

Assessment as qualitative and quantitative of molecular function of protein and peptides in Human 3D Labskin Model comparing with Native Human Skin, using Nano-LC-MS/MS.

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Abstract

The skin is the largest organ of the human body and function as a primary barrier against environmental stressors, xenobiotic and drug metabolism. There is an important need for validated 3D skin models, which can be utilised in the safety assessment of chemicals. Mass spectrometry-based proteomics has been developed as a powerful method for investigating the molecular composition of human skin. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) enables identification and relative quantification of large numbers of proteins and peptides groups from complex biological samples. In this study of proteomics, the level of proteins and peptides in Labskin and NHS have been compared using Nano-LC-MS/MS. Though the expression of proteins level were low in the 3D Labskin model as supplied, significant induction of these proteins were observed after treatment with β -Naphthoflavone, yielded at upregulated of levels in the induced 3D Labskin model compared with 3D Labskin model control. These findings suggest that the proteins of Labskin model appears to be representative of native human skin for metabolism studies of cutaneous exposures to xenobiotic.

Key Words: Human skin - Labskin - metabolism - chromatography

التقييم النوعي والكمي للوظيفة الجزيئية للبروتين والببتيدات في النموذج الجلد البشري ثلاثي

الأبعاد (Labskin) مقارنة بجلد البشري الطبيعي باستخدام Nano-LC-MS/MS.

الملخص

يُعدّ الجلد أكبر عضو في جسم الإنسان، ويعمل كحاجز أساسي ضد العوامل البيئية الضارة، كما يساهم في عمليات أيض المواد الغريبة عن الجسم والأدوية. ولذلك، هناك حاجة مهمة إلى نماذج جلدية ثلاثية الأبعاد مُعتمدة يمكن استخدامها في تقييم سلامة المواد الكيميائية. وقد تطورت تقنيات علم البروتينات المعتمدة على مطيافية الكتلة لتصبح من الأساليب القوية لدراسة التركيب الجزيئي للجلد البشري. وتتيح تقنية كروماتوغرافيا السائل المُقترنة بمطيافية الكتلة تحديد وقياس المستويات النسبية لأعداد كبيرة من البروتينات ومجموعات الببتيدات في العينات البيولوجية المعقدة. في هذه الدراسة، تمت مقارنة مستويات البروتينات والببتيدات في نموذج ثلاثي الأبعاد Labskin والجلد البشري الطبيعي باستخدام تقنية Nano-LC-MS/MS. وعلى الرغم من أن مستويات التعبير عن البروتينات كانت منخفضة في نموذج Labskin ثلاثي الأبعاد، فقد لوحظ تحفيز واضح لهذه البروتينات بعد المعالجة بمادة β -Naphthoflavone، مما أدى إلى زيادة مستويات تعبيرها في نموذج Labskin ثلاثي الأبعاد المُحفّز مقارنةً بنموذج LabSkin ثلاثي الأبعاد غير المعالج Control.

وتشير هذه النتائج إلى أن البروتينات الموجودة في نموذج LabSkin قد تكون مماثلة بدرجة مناسبة للبروتينات الموجودة في الجلد البشري الطبيعي، مما يدعم إمكانية استخدام هذا النموذج في دراسات أيض المواد الغريبة عن الجسم الناتجة عن التعرض الجلدي.

Introduction

The skin is the largest organ of the human body and function as a primary barrier against environmental stressors, xenobiotic and drug metabolism. These function depended on structural proteins, regulatory proteins, and peptides distributed across the epidermis and dermis. Beyond its protective role, the skin is metabolically active, possessing a variety of proteins which break to enzymes that contribute to the biotransformation of endogenous and exogenous compounds. A thorough examination of the native human skin proteome, about 2,948 proteins were presented at low levels (van Eijl, et al., 2012). With over 10,000 proteins measured across several 3D skin model and cell types, quantitative proteomics has shown the significance of cellular variability in the human skin proteome (Dengj el et al., 2020).

In previous review by Oesch et al (2018) an important substitute for animal skin in drug testing has made possible by the design of three-dimensional (3D) human skin. Labskin among these models is a commercially available skin equivalent that reproduce key characterisation of human skin in order to ascertain whether proteins and peptides involved in metabolism, which are present at comparable levels (Oesch et al., 2018).

