing standard deviation for samples within duplicates. Data is given for 2 conditions – Laminin 411- and Collagen I-coated wells.*

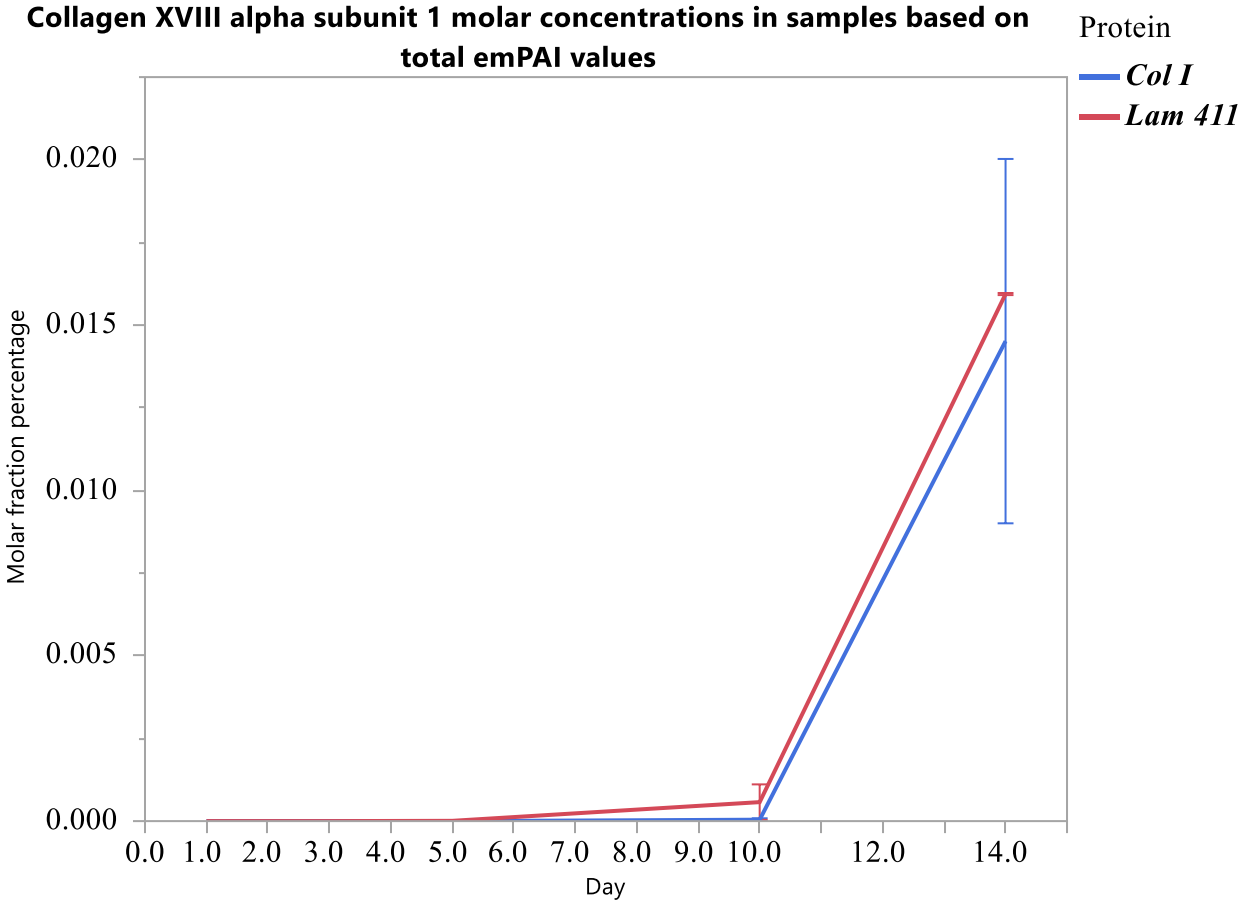


Figure A5. *Molar fraction percentages of Collagen XVIII alpha subunit 1 over 5 time points: Day1, Day3, Day5, Day10 and Day14. Values are calculated based on total emPAI values and taken as average of duplicate samples. Error bars are showing standard deviation for samples within duplicates. Data is given for 2 conditions – Laminin 411- and Collagen I-coated wells.*

