CELL PROTEOMIC FOOTPRINT

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The authentication of mammalian cell cultures and their subpopulations are of tremendous demand in biotechnology and cell therapy. However, current techniques are either not efficient or can be very complex and expensive. Here we report a simple and straightforward approach for authentication of biological cells and their subpopulations with high speed, high throughput, low sample cost, and high sensitivity. We discovered that cell cultures treated with protease at soft, "non-killing" conditions release fragments of cell surface proteins, which composition is a strong characteristic of the cells. Mass spectrometric analysis of the released fragments allows a direct comparison of the produced mass spectrum with the mass spectrum of known cells. As an example, we applied this technique to verify subpopulations of human fibroblasts which have different origins and exhibit different medical characteristics.

Cultivated *in vitro* cells have a great commercial value in a wide range of therapeutic applications, among these are: tissue engineering applications,[1,2] regenerative medicine,[3] cell-based anti-cancer vaccination,[4] burns treatment,[5] etc. However, cultivated cells indicate that cross-contamination between cell lines is widely prevalent, and continues to be a major problem[6–9]. From the existing estimates, it is know that during the cultivation process up to 36% of cell lines have already a different origin from their initial cell lines.[10]

From the known cell authentication methods,[11] short tandem repeat profiling (DNA fingerprinting)[10] is considered to be the most powerful method that provides an international reference standard for authentication of human cell lines.[12] However, this method exhibits some limitations: DNA fingerprinting is usually so complex and labor intensive that the cost of fingerprinting can be up to $200 for each cell line,[12] moreover DNA fingerprinting profiles could be very difficult to interpret and store in computer databases. Another major limitation is that the common cell culture protocols may dramatically affect the fingerprinting profile of certain cell lines thus making the definition of their origin improbable[13]. The control of cell propagation by DNA fingerprinting, when following a GTP (Good Tissue Practice) protocol, may increase the cost of end-product substantially.

Also important application where the authentication of cell subpopulations is essential is primary cultures in cell therapy. For example, fibroblast or cancer cell primary cultures have an extremely high potential value in therapeutic applications and exhibit different useful properties depending on its origin.[4,14-18]

Thus, a simple and routine method for identification of cells and their subpopulations is required. Direct mass-spectrometry is proven to be a good method for that. Previously, a fast and simple approach for cell identifying was proposed where mammalian cells are lysed with 2,5-dihydroxybenzoic acid (DHB) and analyzed by matrix assisted laser desorption ionization time-of-flight (MALDI TOF) mass spectrometry. Using this unique mass spectral protein profile the authors were able to differentiate among several different mammalian cell lines.[19]

Here we report a simple and straightforward approach for authentication of mammalian cells and their subpopulations with high speed, high throughput, low sample cost, and high sensitivity. We discovered that cell cultures treated with protease at soft, "non-killing" conditions release fragments of cell surface proteins, which composition is a strong characteristic of the cells (see Fig.1). Mass spectrometric analysis of the released fragments allows a direct comparison of the produced mass spectrum with the mass spectrum of a known cells. Thus, analyzing peptides instead of the whole cell lysate avoids contamination of mass spectra by high abundant and common for all mammalian cells house-keeping proteins, lipids, nuclear acids and other intracellular contents. As a result, obtained peptide samples ideally suit for MALDI mass analysis, which provides enough information to differentiate cells on subpopulation level.

In this study we applied this technique to authenticate different types of human fibroblasts. All fibroblasts have spindle-like morphology in cultures, but exhibit different medical properties according to their origin: dermal papilla fibroblasts are trichogen cells,[15] adipose-derived fibroblasts are pluripotent,[16] fetal skin fibroblasts have high potency to proliferate and are well suited for allogenic cell therapy,[17] wherever adult skin fibroblasts are well suited for autologouse cell therapy.[18] Fibroblast cultures could be a very good model to test new cell authentication methods, because they have an identical morphology and are propagated under the same conditions.

Fibroblast primary cultures were analyzed by mass spectrometry according to the following protocol (see Methods). Mass spectra were then compared by hierarchical cluster analysis. Figure 2 shows obtained hierarchical tree plot where all mass spectra were split into two main groups: fetal and non-fetal cells. Mass spectra of non-fetal cells were then split into subgroups according to the cell origin: skin fibroblasts and a group of other non-fetal cells, which was again divided in to subgroups of dermal papilla fibroblasts and adipose-derived fibroblasts. Thus all mass spectra were separated into groups and subgroups according to cells origin.

Obtained data allows to define different characteristic of fibroblasts such as: fetality, pluripotency or trichogen properties. So offered protocol can be considered as a new cell authentication technique suitable for primary cultures and their subpopulations. Apart from all other methods only this satisfies all the criteria below:

High throughput: 10 min per sample.

