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Comprehensive proteomic characterization of stem cell-derived extracellular matrices

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ABSTRACT

In the stem-cell niche, the extracellular matrix (ECM) serves as a structural support that additionally provides stem cells with signals that contribute to the regulation of stem-cell function, via reciprocal interactions between cells and components of the ECM. Recently, cell-derived ECMs have emerged as *in vitro* cell culture substrates to better recapitulate the native stem-cell microenvironment outside the body. Significant changes in cell number, morphology and function have been observed when mesenchymal stem cells (MSC) were cultured on ECM substrates as compared to standard tissue-culture polystyrene (TCPS). As select ECM components are known to regulate specific stem-cell functions, a robust characterization of cell-derived ECM proteomic composition is critical to better comprehend the role of the ECM in directing cellular processes. Here, we characterized and compared the protein composition of ECM produced *in vitro* by bone marrow-derived MSC, adipose-derived MSC and neonatal fibroblasts from different donors, employing quantitative proteomic methods. Each cell-derived ECM displayed a specific and unique matrisome signature, yet they all shared a common set of proteins. We evaluated the biological response of cells cultured on the different matrices and compared them to cells on standard TCPS. The matrices lead to differential survival and gene-expression profiles among the cell types and as compared to TCPS, indicating that the cell-derived ECMs influence each cell type in a different manner. This general approach to understanding the protein composition of different tissue-specific and cell-derived ECM will inform the rational design of defined systems and biomaterials that recapitulate critical ECM signals for stem-cell culture and tissue engineering.

1. Introduction

In the body, cells are surrounded by a complex three-dimensional microenvironment, termed the extracellular matrix (ECM), which provides cells with many chemical and biophysical signals required for cell function. Both specific components and biophysical properties of the ECM coordinate intracellular signaling and downstream biological responses through bidirectional interactions with the cells, regulating numerous physiological processes such as cell survival, migration, proliferation and differentiation [1–3]. In the stem-cell niche, cell-matrix interactions influence and modulate stem-cell self-renewal and differentiation. That is, the ECM operates *in vivo* not only as a cellular support but also directs cell fate through coordinated physical and biochemical cues [4–6].

During mammalian stem-cell culture, stem cells are removed from their native microenvironment (*e.g.*, the bone-marrow niche for bone-marrow-derived mesenchymal stem cells (MSC), adipose tissue for adipose-derived mesenchymal stem cells) and need to

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adapt to a relatively foreign environment, that is, tissue-culture polystyrene (TCPS), which is fully synthetic and does not present standard ECM signals. Evidence in the literature has shown that TCPS biases MSC function resulting in lower proliferation rates and a loss of stemness over sequential passages [7–9]. Therefore, ECM-based cell-culture substrates have been developed in an attempt to better recapitulate *in vitro* the native cellular microenvironment [10–14]. MSC are able to deposit an ECM on TCPS over the course of two weeks and this ECM can be used, after decellularization, as a culture substrate for a new batch of MSC. It has been reported that the culture of MSC on *in vitro* cell-derived ECM induces significant biological changes in MSC function compared to standard culture conditions [10,11,15,16]. As ECM components are key players in the regulation of cellular processes, it is critical to gain a better knowledge of ECM composition to decipher how ECM components regulate cell function. In addition, a comprehensive understanding of cell-matrix interactions will provide further insight into the rational design of ECM-mimicking substrates for tissue engineering and regenerative medicine [17,18].

Despite several reports on the development of cell-derived ECMs for MSC culture, detailed data about their molecular composition is limited. Traditional biochemical analysis of ECM is challenging on account of the insolubility and complexity of ECM components [19]. To address these issues, we have applied a proteomic approach initially described for the analysis of tumoral ECM [20]. The method consists of a sequential digestion of the ECM proteins followed by tandem mass spectrometry and bioinformatic analyses, yielding a detailed inventory of the ECM and ECM-associated proteins (termed the “matrisome”). By coupling this method to label-based quantitative proteomics, we were able to characterize and compare the molecular composition of cell-derived ECM produced by different cell types *in vitro*, specifically bone- marrow-derived human mesenchymal stem cells (Bm MSC), adipose-derived MSC (Ad MSC) and human neonatal dermal fibroblasts (NHDF), as well as to evaluate the ECM produced by cells from different donors. Proliferation and mRNA transcriptomic profiling of the cells cultured on the different ECM were performed and compared to standard culture conditions.

We observe that, in addition to a set of common proteins, each cell-derived ECM contains cell-type-specific proteins. Quantitative proteomic analysis reveals a specific matrisome signature for each type of ECM. The matrices lead to differential cell growth and gene expression among the cells as compared to TCPS culture, indicating that the ECM signatures influence each cell type in a differential fashion.