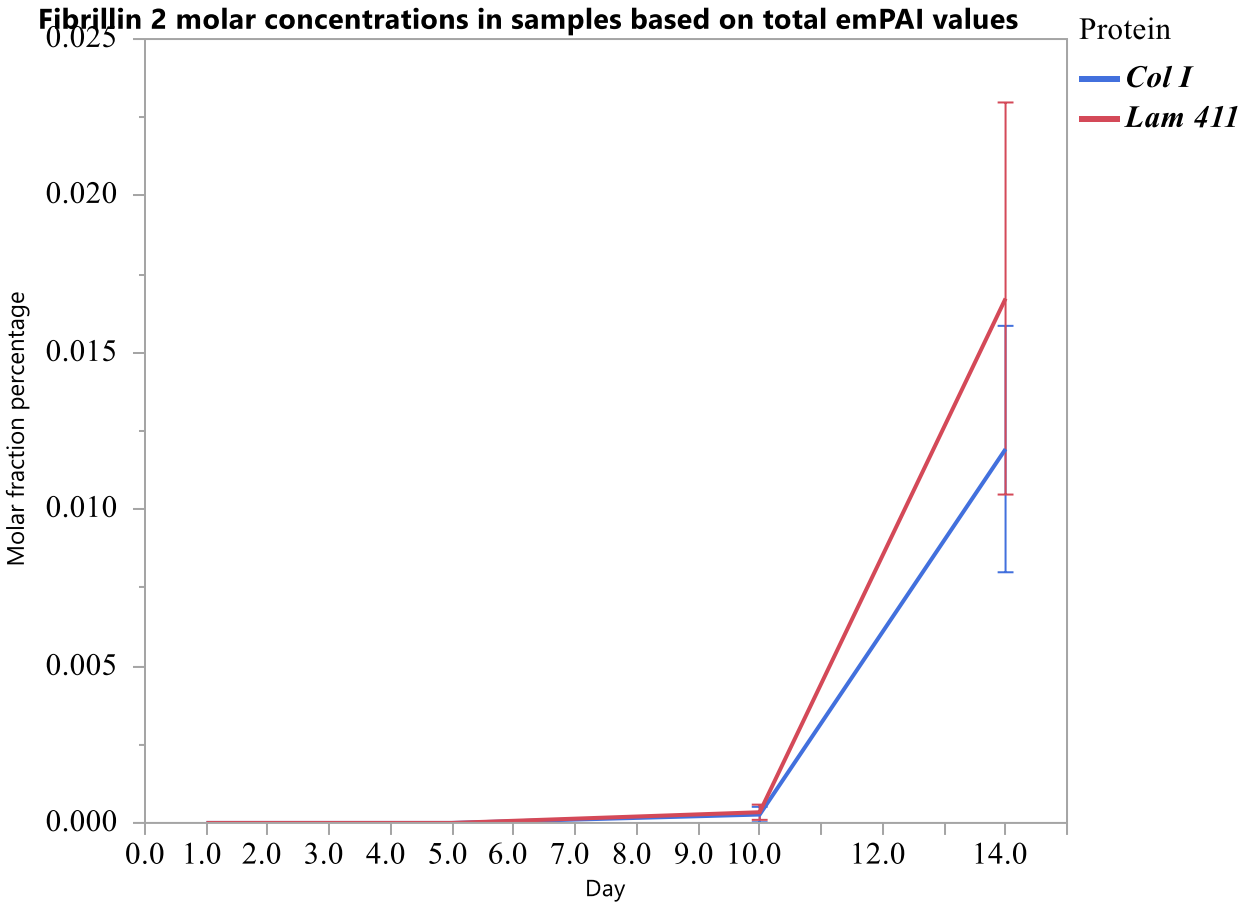


Figure A6. *Molar fraction percentages of Fibrillin 2 over 5 time points: Day1, Day3, Day5, Day10 and Day14. Values are calculated based on total emPAI values and taken as average of duplicate samples. Error bars are showing standard deviation for samples within duplicates. Data is given for 2 conditions – Laminin 411- and Collagen I-coated wells.*

