

Profile of Secreted Proteins In Conditioned Media Which Supports The Prolonged Growth Of Human Embryonic Stem Cells (hESC)

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OVERVIEW

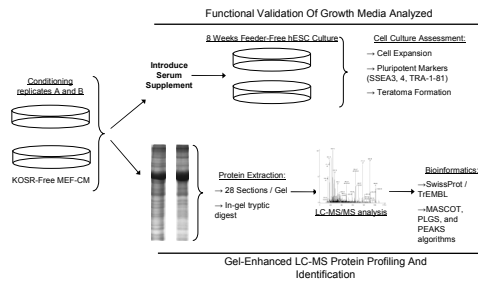
Human embryonic stem cells (hESC), derived from the inner-cell mass of pre-implanted embryos, are stable cell lines that can proliferate indefinitely in culture, and form all primary human cell types.^{1,2} Consequently, they may provide clinical potential for cell replacement based regenerative therapies after tissue or organ injury.³ Unfortunately, little is known with respect to the signals that control their differentiation into different tissue types, and, more importantly, factors that control self-renewal of hESCs in culture. In addition, much of what is known about hESC has proven contrary to previous observations made using similar cells from mice. Understanding the fundamental mechanisms of hESC growth and differentiation will be essential in order to effectively use hESC for future therapeutic and research endeavors. Here, using mass spectrometry-based proteomic approaches combined with biological assays and measures that define hESCs, we take an integrated approach to characterize secreted proteins – specifically, growth factors – introduced to hESCs by mouse embryonic fibroblast feeder cells.

Recently, it was shown that undifferentiated hESC growth is supported by media that is pre-conditioned by replication deficient mouse embryonic fibroblasts (MEFs).⁴ **HYPOTHESIS: there are protein factors released into the growth media by the embryonic fibroblasts which enable hESCs to maintain normal, pluripotent growth.**

Previous attempts have been made at profiling the proteome of MEF conditioned media (MEF-CM), their results have been lacking in terms of both proteome coverage and functional relevance to hESC biology.^{5,6}

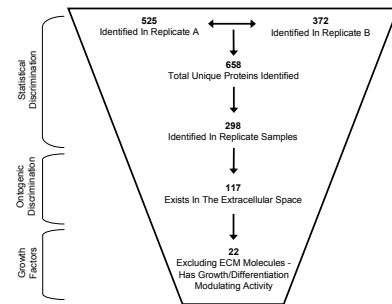
Knockout serum replacement (KOSR) media is a key component of hESC culture media. Like other serum supplements, its components account for the majority of proteins present in media, greater than 10⁷ in excess of those secreted by the MEFs. To facilitate proteomic analysis, MEF-CM was generated free of KOSR and validated for its ability to continue the long-term support of hESC culture under the new formulation.

EXPERIMENTAL STRATEGY



Profiling the proteome of KOSR-free MEF-CM. In two independent procedures MEFs were irradiated and plated on gelatin coated plates. KOSR-free hESC media was conditioned for 24hrs at a time over the course of 5 days and pooled. The KOSR-free hESC media conditioned by MEFs was subject to two parallel lines of investigation: **1) Gel-enhance LC-MS/MS analysis** – Media concentrated and fractionated by SDS-PAGE; each lane was sectioned into 28 segments. Segments were subject to an in-gel digestion with trypsin and extracted peptides analyzed by reverse phase liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). The resulting spectra were searched against SwissProt / TrEMBL protein databases using the indicated algorithms. **2) Validation of KOSR-free MEF-CM functionality** – Conditioned media was re-supplemented with KOSR post-conditioning and used to culture hESC (H9 cells) over multiple passages. Cell expansion as well as hESC surface markers were monitored at each passage. Following 8 weeks of growth, hESCs cultured in KOSR-free MEF-CM were assessed via teratoma formation for their ability to form cell types from all 3 germ layers.

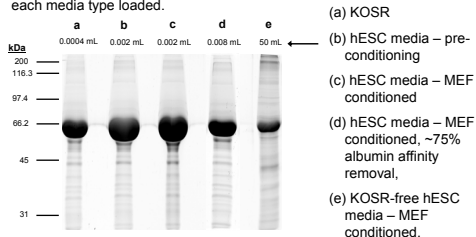
Filtering The Dataset



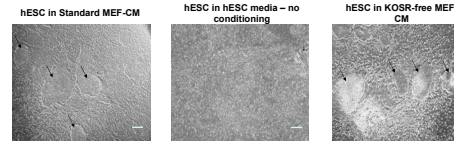
Dataset was filtered based on a duplicate analysis and multiple gene ontology parameters. From a total of 658 unique proteins identified a candidate list of 22 was created for further testing in culture.

Why Remove The Serum Supplement?

