Journal of Dermatological Science 78 (2015) 173–180

Contents lists available at ScienceDirect

Journal of Dermatological Science

journal homepage: www.jdsjournal.com

Quantitative proteogenomic profiling of epidermal barrier formation in vitro

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ARTICLE INFO

Article history:
Received 14 January 2015
Received in revised form 13 February 2015
Accepted 24 February 2015

Keywords:
Skin equivalent
Epidermal differentiation
Barrier
Proteomics

ABSTRACT

Background: The barrier function of the epidermis is integral to personal well-being, and defects in the skin barrier are associated with several widespread diseases. Currently there is a limited understanding of system-level proteomic changes during epidermal stratification and barrier establishment.
Objective: Here we report the quantitative proteogenomic profile of an in vitro reconstituted epidermis at three time points of development in order to characterize protein changes during stratification.
Methods: The proteome was measured using data-dependent "shotgun" mass spectrometry and quantified with statistically validated label-free proteomic methods for 20 replicates at each of three time points during the course of epidermal development.
Results: Over 3600 proteins were identified in the reconstituted epidermis, with more than 1200 of these changing in abundance over the time course. We also collected and discuss matched transcriptomic data for the three time points, allowing alignment of this new dataset with previously published characterization of the reconstituted epidermis system.
Conclusion: These results represent the most comprehensive epidermal-specific proteome to date, and therefore reveal several aspects of barrier formation and skin composition. The limited correlation between transcript and protein abundance underscores the importance of proteomic analysis in developing a full understanding of epidermal maturation.

1. Introduction

The skin carries out a variety of protective functions that must be maintained despite the constant turnover of skin tissue and are collectively termed the epidermal barrier. These functions include water retention, antibacterial action, protection from toxic substances, and initial immune responses [1]. Barrier dysfunction is tied to many acute and chronic conditions, several of which are prevalent in occurrence such as icthyosis vulgaris, atopic dermatitis, and psoriasis [2].

The barrier is initially formed in utero at approximately 34 weeks gestation [2] and is comprised of several functional components. Keratinocyte-derived squames of the outer epidermis are sheathed in a layer of lipids and proteins called the cornified envelope [3]. Disruption of the lipid "mortar" (e.g. with detergents) causes barrier disruption and skin irritation. In lower epidermal layers, protein-based cell–cell junctions are another important component of the barrier. Loss of tight junctions in the central region of the epidermis (the stratum granulosum) leads to death in neonatal mice [4,5]. Additional proteins including Loricrin, Involucrin, Keratins, and Desmosome components also contribute to the barrier function [6]. A comprehensive, quantitative proteomic profile of the temporal differences in protein abundance would aid in understanding barrier health and functionality.

Comprehensive proteomic studies of the skin have been hampered by a number of factors. The dynamic range of proteins in skin, where keratins can comprise 70% of the cells by dry weight [7], complicates detection of lower abundance proteins in the sample. To overcome this issue, past efforts have frequently employed separation of proteins via gel electrophoresis [8–10], a

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http://dx.doi.org/10.1016/j.jdermsci.2015.02.013
0923-1811/

procedure with low throughput as well as poor sensitivity for lower abundance proteins [11]. Several published studies have been carried out using relatively undifferentiated cultured cells which do not suffer from such extreme dynamic range [12–15]. Such studies can produce a large number of protein identifications but do not accurately represent the stratified structure and resulting protein profile of natural epidermis. Here we report a quantitative proteomic time course analysis of a previously described reconstituted epidermis (RE) [16]. Extensive characterization of this model demonstrated many strong biological parallels with natural skin, including stratification, similar lipid and natural moisturizing factor composition, a functional water barrier and gradient, appropriate pH, and proper localization of epidermal marker proteins. Transcript analysis of this model revealed several time points where major changes in RNA patterns were observed for marker proteins of skin functions such as keratinization, desquamation, cell–cell junctions, and lipid metabolism. Many of these marker proteins exhibit most transcriptional changes during the first 10 days of culture, followed by stabilization for the remainder of the time course (to 31 days). Based on this data, we chose to focus our proteomic analyses at culture days 3, 10, and 18 to examine early, mid, and late time points in epidermal maturation.

2. Materials and methods

These experiments on human-derived samples were approved by the Western Institutional Review Board. Reconstituted epidermis cultures were prepared as described previously [16]. Briefly, human skin from surgical waste is treated to remove the endogenous epidermis and render the dermal tissue nonviable. This prepared substrate is then seeded with primary keratinocytes isolated from individual donors (Lonza). Cultures are initially submerged in media then raised to the air–water interface at day 3. To separate the epidermis, samples were first removed from the transwell and placed in a new 6-well plate. The sample was then covered in ammonium thiocyanate (3.8%) and incubated for 15 min at room temperature. Epidermis was peeled off using a dissecting scalpel, and flash-frozen in liquid nitrogen.