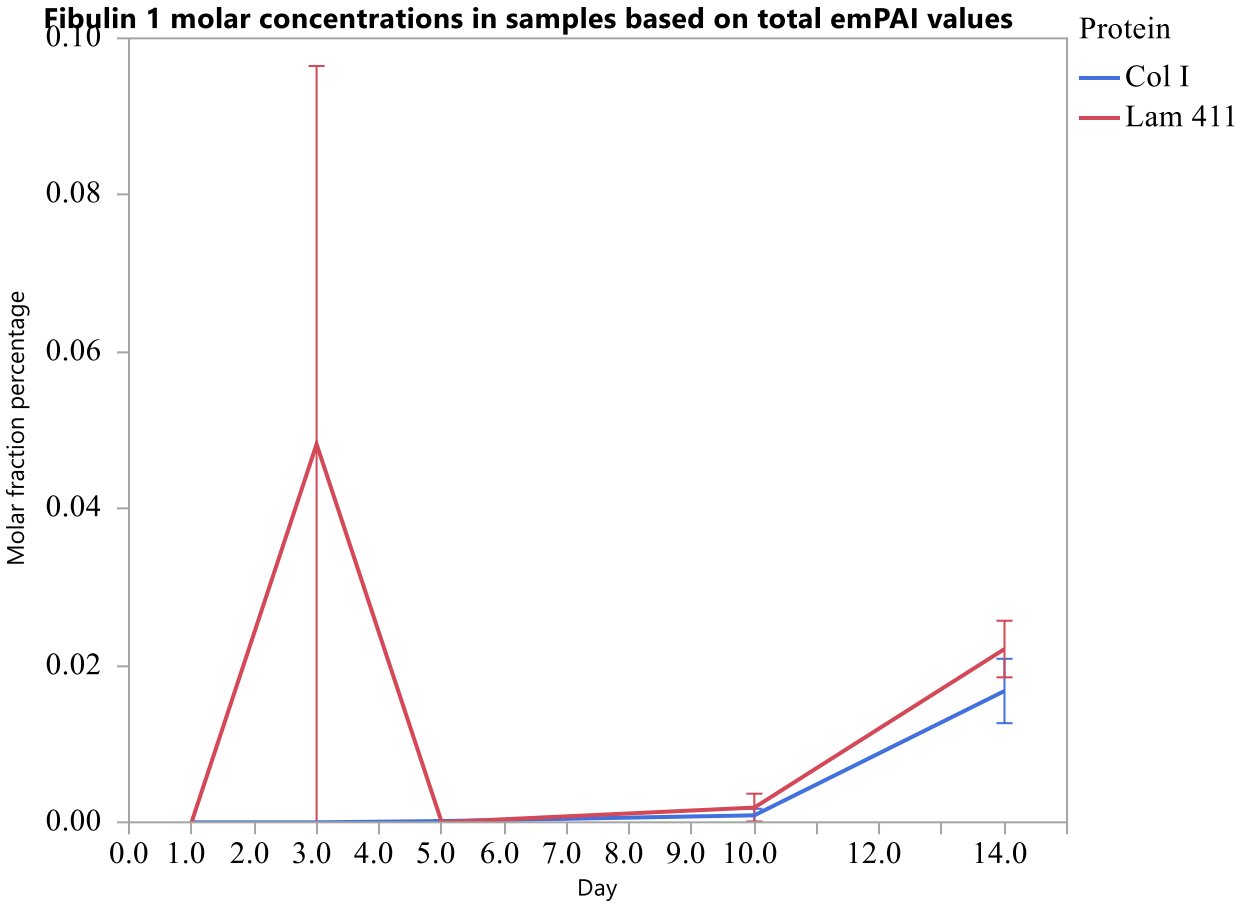


Figure A7. *Molar fraction percentages of Fibulin 1 over 5 time points: Day1, Day3, Day5, Day10 and Day14. Values are calculated based on total emPAI values and taken as average of duplicate samples. Error bars are showing standard deviation for samples within duplicates. Data is given for 2 conditions – Laminin 411- and Collagen I-coated wells.*