In vitro models of skin are required as tools for studying absorption, detoxification and metabolism of topically applied drugs as an alternative to experiments using, hard to obtain, ex vivo human skin or live animals (Madison, 2003; Wickett & Visscher, 2006). The major cell types present in skin: keratinocytes and fibroblasts, when cultured in a scaffold develop into a highly discriminated 3D skin equivalent model containing both dermal and epidermal layers (Hu et al., 2010).

Mass spectrometry-based proteomics has been developed as a powerful method for investigating the molecular composition of human skin. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) enables simultaneous identification and relative quantification of large numbers of proteins and peptides groups from complex biological samples. In dermatological research, Nano-LC-MS/MS-based proteomics has been used to characterize high-efficiency peptide and protein detection. This is particularly relevant to skin samples, where abundant structural proteins can be low abundance regulatory protein. A study by Russo et al (2018) demonstrated that the determination of terbinafine absorption as topical treatment in the epidermal of Labskin tissues by LC-MS/MS approach.

Importantly, previous study has shown that using quantitative proteomics to compare Labskin with native human skin (Couto et al., 2021). A recent research by Couto *et al.* (2021) used label-free quantitative proteomics to investigate xenobiotic-metabolizing enzymes in native human skin and 3D LabSkin model. More than 2,000 proteins were identified and quantified several important proteins which showed at low abundance in LabSkin tissues compared to native human skin tissues, supporting the LabSkin for protein identification (Ryan, et al., 2019).

1. The present study aims to perform a qualitative and quantitative assessment of proteins and peptides in a human 3D LabSkin model (LS) in comparison with native human skin (HS) using Nano-LC-MS/MS-based proteomic analysis. The study focuses

on identifying proteins and peptides present in both skin, determining differences in their relative abundance, and evaluating the molecular and biological functions represented by the identified proteomes.

2. A comprehensive comparison of the LS and HS proteomes may provide a more rigorous molecular basis for evaluating the biological relevance of engineered human skin models. Such information could support the application of 3D models for topical drug development, cosmetic testing as alternative to animal models.

Materials and methods

Skin Sample preparation for protein extraction

After 24h, all Labskin (LSE) was cultured 12 well plate (4.5 ml Labskin medium) at 37 °C and 5% CO₂ incubator. The medium was changed every 24 h. Induction process was made as systematic treatment through the addition of inducers dissolved in dimethyl sulfoxide (DMSO) (Sigma-aldrich, Gillingham, UK). The Labskin was induced with 50ug β -Naphthoflavone (BNF) as systematic treatment for 48 h (Wiegand et al., 2014). LSE samples as control were treated only with DMSO not exceed 0.2%. (without drug inducers) for 48 h. After removed LSE from the well insert and samples snap-frozen with liquid nitrogen cooled isopentane (2-5 min) and then, stored at -80 °C. Samples of human skin were obtained from healthy males (n =8) at the Bradford hospital, UK. Subcutaneous tissue was removed before skin tissues samples were cut into small pieces.

For LC-MS/MS experiments, both Labskin and human skin were then homogenized in 2ml of phosphate buffer (150 mM potassium phosphate buffer, pH 7.25 containing 150 mM KCl and 1 mM EDTA) (van Eijl, et al., 2012).

The dermal and epidermal layers from the 3D skin models were isolated by incubation in phosphate-buffered saline (PBS) at 60 °C for 2 min then homogenization with a manual homogeniser (Ultra-Turrax, Germany) in 0.2 % of MeOH (Capolongo et al., 2010). The homogenate samples were then centrifuged at 13000 rpm at 4 °C for 10 min (Centrifugation, Micro Star17R Microcentrifuge, Germany). The LSE samples stored at -80 °C and ready for LC-MS/MS analysis. According to van Eijl, et al., (2012) for LC-MS/MS experiments, the LSEs control and treated LSEs tissues were placed for 2 min in PBS preheated at 60 °C to isolate epidermis (Götz, et al., 2012).

Microsomal and cytosol fractions of skin were prepared by differential centrifugation as described previously by van Eijl, et al., (2012) and stored at 80 °C until required.

