QUALITATIVE AND QUANTITATIVE PROTEOMIC PROFILING OF CRIPTO-/- EMBRYONIC STEM CELLS BY MEANS OF LC-MS ANALYSIS

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INTRODUCTION

Embryonic stem (ES) cells deriving from the inner cell mass of the blastocyst are able to differentiate into all lineage derivatives of the three germ layers: ectoderm, endoderm, and mesoderm (Figure 1). ES cells are therefore an outstanding model system both for developmental studies as well as for biomedical applications. Recently, particular attention has been paid to molecules and signaling pathways controlling the balance between ES cell self-renewal and differentiation. Cripto is a key regulator of ES cell differentiation and mouse Cripto-/- ES cells have been utilized to investigate the molecular mechanisms underlying early events of mammalian lineage specification and differentiation.

A 2D LC-MS/MS approach has been used to qualitatively profile the cripto–/– ES cell proteome. The method is based on the multidimensional separation of a complex tryptic mixture using a nanoscale LC system connected on-line to a mass spectrometer capable of data-directed switching between the MS and MS/MS modes. The study enabled a large dataset to be recorded from relatively low sample amounts. Qualitative information on the proteins present in the complex mixture was obtained by databank searching of the resulting ESI-MS/MS spectra. Furthermore, the identified proteins have been classified in terms of subcellular localization, molecular function and biological process as defined by their associated Gene Ontology annotation.

A quantitative profile of the Cripto–/– ES cells proteome was obtained by performing a label-free quantitative LC-MS experiment, utilising multiplexed, alternate scanning LC-MSE. The principle of the method is based upon the measurement, and subsequent comparison, of the chromatographic peak area for each peptide, across samples. These peptides are subsequently mapped back to their constituent proteins to determine the relative amounts of each protein, which in this study allows an integral view of the alterations induced in stem cell functions by deleting the cripto gene. Several differentially expressed proteins have been identified and quantified in Cripto-/- versus wild type ES cells.

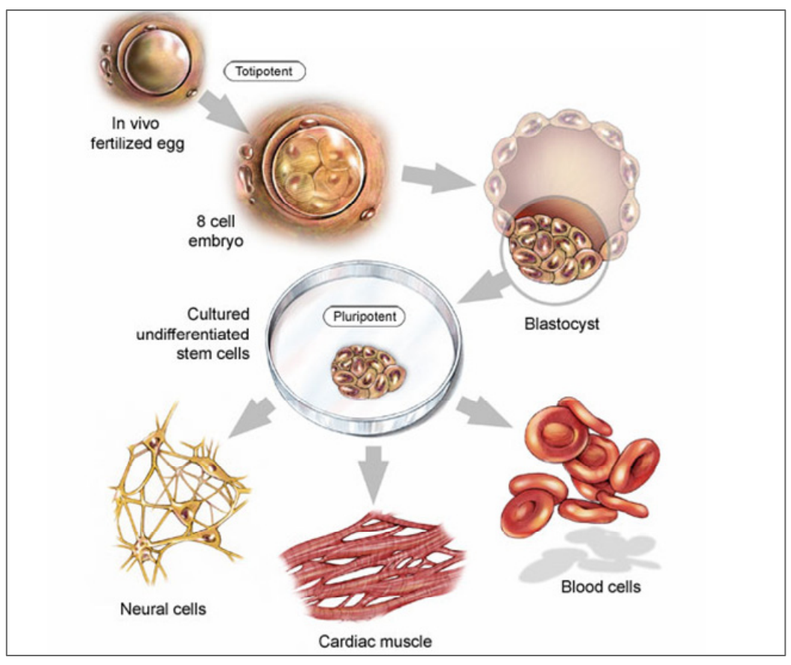

Figure 1. Experimental system.

METHODS

Cell cultures and sample preparation

Wild type (RI) and Cripto-/- ES cells were maintained in the undifferentiated state by culture on mitomycin C-treated mouse embryonic fibroblast feeder layers.