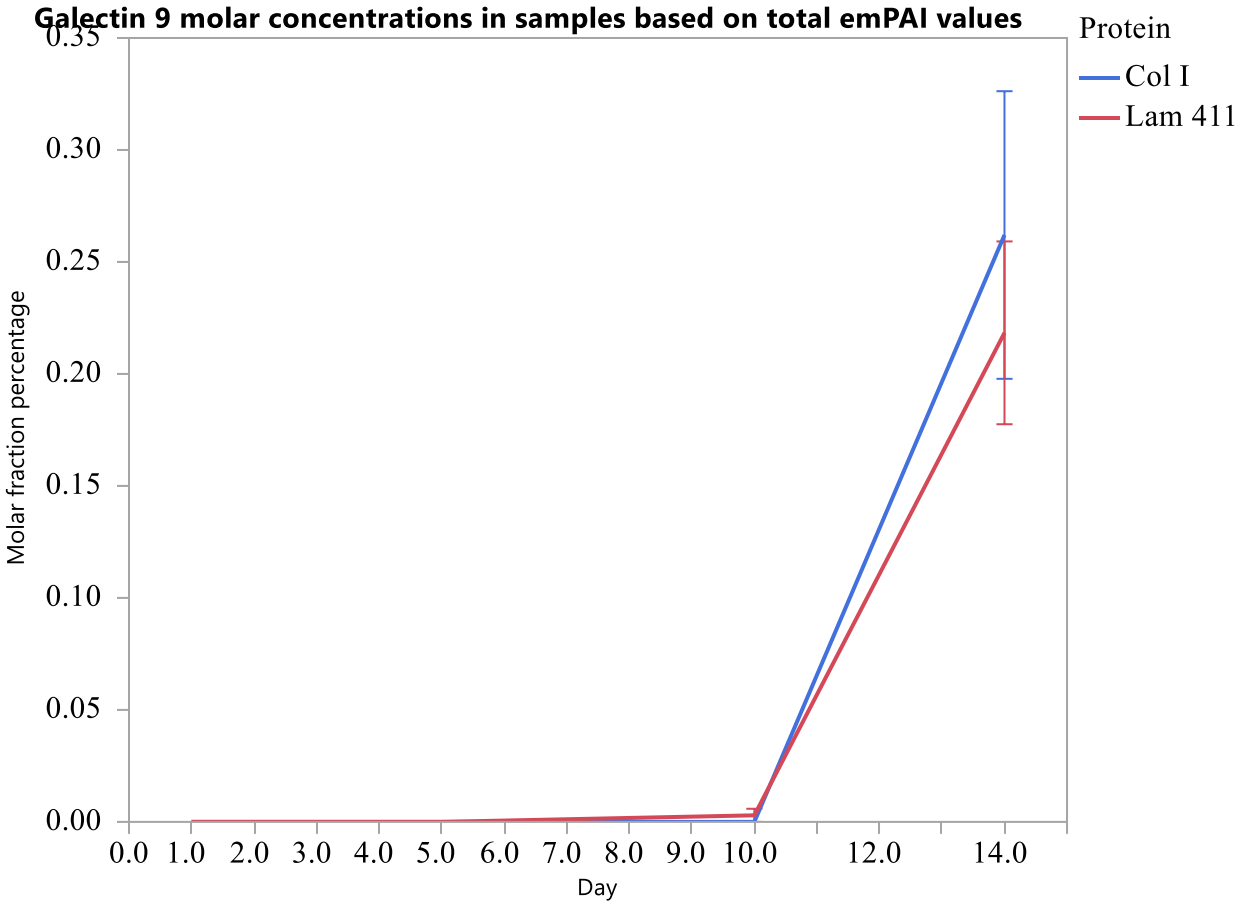


Figure A8. *Molar fraction percentages of Galectin 9 over 5 time points: Day1, Day3, Day5, Day10 and Day14. Values are calculated based on total emPAI values and taken as average of duplicate samples. Error bars are showing standard deviation for samples within duplicates. Data is given for 2 conditions – Laminin 411- and Collagen I-coated wells.*