Labskin model with LC–MS/MS were applied using a Xevo G2-XS QToF (Waters Corporation, Manchester, U.K.) with ionization mode ESI+ ionization mode as described previously (Russo, et al., 2018). The LC separation was performed using an Acquity UPLC® peptide GSHTM-C18 Column (WatersTM, UK) microbore column (2.1 mm x 100 mm, 1.7 μ m) using a 20 min gradient of mobile phase B 80% (acetonitrile) and mobile phase A (20% containing 0.1 % formic acid). The gradient elution was conducted as follows: 0.0–2.0 min (A, 80%; B, 20%), 2.0–12.0 min (A, 20%; B, 80%), 12.0–18.0 min (A, 20%; B, 80%), 18.0 – 20.0 min (A, 80%; B, 20%). The flow rate was 0.2 mL/min. The injection volume 5 μ L. The MS parameters applied: capillary voltage, 3.0 Kv, cone voltage, 40.0 V, source temperature 150 °C, desolvation temperature 500 °C, desolvation gas 1000 L/h, and cone gas 150 L/h. Argon was utilized as a collision gas and the collision energy was set at 20 eV. The retention time was ~10.0 min. Three replicate measurements of each sample were applied.

For Nano LC–MS/MS analysis.

Data were analysed by nanoLC-MS/MS using data dependent acquisition on the Exploris 480. All proteins expression was identified and quantified by searched against reference human proteome database (Proteome Discoverer 2.5. Spectra software). To assess the different level in proteins in Labskin compared with human skin, the protein databases were an interpreted according to Gene Ontology (GO). The quantitative data (performed by nonlabeld peak intensity measurements) indicated that was a significant difference at the level of proteins in 3D skin control and induced 3D skin compared with human skin.

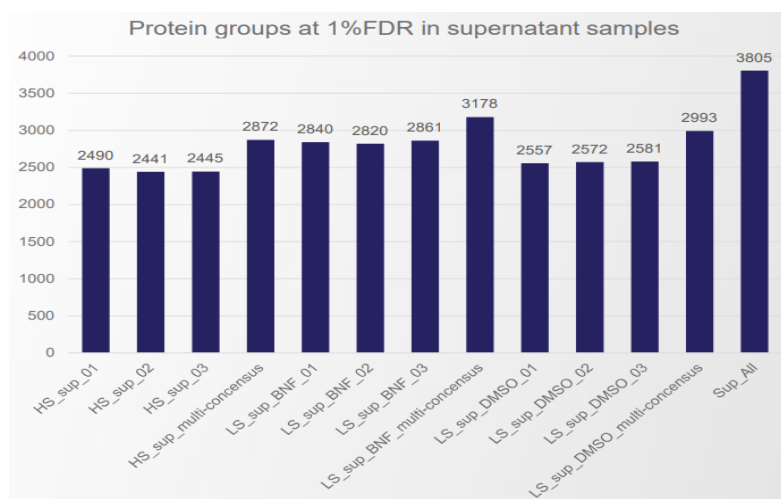
Furthermore, Panther Software (<http://www.pantherdb.org>) was applied to assign protein function using UniProt databases. Statistical analyses were performed using Microsoft Excel 2010 and GraphPad Prism v7.03 (La Jolla, CA).

Results

In this study of proteomics, protein and peptides expression and its function were studied in samples of native human skin (HS), Labskin control (LS) and Labskin treated with β NF (LS_ β NF) (added to the culture medium skin model) after 48h. Label-free proteomics analysis was performed on both the microsomal (supernatant) and cytosol (pellet) fractions. Levels of these proteins and peptides in Labskin and NHS have been

compared using Nano-LC-MS/MS. Our data has been reported at downregulated in 3D skin equivalents model compared to native human skin. Overall, Proteins and peptides were generally higher in human skin than those found in 3D skin model.

Fig. 1. Protein expression groups identified in supernatant samples at 1% FDR



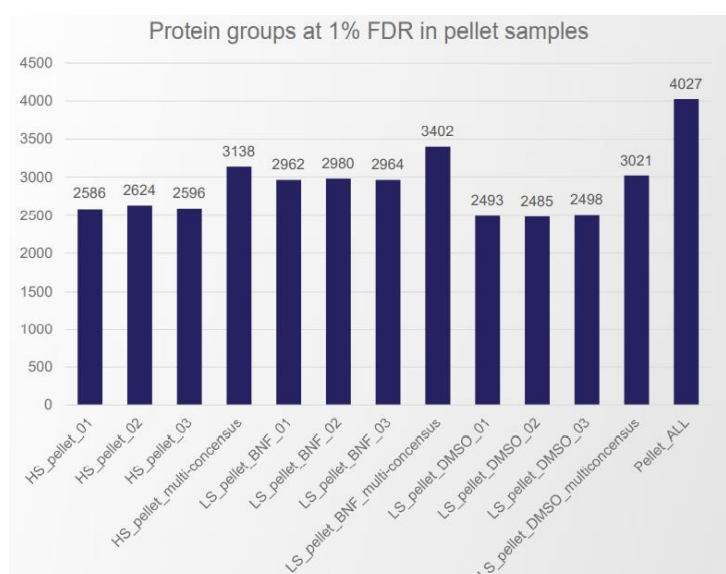
“Figure generated from the authors’ experimental data.”

