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Abstract

The cellular microenvironment often plays a crucial role in disease development and progression. In recessive dystrophic epidermolysis bullosa (RDEB), biallelic mutations of the gene *COL7A1*, encoding for collagen VII the main component of anchoring fibrils, lead to a loss of collagen VII in the extracellular matrix (ECM). Loss of collagen VII in skin is linked to a destabilization of the dermal-epidermal junction zone, blister formation, chronic wounds, fibrosis and aggressive skin cancer. Thus, RDEB cells can serve as a model system to study the effects of a perturbed ECM on the cellular proteome. In this chapter, we describe in detail the combination of stable isotope labeling by amino acids in cell culture (SILAC) of primary skin fibroblasts with reseeding of fibroblasts on decellularized collagen VII-positive and -negative ECM to study the consequences of collagen VII loss on the cellular proteome. This approach allows the quantitative, time-resolved analysis of cellular protein dynamics in response to ECM perturbation by liquid chromatography-mass spectrometry.

Key words: Skin, Fibroblasts, Protein kinetics, Decellularization, Extracellular matrix, Proteomics, GASP, High pH reversed-phase chromatography, SILAC, Mass spectrometry

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1. Introduction

The extracellular matrix (ECM) is of critical importance for cell and tissue homeostasis and its dysregulation is linked to the progression of numerous diseases. In accordance, the influence of the ECM on intracellular signaling has increasingly become the focus of research [1]. Creating an acellular matrix scaffold is a widely used method to study the physiological role and function of the microenvironment on cells, in tissues and organs, and its contribution to diseases [2, 3]. Decellularized ECM can be repopulated by cells promoting cell proliferation and differentiation [4]. Importantly, it can be employed to study the kinetics of cell responses addressing how cells perceive signals, how signal initiation and transduction are orchestrated leading to altered gene expression, and resulting in qualitative and/or quantitative changes of the proteome.

Recessive dystrophic epidermolysis bullosa (RDEB) is an inherited skin fragility disorder caused by biallelic mutations in the gene *COL7A1* resulting in the loss of collagen VII [5]. Collagen VII forms anchoring fibrils which support dermal-epidermal adhesion [6]. Its loss is linked to a complex disease phenotype. Respective individuals suffer from trauma-induced skin blistering, causing subsequent scarring, chronic wounds and aggressive skin cancer [7, 8]. By mass spectrometry (MS)-based proteomics it was shown that RDEB fibroblasts produce a globally altered cellular microenvironment [9], which appears to actively contribute to the progression of aggressive squamous cell carcinoma [7, 9, 10]. However, loss of collagen VII seems to alter not only the cellular microenvironment but also the intracellular proteome [11, 12]. Whether this is due to a perturbed ECM or due to intracellular alterations linked to collagen synthesis and secretion remains to be studied.

Since the molecular consequences linked to the loss of collagen VII in the ECM are known [9], RDEB cells are a suitable model to study how perturbation of the ECM influences cellular signaling and decision finding. Here we describe a strategy to quantitatively analyze intracellular proteome

alterations in response to interactions of cells with decellularized matrices isolated from control or RDEB fibroblast. For quantitation by MS, we implement stable isotope labeling by amino acids in cell culture (SILAC) for primary human skin fibroblasts. Additionally, we present a protocol for the fractionation of complex proteome samples in large volumes prior MS analysis to ensure a deep proteome coverage.