Monolayer cultures of cell lines were harvested, after three washes in ice-cold PBS, by incubating with a solution containing trypsin and EDTA. After centrifugation , the cell pellets were washed three times with PBS and resuspended in 25 mM NH_4HCO_3/0.5% RapiGest for cell lysis and protein extraction. Samples were then sonicated and centrifuged to eliminate cellular debris. The supernatants were collected and protein concentration determined by the Bradford method.

Total protein extracts were subsequently reduced (10 mM DTT), alkylated (10 mM IAA) and enzymatically digested with trypsin, 1:50 (w/w) enzyme:protein ratio.

LC-MS conditions

The qualitative on-line 2D-LC MS/MS set up employed a 180 µm × 23 mm SCX column packed with Polysulfoethyl A 5 µm for the first dimension separation. A combined salt and organic step gradient was applied to the SCX column by sequentially injecting a series of solvent plugs (20 mM to 200 mM ammonium formate pH 3.2, with 5% to 20% CH_3CN) onto the SCX column. A 180 µm × 20 mm Symmetry C18 5 µm reversed phase trap column was used both to collect the peptides that elute from the first dimension SCX column for the qualitative study and for preconcentration and sample desalting for the quantitative profiling study.

The second dimension qualitative separations and single dimension quantitative LC-MS experiments were conducted using a 1.5 reversed phase gradient from 5 to 40% acetonitrile (0.1% formic acid) at 250 nL/min on a nanoACQUITY system. An Atlantis 3 µm C18 75 µm x 15 cm nanoscale LC column was used. Typical on-column sample loads range 2 and 0.5 µg protein digest for the qualitative and quantitative studies, respectively.

Data directed analysis and multiplexed, alternate scanning LC-MSE experiments were performed with a Q-Tof Premier mass spectrometer.

2D gel electrophoresis

Wild type (RI) and $Cr^{-/-}$ ES cells protein extracts were separated in the first dimension on a non linear pH 3-10 gradient and in the second dimension, on homogeneous polyacrylamide gels. The gels were stained with colloidal Coomassie blue stain followed by protein identification using a MALDI L/R mass spectrometer.

RESULTS

Qualitative Profiling

The database search results of the obtained spectra for both cell lines were used to generate qualitative profiles — as shown in Figure 2 — and to conduct Gene Onthology annotation experiments. The results of the latter are shown in Figure 4.

106 and 85 proteins were identified to the IR and $Cr^{-/-}$ cell lines, respectively. 146 proteins were commonly identified.