As shown in Fig 1, the total number of identified protein groups indicated that a higher number proteomic coverage in both skin. The protein group were detected from 2441 to 3805 (in Sup-All as supernatant in both skin) across all conditions. These data were detected and presented as a good relatively proteome coverage. The LS control samples (untreated samples) in three replicates showed the lowest protein identifications, with 2490, 2441, and 2445. These results suggest that LC-MS/MS is a great method for detection of proteins and peptides purification efficiency. In contrast, LS-Sup (LabSkin supernatant) which treated with β NF samples overall increased in the number of identified protein groups, yielded 2872, 2840, and 2820 protein groups. The corresponds to 15–18% increase compared with untreated LabSkin as control samples. While two replicates presented high protein identification, one replicate (2557) was markedly lower than observed in the β NF group.

Although the results were variable between biological replicates samples, this method generally maintained good proteome coverage. Notably, the LS-Sup β NF treated identified about 3,178 protein groups, representing one of the highest identifications. These data demonstrate that BNF inducer was increased the number of proteins and their

level, allowing the mass spectrometer to detect low proteins. Native human skin (HS) replicates showed almost similar number protein with LS-Sup as skin model control which means a good representative to human skin. It can be seen that, LS-DMSO samples have about 2993 proteins groups compared with native skin (about 2872 proteins). However, LS-BNF samples were increased in the number of protein identification compared with LS-DMSO, suggesting improved protein recovery under BNF treatment. Furthermore, the multi-consensus dataset enhanced proteome coverage, while the combined dataset (all Sup) yielded the highest number of protein groups. These findings indicate that both treatment condition contribute to improved protein identification in the supernatant fraction in LS models. These results support the β NF as drug inducer improved and increase the protein identification groups.

Fig. 2. Protein groups identified in pellet samples at 1% FDR



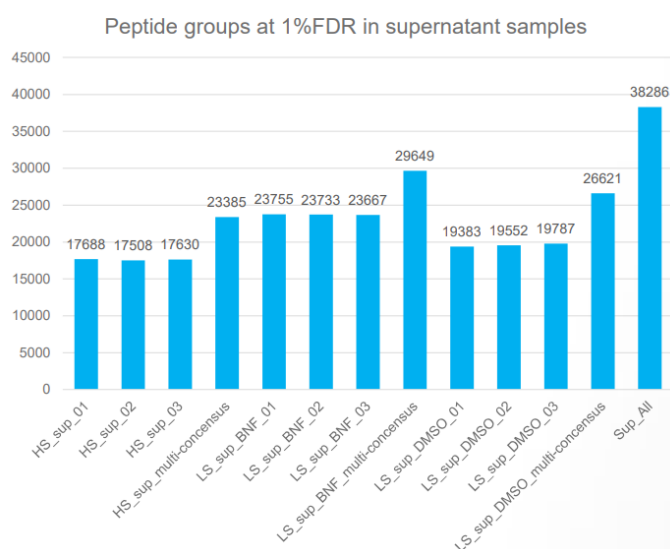
“Figure generated from the authors’ experimental data.”

The results confirm that the depth of proteome coverage, with substantial differences observed among the control and treatment LS pellet fraction compared with pellet in human skin. As shown in Fig. 2, HS pellet (control) indicated excellent reproducibility, with 2586, 2624, and 2596 protein groups recognised across the three biological replicates samples. However, The LS pellet DMSO (control) samples produced the lowest protein group detection between the untreated groups, with 2485, 2493 and 2498 protein groups. The proteins groups in replicates samples produced lower number

with those that found in the HS pellet. These observations suggest that LS DMSO untreatment with inducer samples are less reliable and protein groups for protein extraction which they may not efficiently drug absorption and metabolism.