High sensitivity: 500 cells are enough for the analysis and this parameter mainly is limited by mass spectrometer sensitivity; note that all cells utilized in this analysis can be returned to cultivation, which is extremely important when only few cells are available for the analysis.

Full integration in cell cultivation technology: cell treatment with trypsin in soft conditions is widespread in adherent cell culture maintain protocols for collecting cells from the surface and their further subcultivation. Thus samples of used harvesting solution can be directly used for analysis.

Low cost: ~15$ per sample, that is much lower in comparison with all other offered techniques.

Easy data processing: mass spectra return numerical results, which avoids human factor in cells authentication.

This technique can also be applied for large-scale applications: sample preparation, mass spectrometry analysis can be easily automated by available on the market instruments.

Obtained by offered technique mass spectra characterize cells in a new, previously not described manner. For this reason the term '*cell proteomic footprint*' was introduced for such mass-spectra. Cell culture foot printing offers an excellent alternative to traditional laboratory authentication methods for cells and cells subpopulations. Because the proteomic footprint specific for each individual cell cultures, it is useful not only to authenticate cell culture but also to monitor their identity during propagation in a laboratory. The speed, robustness and minimal costs of sample preparation and measurement for this method makes it exceptionally well suited for routine and high-throughput analysis.

METHODS

42 primary cultures from the Cell Culture Collection (ZAO BioBohemia, Russia) of dermal papilla fibroblasts, adipose-derived fibroblasts, adult skin and fetal skin fibroblast cultures, which were initiated as described by Lü et al.[15], Zuk et al.[16], Rittie and Fisher[20], and Salvatori et al.[21], respectively. Primary cultures were cultivated in identical conditions (DMEM, 10% FBS, 5% CO_2, 37ºC) and between passage 3 and 5 were analyzed by mass spectrometry according to the following protocol:

1. Cells were washed at least three times with 0.9% NaCl to remove serum traces.

2. Cells were quickly rinsed with cold trypsin solution (4-8°C, 1 μg/ml, trypsin activity 3000 U/mg; Sigma, USA) prepared in 0.9% NaCl followed by cell incubation at 37ºC and >95% humidity.
3. Between 5 and 7 min of incubation cells were rinsed with 0.9% NaCl (1 ml per 25 cm^2 of flask surface) to wash off protein fragments released from cell surfaces. Cells at this moment must be attached to flask bottom and have a round shape (see Fig.1). If some cells were detached from flask surface the samples were quickly centrifuged to remove cells from NaCl solution.
4. Obtained NaCl solution with protein fragments was desalted by using $ZipTip_{C18}$ (Millipore Corp., USA) according to protocol of the manufacturer (Millipore Corp., USA). MALDI samples were prepared using a standard "dried droplet" method with 2,5-dihydroxybenzoic acid (DHB) as a matrix. All mass spectra were acquired on a MicroFLEX MALDI-TOF mass spectrometer (Bruker Daltonik, Germany) in linear positive ion mode.

The sets of the obtained mass spectra were encoded into binary format, where 'one' – is a presence of a measured peptide mass in the spectrum, 'zero' – is the absence one. Binary encoded mass spectra were partitioned into different groups by hierarchical cluster analysis using Ward method and correlations between spectra as a distance matrix.

AUTHORS CONTRIBUTION

P.L. designed the study; P.L., B.E., and M.D. performed experiments, P.L. collected and analyzed data; P.L. and M.D. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

P.L. and E.B. declare that they have competing financial interest. Patent value may be affected by publication.