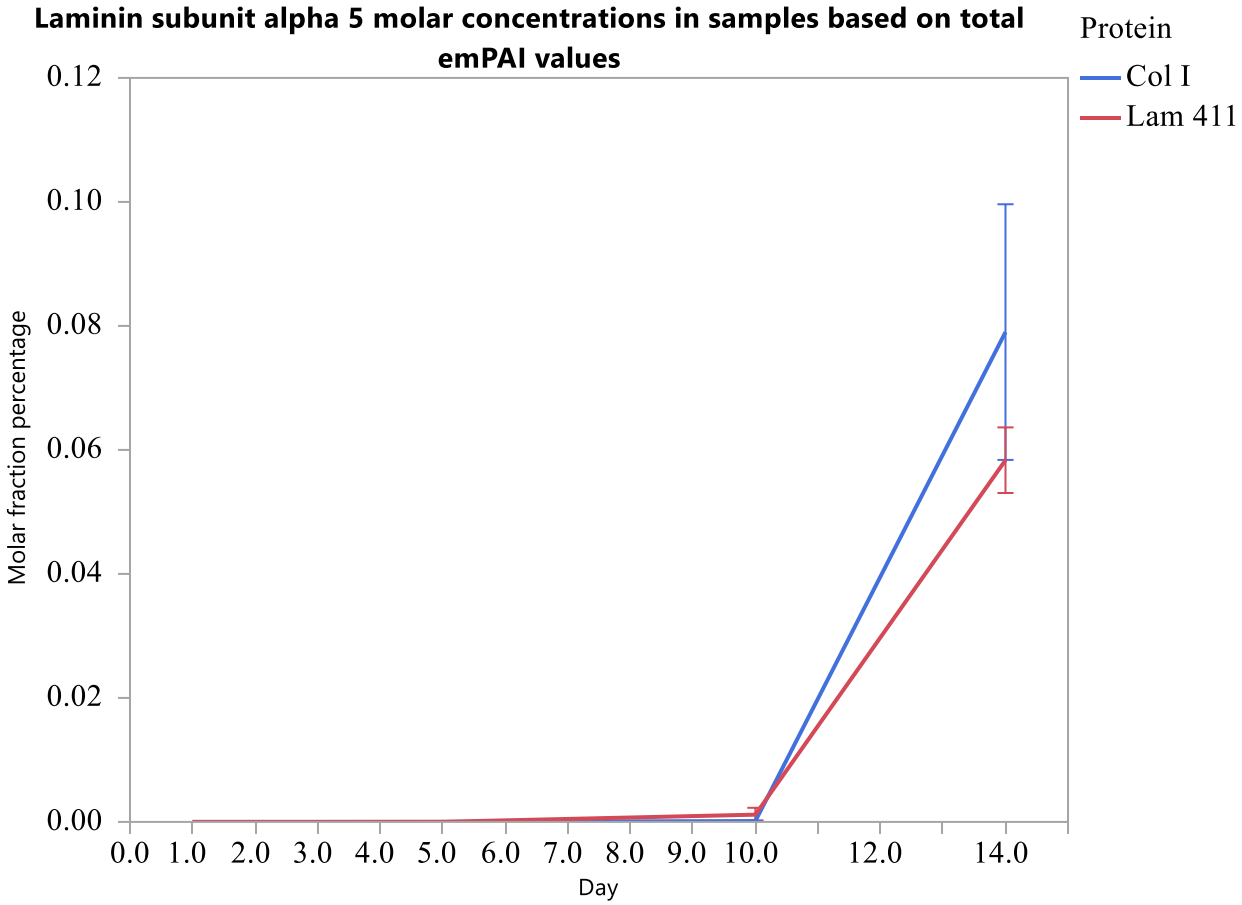


Figure A9. *Molar fraction percentages of Laminin subunit alpha 5 over 5 time points: Day1, Day3, Day5, Day10 and