In contrast, treatment of LS pellet samples using the β -naphthflavon (β NF) was significantly increased the number of identified protein groups in 3D skin models. The three replicates yielded 3138, 2962, and 2980 protein groups respectively, representing an increase of about 14–21% relative compared with the untreated HS pellet samples. These findings show that the β NF drug inducer effectively improves protein number from the pellet fraction. In addition, the Pellet ALL samples of 3D skin models generated the highest number of identified protein groups (4027 protein groups compared with the average HS pellet control (~2602 protein groups), this corresponds to an approximate 55% increase in the protein identification. Overall, the total number of identified proteins ranged from 4027 proteins groups in all pellet. These data clearly demonstrate that the β NF inducer strongly affects proteome coverage in LabSkin pellet samples. Therefore, β NF increased the number of identified protein, confirming good analytical reproducibility in all replicates.

Fig. 3. Peptide groups identified in supernatant samples at 1% FDR.



“Figure generated from the authors’ experimental data.”

Fig. 3 illustrated that the peptide identification groups were detected in supernatant samples, peptide ranged from 17,508 to 38,286 peptides across all datasets. HS replicates showed similar peptide number, indicating high reproducibility. In contrast, peptide

groups were increased in the LS- β NF samples compared with those that found in LS-DMSO as controls using LC-MS/MS. These findings show that the LS- β NF model was the most comprehensive peptide data in 3D skin model, making this it a suitable model for in-depth quantitative proteomic studies. In addition, the multi-consensus data were improved peptide coverage. These results confirm that β NF treatment was a good drug inducer to detect massive peptides groups about 23585 (34%) identification in the supernatant fraction whereas with average 23385 (about 33%) were detect in control sample of skin models.

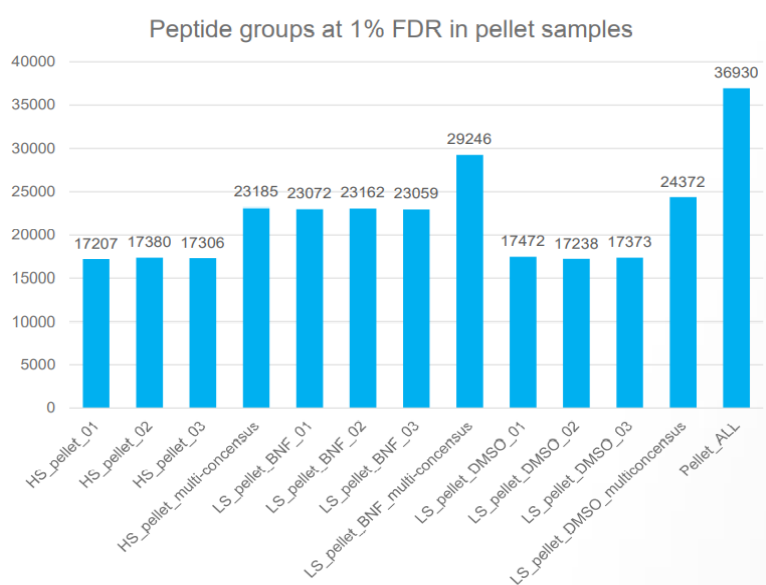
Table 1. Average peptide groups identified at 1% FDR in pellet samples.

Sample	Average Peptide Groups	Change vs HS Control
HS Control	~17,609	—
LS Skin Model Control	~23,385	+33%
LS Skin Model treated with β NF	~23,585	+34%

“Table generated from the authors’ experimental data.”

The HS as normal human skin supernatant were produced about 17,688, 17,508, and 17,630 peptide groups respectively, with an average of about 17,609 peptide groups. These data close agreement between the biological replicates and provides a reliable model for drug testing.

Fig. 4. Peptide groups identified in pellet samples at 1% FDR.



“Figure generated from the authors’ experimental data.”

The results demonstrate significant differences in peptide identification between the skin models and human skin. As we can see that **Peptide groups at 1% FDR in pellet samples** compares the total number of peptide groups identified in pellet fractions. The peptide groups identified in pellet samples at 1% FDR, ranging from 17,207 to 36,930 peptides. HS replicates samples presented consistent peptide groups, supporting experimental reproducibility. LC-MS/MS showed that LS- β NF as skin model treated with inducer revealed high peptides identification groups (about 23111) peptides compared with those that presented (approximately 17373) in LS-DMSO untreated models, indicating β NF improved the skin model for the peptide identification and their levels. These findings confirmed that the pellet fractions of skin model provide deeper peptide coverage in skin, particularly under β NF conditions.