2. Materials

2.1. Cell culture

1. Dulbecco's Modified Eagle Medium (DMEM), high glucose 4.5 g/L
2. 10% Fetal Bovine Serum (FBS)
3. SILAC-DMEM (high glucose 4.5g/L), without L-lysine and L-arginine
4. 10% dialyzed Fetal Bovine Serum (dFBS).
5. 200 mM L-glutamine (100x stock solution)
6. 10'000 U/ml Penicillin, 10 mg/ml Streptomycin (100x stock solution).
7. Following SILAC labels are used: L-lysine-$^{2}H_4$, L-arginine-$^{13}C_6$-$^{14}N_4$ (Lys4, Arg6) and L-lysine-$^{13}C_6$-$^{15}N_2$, L–arginine–$^{13}C_6$–$^{15}N_4$ (Lys8, Arg10) (see Note 1). For primary fibroblasts, we add 84 mg/L L–arginine, 146 mg/L L–lysine (all from EURISO-TOP GmbH) and 164 mg/L proline (Sigma-Aldrich, see Note 2).
8. Ascorbic acid
9. Trypsin-EDTA solution (200 mg/L trypsin, 500 mg/L EDTA)
10. Tissue culture flasks
11. Sterile phosphate-buffered saline (PBS)
12. Syringe Driven Filters, 0.2 μm, 30 mm diameter
13. Primary normal human fibroblasts (NHF) from foreskin

14. Primary fibroblasts from RDEB patients

2.2. ECM decellularization and cell harvest

1. 0.5% Triton X-100 in 20 mM NH_4OH
2. 4% SDS, 0.1 M Tris-HCl (pH 7.6) and 1 µM DTT

2.3. Gel-aided Sample Preparation (GASP)

1. 40% Acrylamide/Bis-acrylamide, Tetramethylethylenediamine (TEMED) and Ammonium persulfate (APS)
2. 50% methanol, 10% acetic acid and 40% dH_2O
3. 6 M Urea in Tris-HCl, adjusted to pH 7.6
4. Acetonitrile, MS grade
5. 100 mM Ammonium bicarbonate, pH 7.5 (ABC buffer)
6. 5 µg lysyl endopeptidase (Waco) in 100 mM ABC buffer per gel-plug
7. 40 µg sequencing grade modified trypsin (Promega) per gel-plug
8. 5% formic acid (MS grade) in dH_2O

2.4 High pH Reversed-Phase Chromatography

1. RP Buffer A, pH 10: 10 mM ammonium formate in dH_2O, adjusted to pH 10 with ammonia
2. ReproSil-Pure C18-basic (Dr. Maisch) in methanol (MS grade)
3. C18 discs (3M Empore)
4. 200 µl pipet-tips
5. Acetonitrile in RP Buffer A, pH 10: 2%, 6%, 10%, 12%, 13%, 14%, 16%, 20%, 25% and 50% acetonitrile for step elution of peptides

6. Buffer A: 0.5% acetic acid (MS grade) in dH_2O
7. Buffer A*: 0.3% trifluoroacetic acid in 3% acetonitrile (all MS grade)
8. Buffer B: 80% acetonitrile, 0.5% acetic acid

3. Methods

In this protocol, we generate control and RDEB ECM *in vitro* and use it as a scaffold for NHF and RDEB fibroblasts (Figure 1a). For quantitation of protein dynamics in cells seeded on decellularized ECM, we implemented SILAC-based MS. MS-samples are prepared by GASP with subsequent high pH reversed-phase chromatography for peptide fractionation (Figure 1b) [13-15]. In our experimental setup, we mixed heavy-labeled NHF, which were cultured on RDEB ECM, with the medium-labeled NHF cultured on control ECM. Complementary, we mixed heavy-labeled RDEB cells, which were cultured on control ECM, with medium-labeled RDEB cells cultured on RDEB ECM. Heavy/medium SILAC ratios imply cells cultured on their own ECM in the denominator. With this approach, protein abundances are normalized to the cellular response to re-seeding and significant outliers should solely reveal the effects of RDEB ECM on NHF and of control ECM on RDEB cells, respectively (Figure 1a). The experimental design can be changed, depending on the questions asked: e.g. NHF cultured on control ECM can be mixed with RDEB cells seeded on control ECM to study the different effects the same ECM has on distinct cell types in single MS experiments.

3.1. SILAC-Labeling of Primary Skin Fibroblasts

1. To transfer cells from standard DMEM to SILAC DMEM, wash cells with PBS, trypsinize and spin down for 3 min at 300 g, RT. Discard the supernatant.