Day14. Values are calculated based on total emPAI values and taken as average of duplicate samples. Error bars are showing standard deviation for samples within duplicates. Data is given for 2 conditions – Laminin 411- and Collagen I-coated wells.*

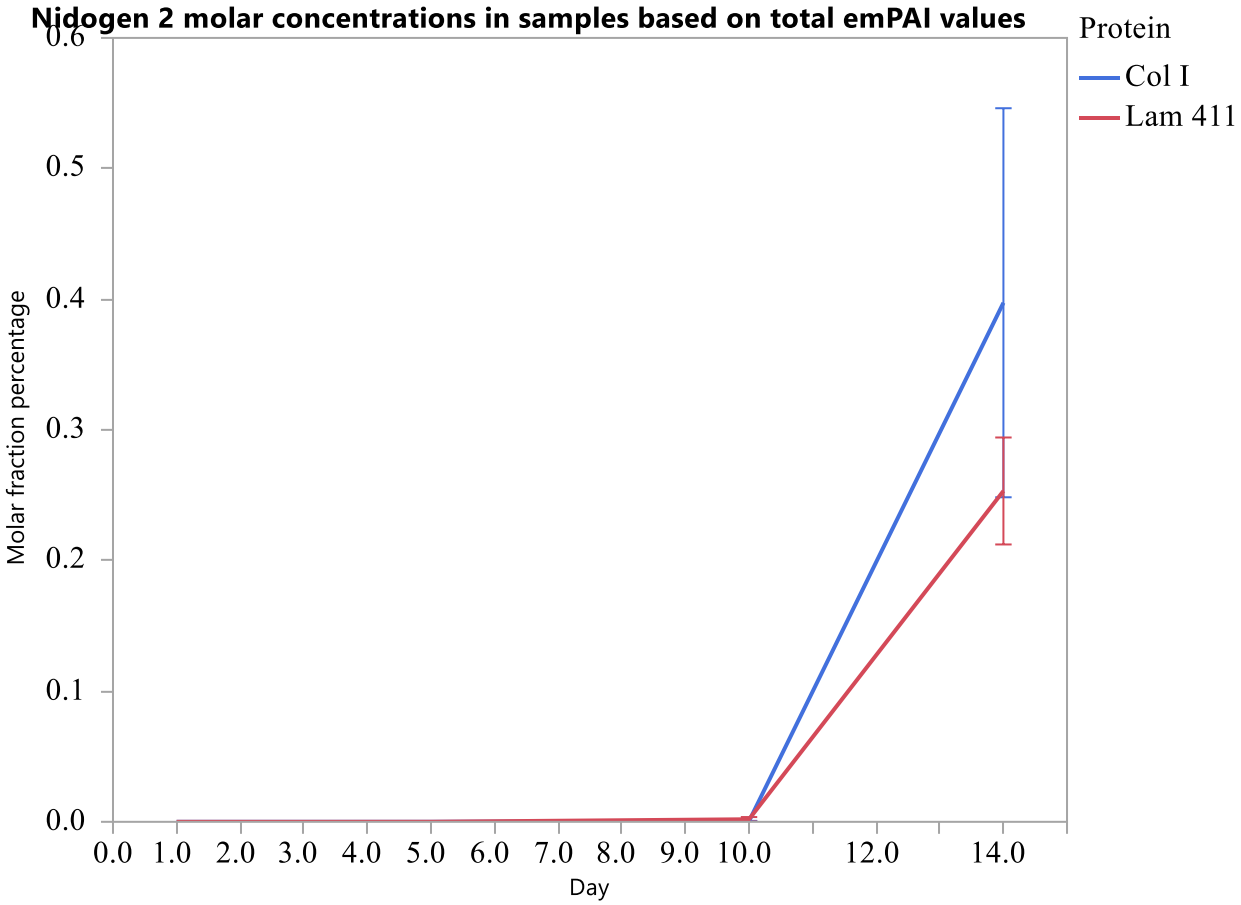
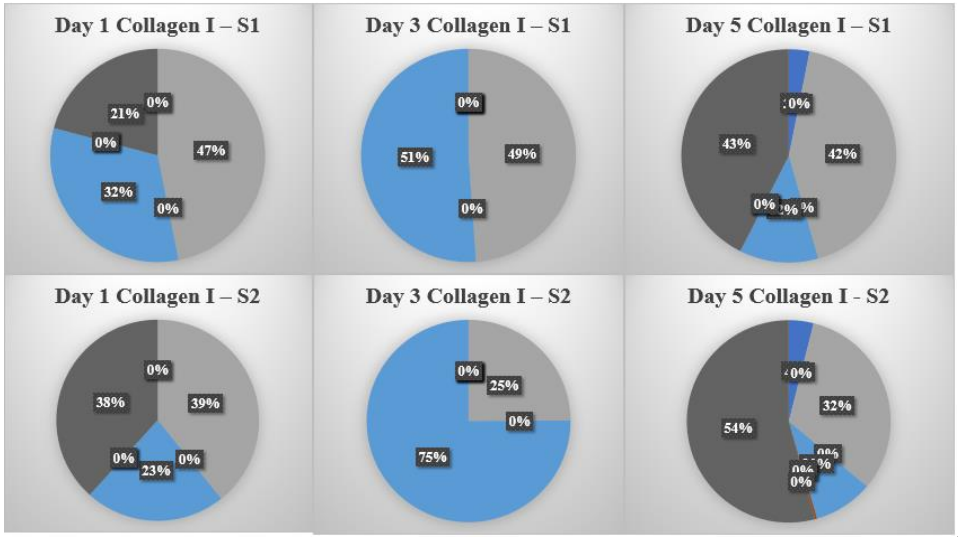