Table 2. Average peptide groups identified at 1% FDR in pellet samples.

Sample	Average Peptide Groups	Change vs HS Control
HS Control	17,207	—
LS βNF	23,111	+34.3%
LS DMSO	17,373	+1.0%

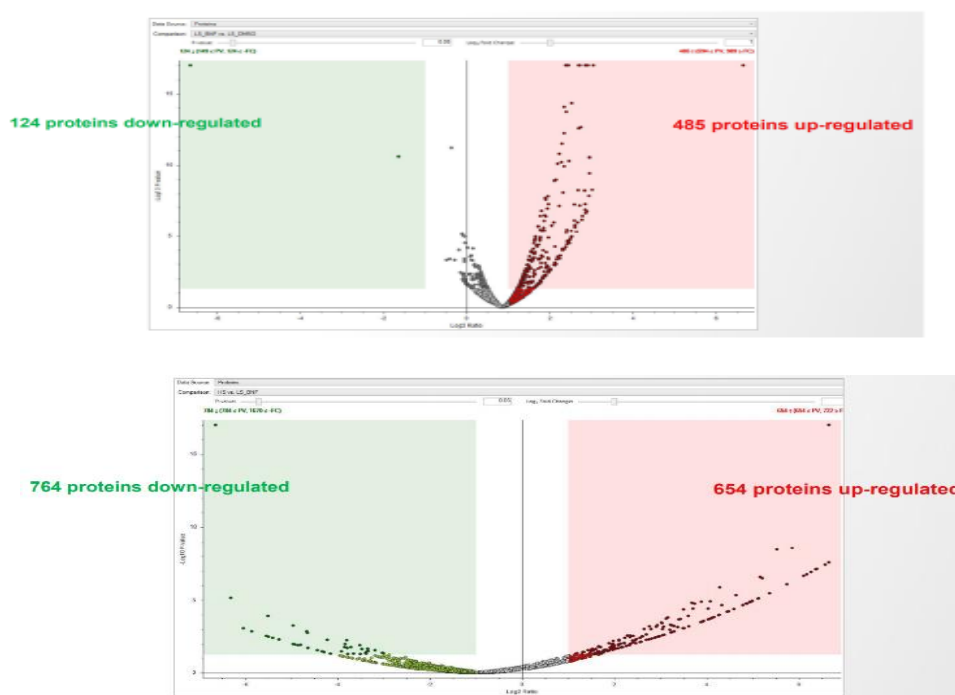
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The percentage changes were calculated relative to the **HS** (17,207 peptide groups). The **LS-DMSO** showed that peptide group numbers comparable to the human skin with +0.1%, whereas the **LS- β NF** sample showed increase about +34.3%, indicating greater proteome coverage compared with the HS Control. The HS Control sample detected as the reference for untreated 3D skin as control, with 17,207 peptide groups identified. This value represents the baseline proteomic profile against which all other experimental groups were compared. The results demonstrate that LS- β NF achieved the greatest increase in peptide identification (+34.3%), suggesting enhanced proteome coverage and potentially greater biological activity compared with the HS. These results indicate that the LS condition, particularly in combination with β NF treatment, provides proteomic coverage, which is advantageous for identifying differentially expressed proteins and biological pathways in subsequent analyses.

Fig. 5: shows the volcano plots for supernatant comparisons (LS-DMSO vs LS- β NF and HS vs LS- β NF).

Volcano plot for supernatant LS_DMSO/LS_BNF

PValue ≤ 0.05 and log2 fold change = 1



“Figure generated from the authors’ experimental data.”

The differential expression analysis was performed using a significance threshold of $p \leq 0.05$ and $\log_2$ fold change ≥ 1 . The vertical threshold ($\log_2$ fold change ± 1) corresponds to at least a two-fold change in protein abundance, while the horizontal threshold ($p \leq 0.05$) indicates statistically significant differences between groups. Proteins located in the upper right region of the volcano plot represent significantly up-regulated proteins 485 proteins in the LS- β NF condition, whereas proteins in the upper left region represent significantly down-regulated with 124 proteins. The LS-DMSO compared with LS- β NF were showed several proteins exceeding both thresholds, indicating statistically significant changes in protein abundance following β NF treatment. Similarly, the HS compared with LS- β NF were revealed both up-regulated proteins, which confirming that LS- β NF treatment induces measurable proteomic level. Proteins close to the centre of the plot did not reach statistical significance, indicating minimal or no change in expression.