2. Take up fibroblasts in SILAC-DMEM, supplemented with 10% dFBS, 2 mM L–glutamine, 100 U/ml Penicillin, 0.1 mg/ml Streptomycin, 84 mg/l L–arginine, 146 mg/l L–lysine, and 164 mg/l proline (see Notes 2 and 3).
3. For sufficient labeling, cells should be cultured in SILAC-DMEM for at least seven cell doublings [16]. Cells should not reach a confluence of 100% to ensure an active cellular metabolism and incorporation of the isotopically labeled amino acids.
4. Change media every other day.
5. Check labeling efficiency and proline conversion before starting large-scale experiments.

3.2. ECM Generation and Isolation

1. For ECM generation culture cells in standard DMEM with high glucose, 10% FBS, 2 mM L- glutamine, 100 U/ml Penicillin, 0.1 mg/ml Streptomycin.
2. We recommend the use of 10 cm plates with $5*10^5$-$1*10^6$ cells per plate, depending on the cell type.
3. For proper collagen production, treat cells for seven days in total with 50 µg/ml ascorbic acid [9, 16] (see Note 4). Change medium every other day with freshly added ascorbic acid.
4. For ECM isolation work on ice: wash the cells three times with PBS.
5. Remove cells from ECM by washing with 0.5% Triton X-100 and 20 mM NH_4OH in dH_2O [17]. Add 1 ml per 10 cm plate for approximately 30 seconds. Check the efficiency of cell removal under the microscope.
6. Add 10 ml PBS and aspirate the solution.
7. Wash three times with PBS and add again 1 mL of 0.5% Triton X-100 and 20 mM NH_4OH in dH_2O.
8. Immediately add PBS and aspirate the solution.

9. Wash again five times with PBS to remove remaining intracellular debris, detergent and ammonium hydroxide. Add DMEM to plates and store them for further use.

3.3. Reseeding of Fibroblasts on ECM

1. Trypsinize the fully labeled SILAC fibroblasts for 10 min. at 37°C, 5% CO_2.
2. Carefully aspirate the medium from the ECM
3. Seed $5*10^5$-$1*10^6$ cells in 10 ml SILAC DMEM per 10 cm plate on top of the ECM (see Note 5). As example, we seed NHF of one SILAC label on control ECM and NHF of another SILAC label on RDEB ECM. Do the same with RDEB cells.
4. Keep cells and ECM in SILAC media at 37°C, 5% CO_2 until lysis.

3.4. Gel-aided sample preparation

1. Harvest samples with 0.5 ml 4% SDS, 0.1 M Tris-HCl (pH 7.6) and 1 μM DTT
2. Heat and shake lysates for 5 min at 95°C
3. Mix opposing SILAC labels with each other in a 1:1 ratio. Each vial should contain three SILAC labels from two conditions, i.e. Arg0, Lys0: ECM; Arg6, Lys4: NHF on control ECM; Arg10, Lys8: NHF on RDEB ECM (Figure 1).