Figure A10. *Molar fraction percentages of Nidogen 2 over 5 time points: Day1, Day3, Day5, Day10 and Day14. Values are calculated based on total emPAI values and taken as average of duplicate samples. Error bars are showing standard deviation for samples within duplicates. Data is given for 2 conditions – Laminin 411- and Collagen I-coated wells.*



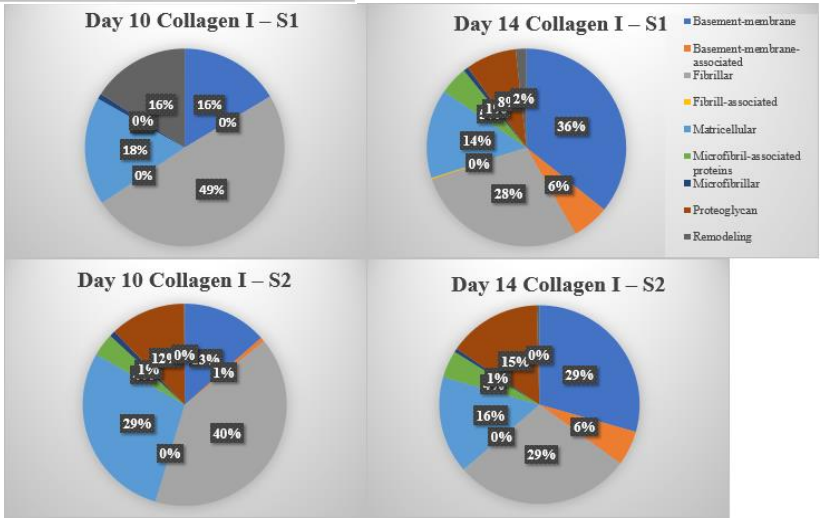
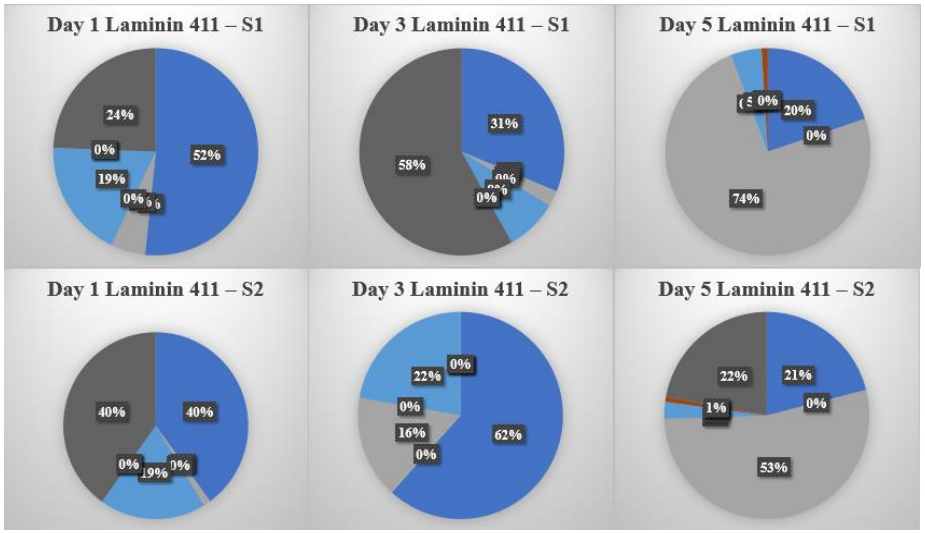


Figure A11. *Pie charts of ECM families, based on fraction percentages of ECM proteins, calculated via total protein emPAI values. Data is given for Collagen I-coated wells at 5 distinct time points, with duplicate samples for each time point (S1-Sample 1, S2- Sample 2).*



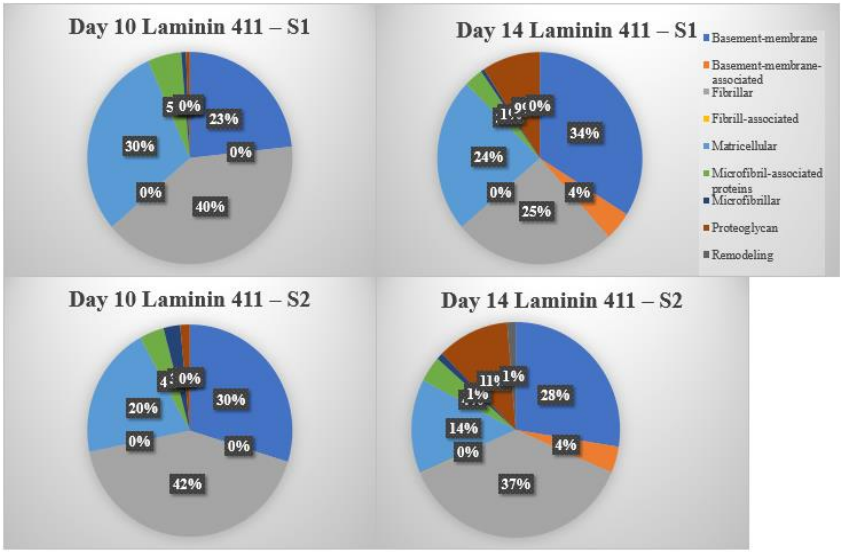


Figure A12. *Pie charts of ECM families, based on fraction percentages of ECM proteins, calculated via total protein emPAI values. Data is given for Laminin 411-coated wells at 5 distinct time points, with duplicate samples for each time point (S1-Sample 1, S2- Sample 2).*