Table 2: presents statistically significant quantified proteins with abundance values and protein ratios. The quantification rate represents the proportion of identified proteins for which reliable abundance values were obtained by LC–MS/MS analysis.

Sample	Identified Proteins	Quantified Proteins	Quantification Rate (%)
LS_DMSO Supernatant	2,993	2,710	90.54
LS_DMSO Pellet	3,021	2,687	88.94
LS_βNF Supernatant	3,178	2,819	88.70
LS_βNF Pellet	3,402	2,965	87.15
HS Supernatant	2,872	2,554	88.93
HS Pellet	3,138	2,803	89.32

“Table generated from the authors’ experimental data.”

Overall, statistical assessment of protein abundance was performed through biological samples, and only proteins quantification was included. The large number of quantified proteins suggests high proteome in skin. All samples showed high quantification between **2,872 and 3,402 proteins** were identified, while **2,554–2,965 proteins** were reliably quantified in skin models. The proportion of quantified proteins relative to high proteins abundance detected from **87.15% to 90.54%**, indicating high quantification through 3D skin fraction (both supernatant and pellet) compared with human skin.

Within the supernatant fractions, the LS_DMSO supernatant was quantified around 2,710 quantified proteins of 2,993 identified proteins (90.54%-fold change). However, the LS_BNF supernatant contained a large number of identified proteins (3,178) and quantified proteins (2,819) compared with the LS_DMSO supernatant, even though its quantification rate was slightly lower about 88.70%. The HS supernatant produced the 2,872 identified proteins and 2,554 quantified proteins, with a quantification rate of 88.93%.

Among the pellet fractions, the **LS_βNF pellet** was produced and demonstrated the highest protein identification, with **3,402 identified proteins** and **2,965 quantified proteins**, representing the largest proteomics coverage between all fractions samples. Nevertheless, its quantification rate about **87.15%** was the lowest observed. In addition, about **3,138 were identified proteins** and **2,803 quantified proteins in HS pellet**, corresponding to a quantification rate of **89.32%**, whereas the **LS_DMSO pellet** was produced with approximately **3,021 identified proteins** and **2,687 quantified proteins**, with **88.94%** quantification rate.

Discussion

The current study demonstrates that Nano-liquid chromatography coupled with tandem mass spectrometry (Nano-LC-MS/MS) is a good for the qualitative and quantitative classification of proteins and peptides in reconstructed 3D Labskin (LS)

compared with native human skin (HS). The overall results indicate that the 3D model contains a substantial proportion of the proteins and peptides groups detected in human skin, while also demonstrating clear differences in proteomic abundance and coverage among the 3D reconstructed model and native skin tissue. These findings are particularly relevant to the potential use of reconstructed skin as an *in vitro* model for investigating cutaneous xenobiotic metabolism.

Proteomic coverage of Labskin and human skin

A major finding of this study was the high level of protein identification obtained from both LS and HS samples. Across the different fractions, about thousands and more of protein groups were identified at a 1% false discovery rate (FDR), demonstrating the high analytical coverage achieved by LC-MS/MS.

The LS-DMSO supernatant was produced approximately 2,993 proteins, whereas LS-BNF supernatant increased to about 3,178 proteins. Similarly, the pellet fraction of LS-BNF contained approximately 3,402 identified proteins. These results indicate that β -Naphthoflavone (β NF) was increase the number of detectable proteins in the 3D skin model. The high level in protein may reflect induction or increased abundance of proteins in cellular responses to xenobiotic and drug metabolism. This is in agreement with previous work where proteins that was detected in whole human skin (comprising both epidermis and dermis) and in four *in vitro* epidermal skin models (van Eijl et al., 2012; Ahmad & Mukhtar, 2004). However, an increase in the total number of proteins may not necessarily be interpreted as an equivalent rise in functional metabolic capacity. Proteins finding by proteomics techniques provides evidence of relative abundance, however enzymatic activity needs independent functional validation.

Also, the pellet fraction of skin model demonstrated greater proteomic coverage by β NF exposure. The LS-BNF in pellet fraction produced about 3,402 detection proteins compared with around 3,021 in LS-DMSO and 3,138 in HS as control. This observation suggests that β NF inducer may have increased the abundance of proteins that were previously below the detection. This shows a similar pattern to that previously reported for different skin models such as Phenion FT Skin Model, OS-REp and Monolayers (Baron et al., 2001; Wiegand et al., 2014). Nevertheless, the lower quantification rate observed in the LS- β NF pellet fraction compared with the other groups shows that a higher number of identified proteins does not result in a consistent improvement in quantitative protein.