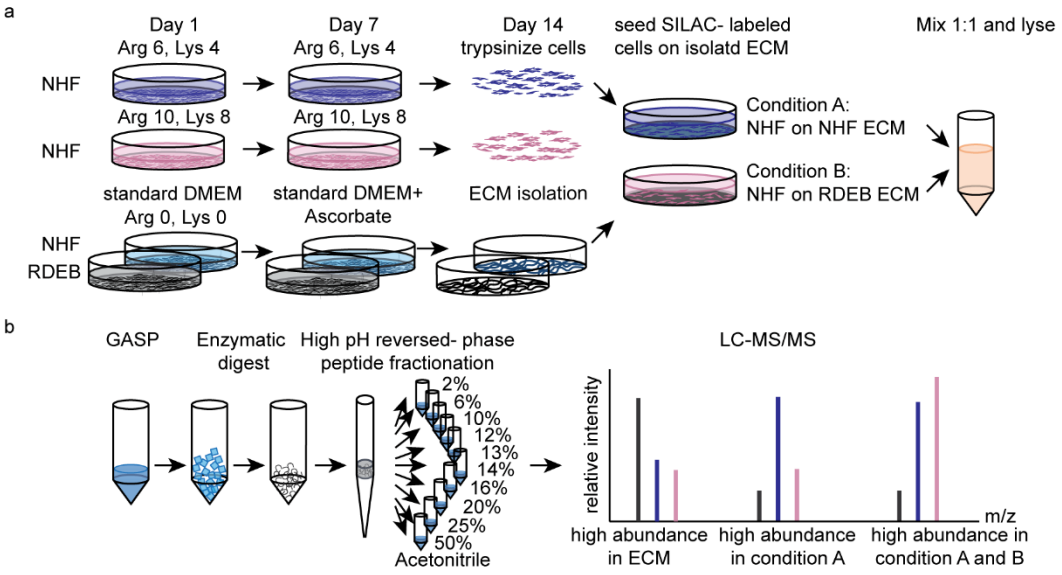

Figure 1: Schematic representation of the experimental procedure for a SILAC-based ECM- reseeding experiment followed by LC-MS/MS analysis. **(a)** Cells are SILAC-labeled and seeded onto fibroblast-generated ECM. After a defined incubation period, the different conditions are mixed and samples are prepared for LC-MS/MS analysis. **(b)** Proteins are digested by Gel-aided sample preparation (GASP) using trypsin and lysyl endopeptidase. High pH reversed-phase chromatography with 10 fractions is utilized for sample fractionation prior to LC-MS/MS analysis.

4. Add Acrylamide/Bis-acrylamide to the lysate up to a final concentration of 20%. Mix sample by pipetting and incubate for 20 min at RT.
5. Add Tetramethylethylenediamine (TEMED) to a concentration of 5% and ammonium persulfate to a final concentration of 0.5% w/v. Let the sample rest until it solidifies.
6. Shred the gel-plug (see Note 6)
9. Fix gel pieces with 50% methanol, 10% acetic acid and 40% dH_2O for 10 min. Samples should be entirely covered by the fixing solution. Shake carefully while fixing.

7. Wash once with 6 M Urea and once with acetonitrile to remove detergents
8. Wash twice, alternatingly with 100 mM ABC buffer and acetonitrile
9. This step is optional to increase identification rates: incubate dehydrated gel-pieces with 5 µg lysyl endopeptidase in 100 mM ABC buffer for 1 h at 37°C [18].
10. Add 40 µg trypsin per gel-plug and incubate samples overnight at 37°C.
11. Collect all the peptides by extracting them from the gel: add 1 ml of 5% formic acid to stop the tryptic reaction. Dehydrate gel pieces twice with 1 ml acetonitrile. Collect all the supernatants in a single reaction tube.
12. Reduce the samples to less than 300 µl in a vacuum concentrator and add 1.5 ml RP Buffer A pH 10.

3.5. High-pH reversed-phase chromatography

1. Reversed-phase columns are self-packed: stack four layers of C18 discs in a 200 µl pipet-tip. Add slurry of reproSil-pure C18-basic in methanol on top to form a layer of approx. 3 mm C18 material (See Note 7). The pipette-tip column should not get dry.
2. Wash column twice with 100 µl Buffer B by centrifuging tips in reaction tubes in a tabletop centrifuge (~2 min, 3'000 g)
3. Equilibrate twice with 100 µl RP Buffer A, pH 10 before the sample is loaded onto the column.
4. Load the sample onto the column by centrifugation.
5. Wash the column with 100 µl RP Buffer A, pH 10.
6. Prepare 10 tubes with 3 µl of 5% formic acid.