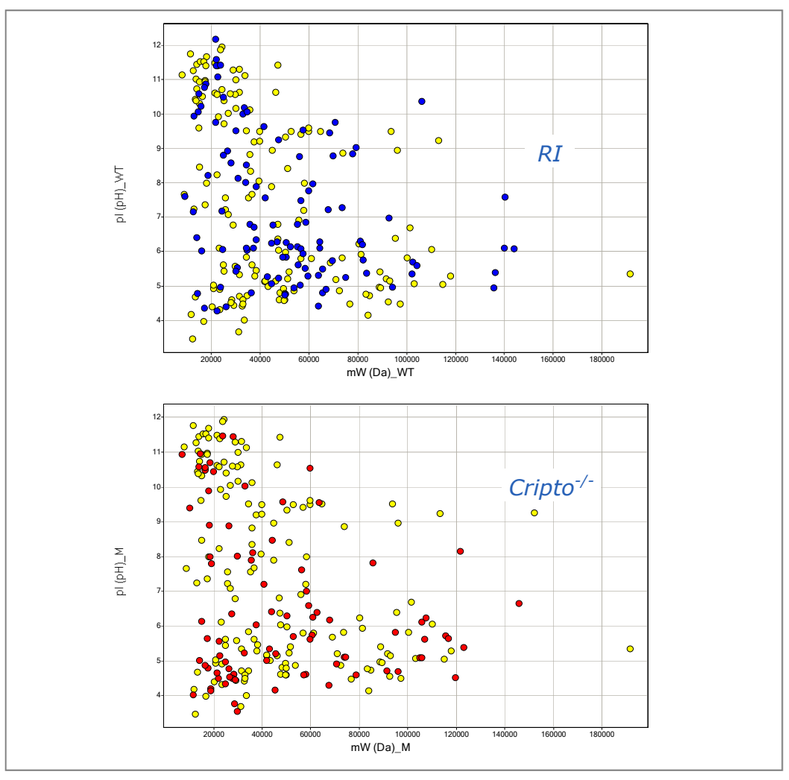

Figure 3. Qualitative proteomic profile of RI (top) and Cripto-/- cells.

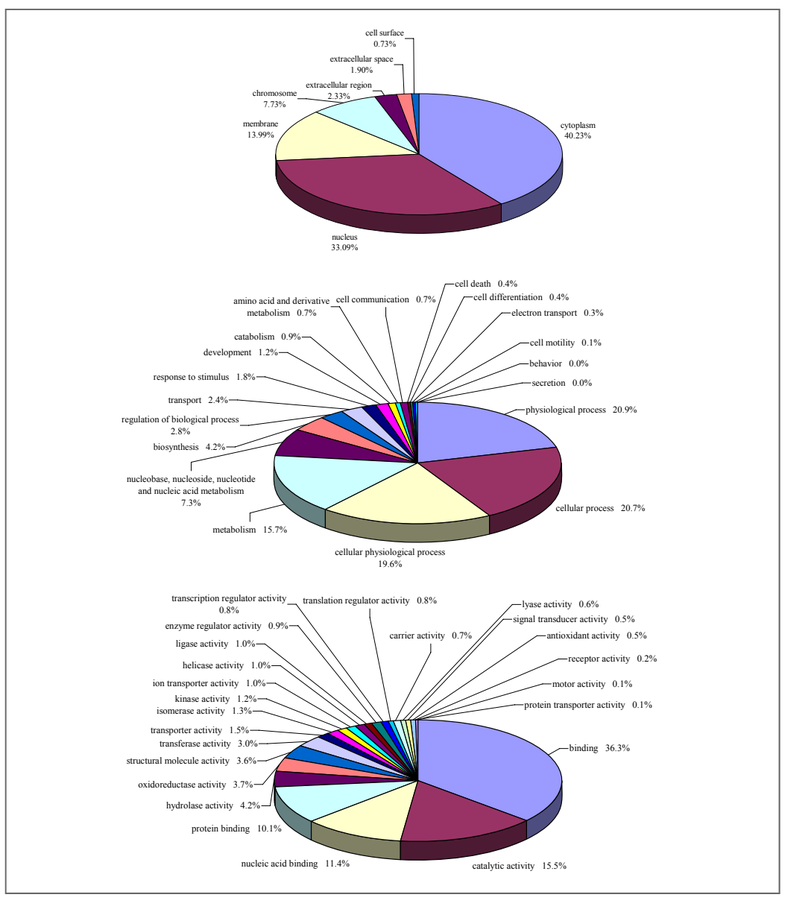

Figure 4. Cripto$^{-/-}$ cell line GO annotation. Cellular component (top), biological processes (middle) and molecular function (bottom).

Quantitative Profiling

The analytical reproducibility was accessed prior to conducting label-free quantitative analysis. The figures of merit for one of the investigated conditions are shown in Figure 5.

Figure 5. Mass precision, %CV retention time and %CV intensity for three replication injections of the Cripto$^{-/-}$ cell line.

Quantitative analysis was conducted at the protein and peptide level. Figure 6 demonstrates an example of filtering on the basis on peptide regulation probability.