Peptide identification and reproducibility

The peptide-level data analysis is further supports for the ability of Nano-LC-MS/MS to provide comprehensive proteomic coverage. The human skin control confirmed relatively consistent peptide identification among replicates samples, with an average of about 17,609 peptide groups in the supernatant fraction and about 17,207 peptide groups in the pellet fraction of skin. This provides evidence for good analytical reproducibility.

Interestingly, the Labskin presented higher numbers of identified peptides compared with the human skin. In the supernatant fraction, LS-DMSO and LS- β NF produced around 23,385 and 23,585 peptide groups, respectively, representing rises of about 33–34% compared to HS. Although these proteins were at low level in the skin model control compared to human skin, BNF induction were increased after 48 h as systematic incubation (Table 1). In addition to that, in the pellet, the LS- β NF produced 23,111 peptide groups, corresponding to rises of about 34.3% compared to HS. These observations suggest that the 3D skin reconstructed model, particularly LS- β NF could provide extensive peptide coverage (van Eijl et al., 2012). However, higher peptide identification did not indicate that Labskin is biologically representative of human skin because human skin contains more cell types than the reconstructed model.

Quantitative protein analysis in Labskin and Human Skin

The quantitative analysis in skin tissue confirmed that a high proportion of the identified proteins could be reliably quantified. The rate of protein quantification ranged between approximately 87.15% and 90.54% across the experimental groups. The LS-DMSO supernatant displayed the highest rate of proteins groups with 90.54%, whereas the LS- β NF pellet revealed the lowest rate quantification with about 87.15%. The level of proteins group was extremely low in the Labskin model studied here and this has been previously reported in the ORS-RHE model (Neis et al., 2010). Despite this difference, the overall great quantification rates determine the robustness of the Nano-LC-MS/MS for comparative investigation of the skin samples.

The LS- β NF in pellet fraction showed the highest number of quantified proteins, 2,965 quantified from 3,402. This support the data was analysed previously for investigating proteins in metabolism (Bacqueville et al., 2017). Therefore, the results revealed that β NF induction can increase the proteomic data of the Labskin model, providing additional

opportunities to investigate changes in metabolic pathways. In a previous study, almost all of proteins observed in Labskin models and native human skin were detected as proteins and genes in skin by Oesch et al., (2018). Support for the abundance of peptides reported by Couto et al., (2021), who first time used a proteomics approach to study Labskin and human skin, but here none of the CYPs proteins were detected.

Also, the volcano-plot analysis further confirmed measurable differences in protein abundance among LS-DMSO, LS- β NF and HS. According to using a statistical threshold of $p \leq 0.05$ and a $\log_2$ fold change threshold of ± 1 , numerous proteins showed statistically significant changes. These results indicate that β NF exposure produced a measurable molecular response in the reconstructed skin model rather than simply increasing the total number of proteins discovered as confirmed by Hansen, et al. (2016).

Conclusion

The comparison between reconstructed skin model and native human skin is particularly significant for determining whether 3D model can be used as an alternative experimental model for drug metabolism. Overall, the current findings validate that reconstructed 3D Labskin has proteomics data similarity to native human skin and contains a variety of proteins including in xenobiotic metabolism. It can be showed that β NF as drug inducer increased protein and peptide identification. However, the lower abundance of many proteins in Labskin compared with native human skin indicates that the 3D skin model does not fully reproduce the metabolic phenotype of native tissue. The study therefore supports the use of Labskin as a valuable complementary *in vitro* model for investigating protein expression, xenobiotic metabolizing pathways, and responses to chemical exposure. The combination of reconstructed skin models with high-sensitivity Nano-LC-MS/MS can provide a powerful method for identifying molecular comparison between native human tissue and skin models as alternative to human skin.

Recommendation

Based on the proteomic data, Labskin can be considered a valuable *in vitro* model for investigating human skin protein expression and molecular functions. We recommend combining quantitative Nano-LC-MS/MS with proteins analysis and validation of using complementary techniques such as western blotting or immunohistochemistry. Future studies should focus on identifying conserved and differentially expressed proteins involved in skin barrier function, and xenobiotic metabolism.



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