2. Materials and methods

2.1. Preparation of in vitro cell-derived ECM

ECM plates were provided by StemBioSys (San Antonio, Texas) and prepared according to a published protocol [16]. Briefly, human mesenchymal stem cells derived from bone marrow (Bm MSC, Lonza) or from adipose tissue (Ad MSC, Life Technologies), or neonatal dermal fibroblasts (NHDF, Life Technologies) were seeded onto a 75 cm^2-cell culture flask coated with human fibronectin (1 h at 37 °C) at a cell density of 3500 cells/cm^2 and cultured in α-MEM medium supplemented with 15% fetal bovine serum (FBS) and 1% penicillin-streptomycin for 14 days. The medium was refreshed the day after initial seeding and then every 3 days. At day 7, ascorbic acid 2-phosphate (A2P, Sigma) was added to the medium at a final concentration of 50 μM, and A2P-supplemented medium was used until the end of ECM production, with medium changes every other day. At day 14, plates were decellularized using 0.5% Triton in 20 mM ammonium hydroxide for 5 min, rinsed two times with Hank's Balanced Salt Solution containing both calcium and magnesium (HBSS +/+), and once with ultra-pure H_2O. Plates coated with cell-derived ECM were stored dry at 4 °C until use for cell culture. *In vitro* ECM produced by commercially available Ad MSC (two different 35- and 45-year-old female donors), Bm MSC (six different 19- to 22-year-old males and female donors) and NHDF (two different new born male donors) are designated as Ad ECM, Bm ECM and Der ECM, respectively. The donor characteristics listed above were provided by the companies.

2.2. Proteomic analysis of ECM samples

The ECM was mechanically detached from the 75 cm^2-cell culture flask using a cell scraper in 2 ml of HBSS +/+, centrifuged at 16,000×*g* for 5 min, washed with 1 ml of HBSS +/+, centrifuged, and dried in a Speed-Vac (Savant) for 15 min. The ECM pellet was then processed as described previously [20,21]. Briefly, the ECM pellet was resuspended and reduced in a solution of 8 M urea, 100 mM ammonium bicarbonate, and 10 mM dithiothreitol at pH 8 under agitation at 37 °C for 2 h. After cooling, cysteines were alkylated by adding iodoacetamide at a final concentration of 25 mM for 30 min. The ECM sample was then diluted to 2 M urea, 100 mM ammonium bicarbonate (pH 8), and deglycosylated with PNGaseF (2000 U, New England BioLabs, Ipswich, MA) for 2 h under agitation at 37 °C, followed by digestion with Lys-C (Wako Chemicals USA, Richmond, VA), at a ratio of 1:100 enzyme:substrate, under agitation at 37 °C for 2 h. Final digestion was done using trypsin (Sequencing Grade, Promega, Madison, WI), at a ratio of 1:50 enzyme:substrate, under agitation at 37 °C overnight, followed by a second aliquot of trypsin, at a ratio of 1:100 enzyme:-substrate, and an additional 2 h of incubation. Digests were acidified and desalted using 30 mg HLB Oasis Cartridges (Waters Corp., Milford, MA) eluted with 50% acetonitrile with 0.1% trifluoroacetic acid (TFA), followed by concentration in a Speed-Vac.

2.3. Analysis by mass spectrometry (LC-MS/MS)

Each sample was separated by reverse-phase HPLC using an EASY-nLC1000 liquid chromatograph (Thermo Fisher Scientific, Waltham, MA) over a 140-min gradient before nanoelectrospray using a Q Exactive mass spectrometer (Thermo Fisher Scientific). The mass spectrometer was operated in a data-dependent mode. The parameters for the full-scan MS were: resolution of 70,000 across 350–2000 *m/z*; AGC 3e6; and maximum IT 50 ms. The full MS scan was followed by MS/MS for the top 10 precursor ions in each cycle with a normalized collision energy (NCE) of 28 (unlabeled samples) or 32 (labeled samples) and dynamic exclusion of 30 s. Raw mass spectral data files (.raw) were searched using Proteome Discoverer (Thermo Fisher Scientific) and Mascot version 2.4.1 (Matrix Science) using the SwissProt *Homo sapiens* database (*SwissProt_2016_02*, *Homo sapiens 20199 sequences*) containing 20,199 entries. Mascot search parameters were: 10 ppm mass tolerance for precursor ions; 0.8 Da for fragment-ion mass tolerance; 2 missed cleavages of trypsin; fixed modifications were carbamidomethylation of cysteines and for the quantitative experiments: Tandem Mass Tag (TMT) 6-plex modifications of lysines and peptide N-termini; variable modifications were oxidized methionines, deamidation of asparagines, pyro-glutamic acid modification at N-terminal glutamines; and hydroxylation of prolines and lysines. Only peptides with a Mascot score greater than or equal to 25 and an isolation interference less than or equal to 30 were included in the quantitative data analysis. The average false discovery rate was 0.0080 (ranging from 0.0033 to 0.0107). Proteins were identified as being ECM-derived or not using the human matrisome annotations as previously described [22,23].