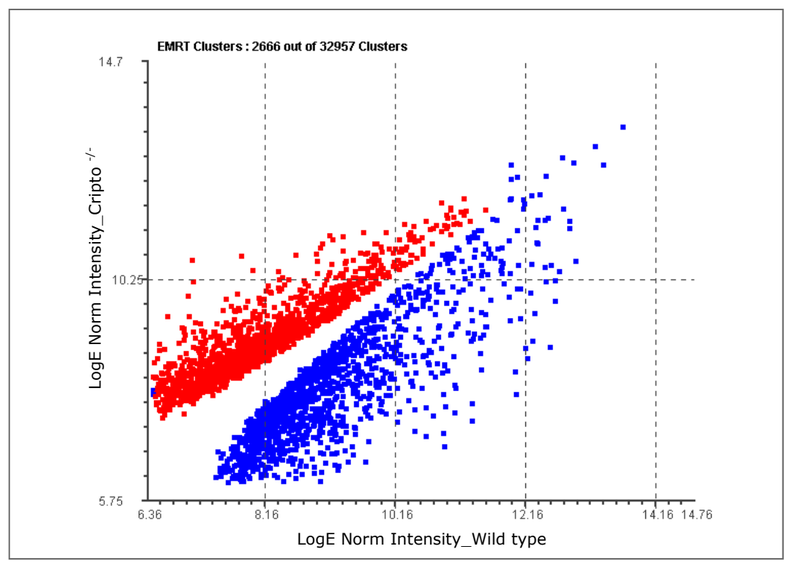

Figure 6. Log-log intensity distribution of significantly regulated peptides (red = up-regulated peptides in the cripto$^{-/-}$ cell line).

Further filtering of the quantified proteins was conducted on the basis of replication (≥ 2 out of 3 replicate injections), significance of regulation (probability of regulation > 95%) and regulation co-efficient of variation (CV < 0.02). An example of exclusively up-regulated protein in the cripto-/- cell line is shown in Table one.

accession	*description*	*ratio**	*CV*
P57780	Alpha-actinin-4	0.44	0.012
P56480	ATP synthase betasubunit	0.38	0.007
Q9D2U9	Histone H2B type 3-A	0.96	0.011
Q8CGP0	Histone H2B type 3-B	1	0.011
P62806	Histone H4	1.39	0.009
Q61696	Heat shock 70 kDa protein 1A	0.49	0.012
P17879	Heat shock 70 kDa protein 1B	0.46	0.011
P16627	Heat shock 70 kDa protein 1L	0.39	0.007
P14602	Heat-shock protein beta-1 (HSP25)	0.56	0.011
P60335	Poly(rC)-binding protein 1	0.68	0.008
P17742	Peptidyl-prolyl cis-trans isomerase A	0.43	0.007

Table 1. Significantly regulated protein in the cripto$^{-/-}$ line.
**RI vs. $Cr^{-/-}$ ES ^{e}log ratio.*

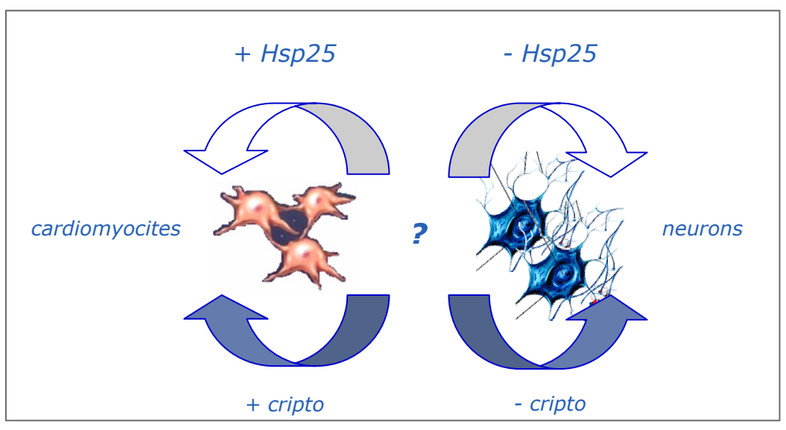

Figure 7. Cripto and Hsp25 as key regulators of embryonic stem cell fate.