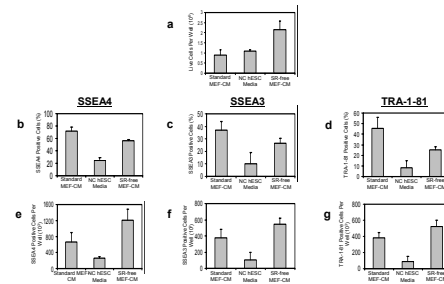
Under standard culture conditions only proteins inherent in the KOSR supplement can be visualized in a standard SDS-PAGE protein analysis even following the removal of ~75% of the most high abundant species. By removing the KOSR from culture the volume of media assayed can be increased 10⁴-10⁵ fold. Loadings were normalized to approximate 40µg of total protein per lane. The bold arrow indicates the effective volume of each media type loaded.



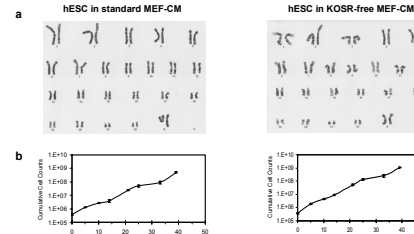
Testing The Function Of KOSR-Free MEF-CM



KOSR-free MEF-CM maintains hESCs in an undifferentiated state. hESCs were cultured in parallel over 10 passages in either standard MEF-CM (left), hESC media without conditioning (middle), or MEF-CM conditioned KOSR-free and re-supplemented with KOSR prior to culture (right). Shown here is the cell culture morphology by phase contrast microscopy during the final week of parallel culture. Arrows indicate typical hESC colony structures. Scale bars represent ~250µm.

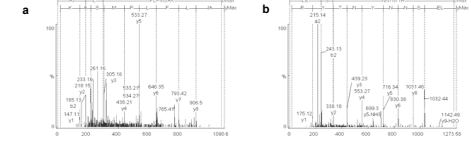


Cells grown in KOSR-free MEF-CM display similar hESC markers and cell numbers as compared to those grown in standard MEF-CM. Live cell counts and hESC surface markers were averaged and compared for weeks 7 and 8 of parallel culture for the three medias tested. **NC** – no conditioning. (a) Total number of live cells per well at passage. (b-d) Frequency of cells expressing hESC surface markers: (Stage Specific Embryonic Antigen) SSEA4, SSEA3, (Tumor Rejection Antigen) TRA-1-81, respectively. (e-g) Total number of cells per well expressing hESC surface markers SSEA4, SSEA3, TRA-1-81, respectively.



hESC grown in KOSR-free MEF-CM proliferate at a similar rate and maintain a normal karyotype when compared to those grown in standard MEF-CM. hESCs were cultured in either standard MEF-CM (left), or MEF-CM conditioned KOSR-free and re-supplemented with KOSR prior to culture (right). (a) Karyotype of cells following after 12 passages of parallel culture. (b) Proliferation capacity.

Functionally Significant Proteins Identified



Summary Of The Protein Profile

(a) Cellular distribution of the 298 proteins identified between the duplicate media profiles. (b) Functional distribution of the 117 extracellular proteins identified in duplicate analyses.

The cellular distribution of proteins identified in MEF-CM is predominantly extracellular. Based on their gene ontology the above pie charts show the functional distribution of proteins identified in duplicate analyses. (a) Cellular distribution of the 298 proteins identified between the duplicate media profiles. (b) Functional distribution of the 117 extracellular proteins identified in duplicate analyses.

Summary:

- Duplicate batches of serum-free hESC MEF-CM were created.
- All modified media showed an equivalent ability to support normal, long-term hESC growth when compared to standard MEF-CM.
- Gel-enhanced LC-MS/MS characterized over 2500 different peptides resulting in the identification of 658 unique proteins – 287 in duplicate.
- 40% of the proteins identified in duplicate are known to be or are predicted to be secreted.
- From the secreted proteins found in the duplicate analyses, a candidate list of 22 soluble growth factors was determined.

Future Directions:

Currently, the candidate factors identified are being evaluated via gain and loss of signaling function experiments. This shall define and validate the mechanism of action of these factors on hESC maintenance, as well as on the orchestration of differentiation into functional mature cell types.

References:

- ¹Thompson JA, et al. (1998) *Science*, **282**: 1145-47. ²Reubinoff BE, et al. (2000) *Nat Biotech*, **18**: 399-404. ³Weissman IL, et al. (2000) *Cell*, **100**: 157-68. ⁴Xu C, et al. (2001). *Nat Biotech*, **19**: 971-74. ⁵Lim J & Bodnar A. (2002) *Proteomics*, **2**: 675-86. ⁶Prowse ABJ, et al. (2005) *Proteomics* **5**: 1-11. ⁷Xu RH et al. (2005). *Nat Meth*, **2**: 185-190. ⁸Wang L, et al. (2005) *Blood*, Feb 17.