7. Add 50 µl of RP Buffer A, pH 10 and 2% acetonitrile to the column. Centrifuge the tip for 1.5-2 min at 3'000 g and collect the flow through in a tube with 5% formic acid. Formic acid acidifies and thus stabilizes the peptides and potential posttranslational modifications.
8. Continue adding 50 µl RP Buffer A, pH 10 with 6%, 10%, 12%, 13%, 14%, 16%, 20%, 25% and 50% acetonitrile. Collect the respective flow-through in separate tubes containing 5% formic acid.
9. Evaporate solvents in a vacuum concentrator to remove ammonium formate and acetonitrile.
10. Suspend the peptides in 15 µl of 30% Buffer A* and 70% Buffer A and store at -80°C for LC-MS/MS analysis [19].

3.6. Data analysis

1. Decellularization of fragile ECM is technically challenging and has to be optimized. Western blot analysis of different cellular components to analyze the purification efficacy is advisable (Figure 2a). We used Histone H2B, Tenascin-C and GAPDH as readout for nuclear, extracellular and cytoplasmic cell fractions, respectively. Decellularized ECM should contain Tenascin-C and be free of GAPDH or Histone H2B.

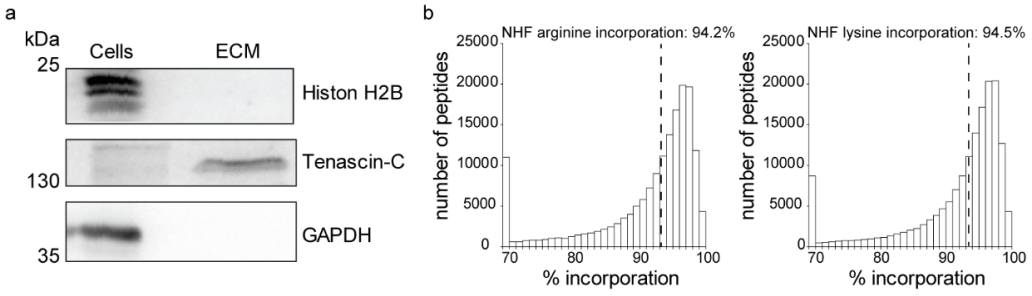

Figure 2: Quality assessment of decellularized ECM and labeling efficiency. **(a)** Western blot analysis of trypsinized cells and isolated ECM. **(b)** Incorporation efficacy of isotopically

labeled arginine and lysine of NHF, which were cultured on decellularized, non-labeled ECM. Dotted lines represent median label incorporation.

2. Bioinformatics interpretation of MS data and statistical analyses can be done by the freely available software Perseus [20].
3. The SILAC labeling efficiency should be determined to ensure complete labeling and accurate MS-based quantification (see Note 8). All non-labeled peptides should be associated with the decellularized ECM and are excluded from the subsequent data analysis as follows. All peptides are annotated according to the matrisome classification [21]. Peptides associated with "matrisome protein", "basal membrane protein" and "collagen" are removed for the determination of labeling efficiency. For the remaining peptides, the ratio of the intensity of the labeled peptide compared to the total intensity is calculated (Figure 2b). Incorporation rates of 94% are sufficient since technical MS-quantitation errors of 10-20% are common.
4. Here we cultured cells on control and decellularized RDEB ECM for 30 min, 6 h, 12 h and 24 h. All time points are normalized relative to the 30 min time point. Ratios are analyzed by a two-sided t-test with a Benjamini-Hochberg corrected false discovery rate of 0.05. Only proteins with a significant ratio in at least one time point are submitted to z-normalization and k-means clustering. K-means clustering is performed with the freely available "Multi Experiment Viewer, MeV" [22].
5. GO-term enrichment of the different clusters can be done by STRING v10.5 [23]. Depicted in Figure 3 are six out of 15 clusters. Proteins in clusters 1 and 11 are upregulated in RDEB fibroblasts in response to NHF ECM but show a negative or no response in NHF cultured on RDEB ECM. These clusters mainly contain proteins regulating ECM-receptor

interaction, proteoglycans in cancer, focal adhesion, amoebiasis and the PI3K-Akt signaling pathway. Cluster 1 contains DCN, TGFBI, COL6A2 and SERPINE2, which are known to be upregulated in RDEB fibroblasts [9], indicating that the increased TGFβ signaling observed in RDEB [24, 25] is cell autonomous and might depend on intracellular sensing of collagen VII. On the other hand, the basement membrane associated proteins COL4A2 and LAMB1 are increasingly expressed in RDEB fibroblasts in response to NHF ECM. COL4A2 and LAMB1 are known to be downregulated in RDEB ECM [9], which seems to be rescued by culturing RDEB fibroblasts on healthy collagen VII-positive ECM.