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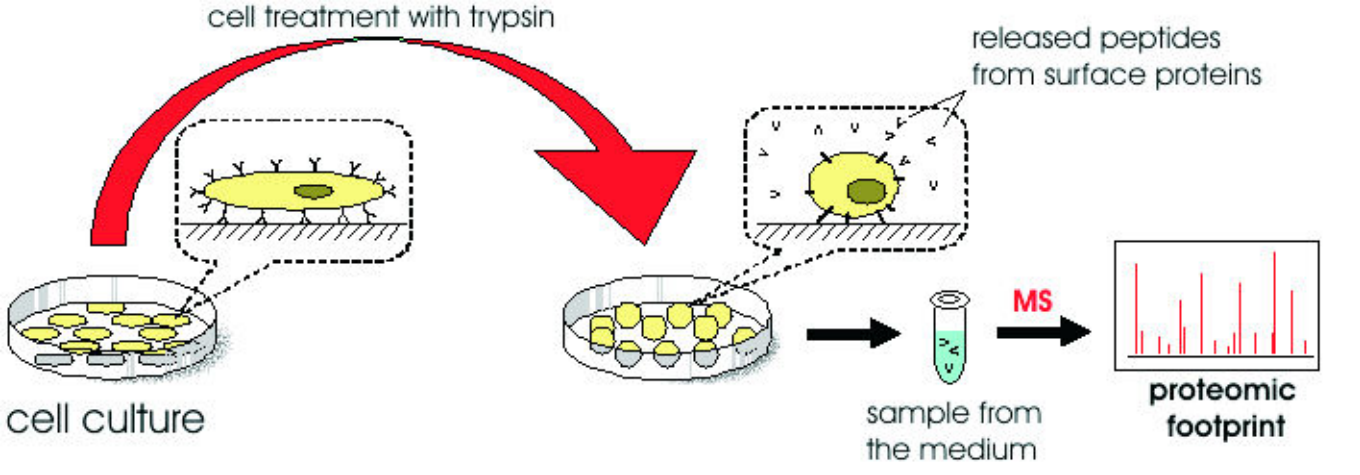
Figure 1. Main steps of cell culture proteomic footprinting.

Culture of adherent cells after washing from traces of culture medium is treated with protease at conditions nonkilling for cells. Released fragments from cell surface proteins are collected and submitted to mass spectrometry. The set of obtained peptide molecular weights represents cell culture proteomic footprint.

Figure 2. Cluster analysis of 42 footprints obtained for fibroblasts primary cultures.

Fibroblast cultures were treated with trypsin at nonkilling for cells conditions. The masses of peptides, released from cell surfaces, were measured by mass spectrometry. Obtained mass spectra (cell proteomic footprints) were encoded in binary format, where ‘one’ is a presence of measured peptide mass in spectrum and vertical dash in figure, ‘zero’ - is the absence one. Footprints were clusterized using Ward method. Footprints of dermal papilla fibroblasts, adipose-derived fibroblasts, skin and fetal skin fibroblasts form separate clusters due to their similarity. The length of branches of hierarchical tree reflects the distance between footprints in similarity units.


cell treatment with trypsin
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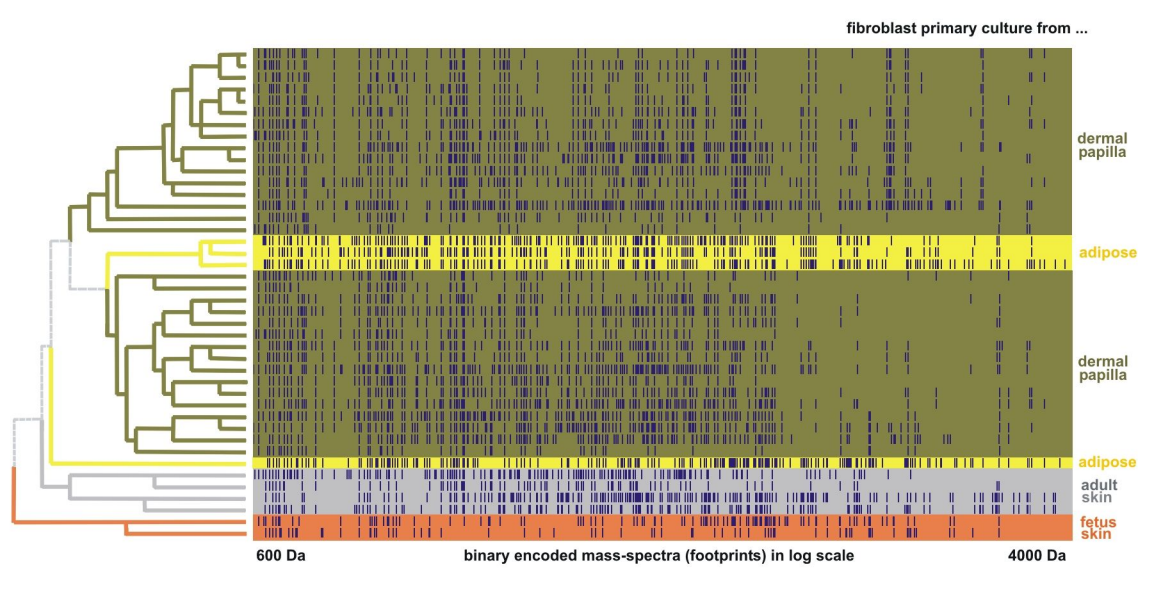
fibroblast primary culture from ...
dermal papilla
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dermal papilla
adipose
adult skin
fetus skin
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binary encoded mass-spectra (footprints) in log scale
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