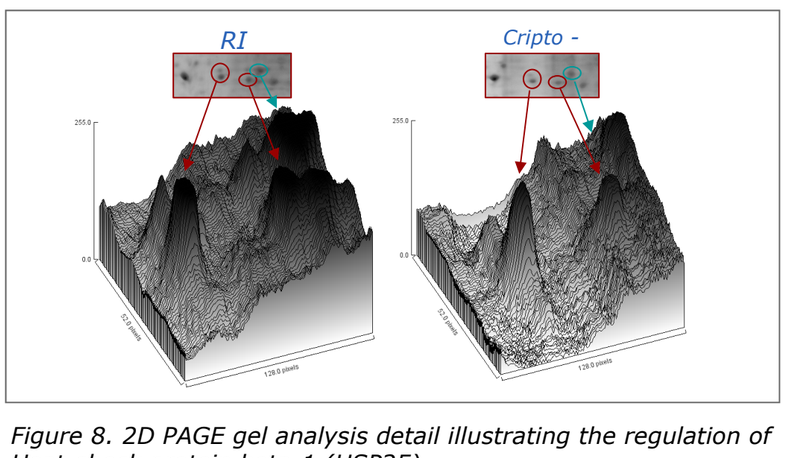

Figure 8. 2D PAGE gel analysis detail illustrating the regulation of Heat-shock protein beta-1 (HSP25).

As an example, HSP25 is essential for functional differentiation into beating cardiomyocytes but is not required for neuronal differentiation — see Figure 7 — and has been positively identified by quantitative label-free LC-MS and 2D gel analysis as shown in Table 1 and Figure 8.

CONCLUSION

- **Proteomic profiling of cripto-/- and wild type embryonic mouse stem cells by 2D LC-MS/MS**
- **Around 300 proteins have been identified in each separate sample**
- **Large fractions appear expressed univocally in the wild type or the cripto-/- cell lines**
- **LC-MSE analysis has been performed to obtain a quantitative comparison of proteins differentially expressed other than a view of the proteomes of the two cell lines**
- **Quantitative results in agreement with protein described in literature that are affected by cripto ablation and 2D gel analysis**
- **the obtained data indicate that a hallmark of neuronal differentiation is already established in undifferentiated ES cells; hence, in the absence of cripto**

References

1. Silva, J.C., Denny R., Dorschel, C.A., Gorenstein, M., Kass, I.J., Li, G.-Z., McKenna, T., Nold, M.J., Richardson, K., Young P., and Geromanos, S. (2005) "Quantitative proteomic analysis by accurate mass retention time pairs" Anal. Chem. 77, 2187-2200
2. Silva, J.C., Gorenstein, M.V., Li, G.-Z., Vissers, J.P.C., Geromanos, S.J. (2006) "Absolute quantification of proteins by LCMSE; a virtue of parallel MS acquisition" Mol. Cell. Proteomics 5, 144-156
3. Battersby A. *et al.,* (2007) "Comparative proteomic analysis reveals differential expression of Hsp25 following the directed differentiation of mouse embryonic stem cells" Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1773
4. Winger Q.A. *et al.,* (2007) "Heat shock protein 1 and the mitogen-activated protein kinase 14 pathway are important for mouse trophoblast stem cell differentiation" Biol. Reprod. 76 (5):884-91.
5. Parisi S., D'Andrea D., Lago C.T., Adamson E.D,. Persico M.G., Minchiotti G. (2003) "Nodal-dependent Cripto signaling promotes cardiomyogenesis and redirects the neural fate of embryonic stem cells" J. Cell Biol. 27, 163(2), 303-314
6. Minchiotti G. (2005) "Nodal-dependant Cripto signaling in ES cells: from stem cells to tumor biology" Oncogene 29, 24(37), 5668-567

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