On the contrary, Clusters 5 and 10 show proteins which are increasingly expressed in NHF in response to RDEB ECM but negatively or do not respond in RDEB fibroblasts cultured on control NHF ECM. These proteins are mainly involved in DNA replication, cell cycle, and poly(A) RNA binding. Examples are MCM3, MCM4, MCM6 and MCM7 of the minichromosome maintenance protein complex, which regulates genomic DNA replication [26]. Another candidate found in this cluster is the dermal basement membrane protein Extracellular Matrix Protein 1 (ECM1). ECM1 is part of suprastructures in the dermal-epidermal junction and thus regulates skin homeostasis [27, 28]. Its overexpression induces cell proliferation by activating EGFR and is linked to enhanced metastasis and poor prognosis in cancer [29-32]. Since RDEB patients develop aggressive squamous cell carcinomas [33], this might indicate a proliferation supporting, pro-cancerogenic effect of collagen VII-negative RDEB ECM [10]. Thus, this experimental strategy reveals new insights into cell-ECM interactions and allows the discrimination between cell-intrinsic and ECM-specific influences on disease progression.

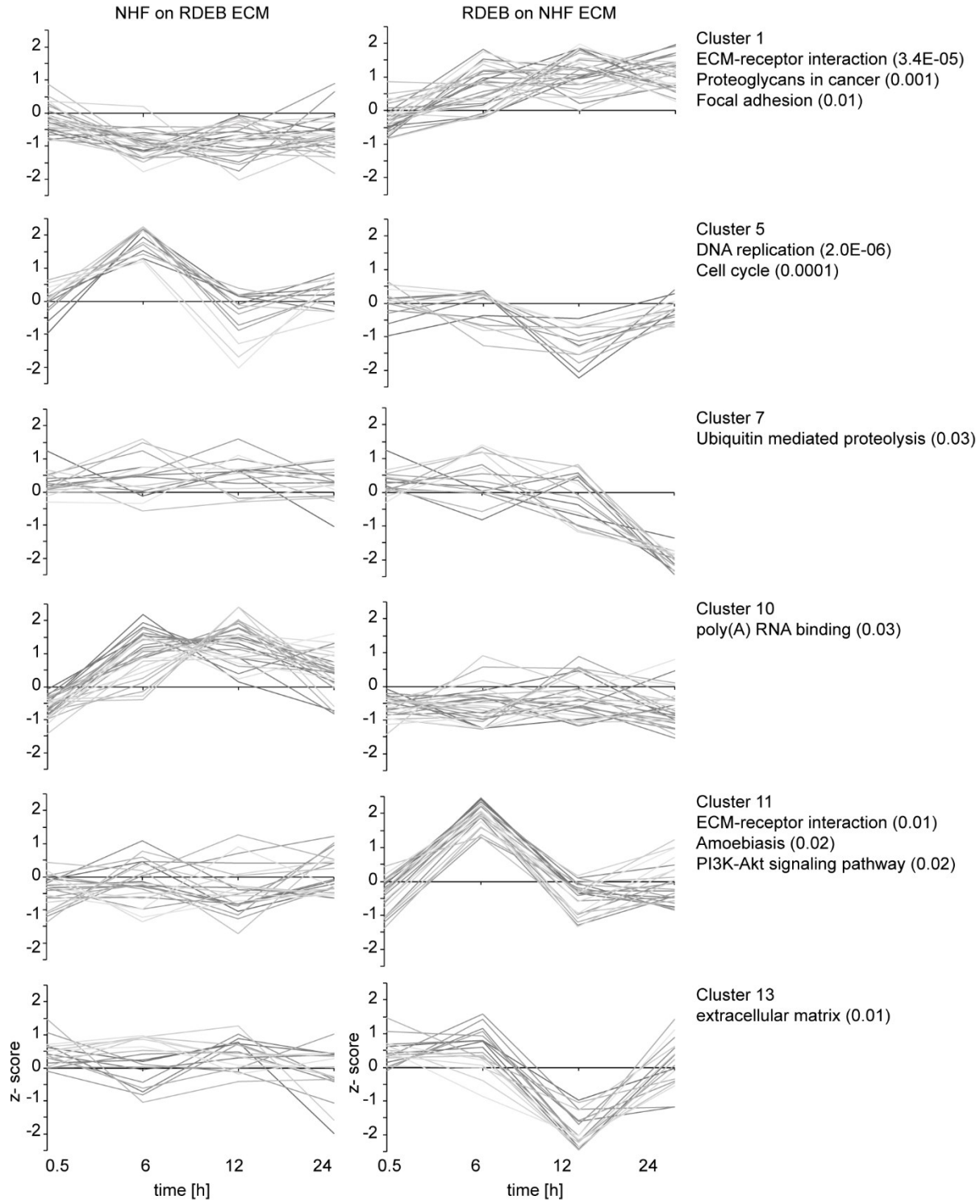

Fig.3. K-means cluster analysis of significantly regulated proteins in NHF and RDEB fibroblasts (FDR: 0.05). Clusters were analyzed for enriched GO-terms. Respective p-values are noted in brackets (Fisher`s exact test, $p<0.05$, BH corrected) [34].

4. Notes

1. The third, light SILAC label is not used, as we use non-labeled ECM as a scaffold. With this approach, one can discriminate ECM proteins synthesized by reseeded, labeled cells from ECM proteins of the initially prepared matrix scaffold. Since the ECM is not used for data evaluation, it is generated in standard DMEM and not in SILAC DMEM.