2.1. Sample preparation

Isolated epidermis was incubated in 50% trifluoroethanol (TFE), 1% SDS, 100 mM ammonium bicarbonate (AMBIC) at 60 °C for 30 min. Samples were vortexed and then sonicated for 10 min total process time using a Misonix 3000 cup-horn sonicator on a 30% duty cycle at a power output of 75 W at 4 °C. Samples were vortexed again and cleared via centrifugation. Protein content of cleared extracts was measured in triplicate with the μBCA assay (Thermo Fisher, USA).

Experimental blocks generated consisting of one sample from each of the three time points selected by a pseudo-random number generator (*random* function in Python 2.7). Pools were then randomly generated in a similar fashion consisting of 5 blocks. The 60 initial samples were therefore combined into 20 blocks and 12 pools. Protein pools were generated by combining 50 μg aliquots of the five component samples, yielding a 250 μg pool. In addition, 5 μg aliquots of each individual sample were processed separately.

Yeast alcohol dehydrogenase (Sigma Aldrich, USA) was added to each sample at 10 fmol/μg protein. Samples were then reduced with 5 mM DTT at 60 °C for 30 min and alkylated with 10 mM iodoacetamide at room temperature for 30 min in the dark. 100 mM AMBIC was added to dilute TFE to 5%, and trypsin was added at 1:100 enzyme:protein, to a final concentration of 2.5 μg/ml. Digestions were performed at 37 °C for 16 h, and halted by addition of trifluoroacetic acid (TFA) to pH < 2. Peptides were purified/desalted on tC18 columns (Waters, USA) and dried to completion.

Individual samples were resuspended to 2.5 μg/μl in 2% acetonitrile (ACN), 0.1% TFA (loading buffer) and run on LC–MS/MS. Pooled samples were resuspended in H_2O and fractionated on 13 cm immobilized pH 3–11 strips (GE Healthcare, USA) using a 3100 OFFGEL Fractionator (Agilent, USA) according to the manufacturer specifications. The 12 fractions were again purified on tC18, dried to completion, and resuspended in loading buffer prior to injection.

2.2. LC–MS/MS

Chromatography consisted of a 2 cm trap column with 100 μm I.D. followed by a 20 cm analytical column with 75 μm I.D. packed with 3 μm ReproSil-Pur C18-AQ (Dr. Maisch, Germany). The LC gradient was carried out on a Nano 2D Plus nanoLC (AB Sciex, Canada) from 0 to 20% B (0.1% formic acid in acetonitrile) over 65 min, then from 20 to 40% B over 25 min, for a total gradient length of 90 min. Buffer A was 0.1% formic acid in water, and the flow rate was set to 200 nl/min. Samples were injected onto the instrument in a random order, again selected via random number generator.

Eluted peptides from the capillary RP-HPLC column were analyzed by shotgun MS using an LTQ Velos Orbitrap (Thermo Fisher, USA). The instrument was run in data-dependent mode, with up to 20 MS^2 scans with CID fragmentation per MS^1 event. Dynamic exclusion was activated for 30 s after two observations of a given precursor ion, with a maximum exclusion list length of 500 precursors.

2.3. Mass spectrometry data analysis

All data processing was performed using the Trans-Proteomic Pipeline, version 4.7 POLAR VORTEX rev. 1 [17]. Raw files were converted to mzML using ProteoWizard msConvert [18]. Resulting mzML files were searched with four separate proteomics search engines, namely Comet [19], OMSSA [20], MS-GF+ [21], and X!Tandem [22]. The search database consisted of UniRef90 human proteins [23] plus yeast alcohol dehydrogenase (spike-in standard), glu-1-fibrinopeptide (QC standard), trypsin, and bovine serum albumin (contaminants). Decoys were generated via pseudo-randomization and interleaved with target sequences. Data were also searched by MS2 spectral matching using SpectraST [24] against a consensus spectral library built from 6 reconstituted epidermis samples from a set of test cultures. Search results were processed with PeptideProphet [25] to return peptide identifications as a pepXML file. Resulting PepXML files from all search engines were combined with iProphet [26], and proteins were inferred using ProteinProphet. Identifications were filtered at a 1% false positive error rate according to iProphet (peptide) or ProteinProphet (protein) error models. All raw data and search results have been deposited in the PeptideAtlas [27] and are accessible at http://www.peptideatlas.org with the database identifier PASS00363.

The normalized spectral index algorithm [28] was implemented in Python 2.7 and extended to support TPP files as input. Protein identifications were filtered at a 1% FDR based on ProteinProphet error models. Proteotypic peptides were parsed based on the ProteinProphet nondegenerate evidence flag. Fragment ion intensities for +1 charged b- and y-ions were matched and summed, then compiled to protein-level intensities. Values were then normalized based on global matched intensity and protein length. All values reported here have been $\log_2$ transformed.

Power analysis on the pilot RE quantification was performed for a variety of ΔSI_N values using the "pwr" package in *R* with the