2. Fibroblasts need proline for proper collagen fibril synthesis. The absence of proline will stimulate cells to convert heavy arginine into heavy proline, leading to protein quantification artifacts in MS data analysis. We add additional proline to reduce the arginine-to-proline conversion without interfering with the incorporation of heavy arginine. Also, an excess of arginine will enhance arginine-to-proline conversion. Thus, optimal arginine and proline concentrations should be titrated for each cell type.
3. dFBS is critical to ensure that only labeled variants of arginine and lysine are metabolized by cells.
4. Ascorbic acid is an essential co-factor of enzymes that catalyze proline and lysine hydroxylation [35], which in turn are critical for collagen stability. It can be prepared in a 5 mg/ml stock solution in dH_2O. The solution is sterile-filtered with a syringe driven filter (0.2 µm, 30 mm diameter). The solution should be kept in the dark. Freeze-stocks are stored at -20°C.
5. Practicing the isolation of ECM in advance is advisable. Plates should be checked for efficacy under the microscope after each step. Backup plates for ECM isolation are desirable, in case the ECM is lost during the purification procedure. The suitable amount of produced ECM needs to be studied beforehand: thick ECM will easily detach from the culture dish.

6. The plug is shredded by centrifuging it through a nitrocellulose filter support grid. Dissolve the nitrocellulose filter membrane by acetone and wash the grid prior to shredding the gel plug. Place the grid in a new, clean tube and centrifuge the plug at maximum speed through the grid.
7. STAGE-tip syringes to stack C18 discs into a pipet tip can be built according to the online video instructions from the Max Planck Institute of Biochemistry (http://www.biochem.mpg.de/226863/Tutorials). The slurry volume of reproSil-pure C18-basic depends on the amount of protein: samples with high protein concentrations require larger C18 volumes.
8. To identify false-positive hits, SILAC labels should be swapped between biological replicates.

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