Comparative proteomic analysis of nuclear and cytoplasmic compartments in human cardiac progenitor cells.

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Expanded METHODS

Cells and culture conditions

Human cardiac biopsies were obtained from patients suffering from an open-chest surgery, usually for valve replacement. Starting material was obtained from the right atria appendage, which is routinely removed in order to place the cannulae for the extracorporeal circulation. Tissue samples were minced into small pieces (<1 mm³) and treated with collagenase type 2 (Worthington Biochemical Corporation, Lakewood, NJ, USA) for 3 cycles of 30 min each to obtain a cellular suspension and processed as previously described [1]. In brief, cardiomyocytes were removed by centrifugation and filtration using 40 μm cell strainers. Human cardiac progenitor cells (CPC) were purified from 3 human myocardial samples after immunodepletion of CD45-positive cells by c-kit immunoselection, following manufacturer recommendations. hCPC were maintained in DMEM/F12 and neurobasal medium (1:1), (both from Invitrogen; Madrid, Spain), supplemented with 10% fetal bovine serum embryonic stem cell-qualified (FBS ESCq, Invitrogen), 2 mM L-glutamine (Lonza; Belgium), penicillin-streptomycin (100 U/mL and 1000 U/mL, both from Lonza), 0.5X B27 supplement, 0.5X N2 supplement, 10 ng/mL bFGF and 0.5X ITS (all from Invitrogen), 30 ng/mL IGF-II and 20 ng/mL EGF (both from Peprotech; Neuilly-sur-Seine, France). Growth medium was exchanged weekly. hCPC were maintained in a 3% O₂/5%CO₂ atmosphere.

Human bone marrow-derived mesenchymal stem cells (MSC) were obtained from cadaveric bone marrow, harvested from brain-dead donors under the supervision of the Spanish National Transplant Organization (in Spanish, Organización Nacional de Trasplantes) under specific regulations (R.D. 1301/2006). Relatives gave written informed consent. Each sample donor was tested and found negative for HIV-1/2, hepatitis B-C, cytomegalovirus and mycoplasma. All cells were obtained from the Inbiobank Stem Cell Bank (www.inbiobank.org) following good manufacturing procedures based on ISO9001:2000.
hMSC and fibroblasts (obtained from Inbiobank) were maintained and expanded under optimal conditions, in low-glucose DMEM supplemented with 10% FBS (both from Sigma-Aldrich, Madrid, Spain), 2 mM L-glutamine (Lonza) and penicillin-streptomycin (100 and 1000 U/mL, respectively, Lonza), also in a 3% O₂/5%CO₂ atmosphere.

Proteomics analyses

Label-free proteomics analysis. hCPC3 and hMSC19 were used for proteomics analysis. Cells were expanded to passage 7–8, recovered (5–8 × 10⁷) and washed several times in PBS. Subcellular cytoplasmic and nuclear protein fractions were obtained (n=3) using the Qproteome Cell Compartment Kit (Qiagen, Barcelona, Spain). When needed equivalent fractions were obtained from human fibroblasts (HDF).

Samples (~500 µg) were digested using an in-gel digestion protocol, as described [2]. Briefly, samples were resolved by conventional SDS-PAGE until the electrophoresis front entered 3 mm into the concentrating gel. The protein band containing the whole proteome was visualized by Coomassie staining, excised, cut into cubes, subjected to reduction conditions using 10 mM dithiothreitol (DTT), alkylated with iodoacetamide (50 mM), and digested (overnight 37°C) with 60 ng/mL modified trypsin (Promega, Madison, WI) at a 12:1 protein:trypsin (w/w) ratio in 50 mM ammonium bicarbonate (pH 8.8) containing 10% acetonitrile. The resulting tryptic peptides were extracted by incubation in 12 mM ammonium bicarbonate pH 8.8 followed by 0.5% trifluoroacetic acid (TFA). TFA was added to a final concentration of 1% and peptides were desalted on C18 Oasis-HLB cartridges and dried. Tryptic peptides were dissolved in 0.1% formic acid (FA) and loaded on a liquid chromatography-mass spectrometry (LC-MS/MS) system for online desalting on C18 cartridges and further analysis by LC-MS/MS, using a reverse-phase nanocolumn (75 µm inner diameter × 50 µm, 3 µm-particle size, Acclaim PepMap 100 C18; Thermo Fisher Scientific, San Jose, CA) in a continuous (0–30%) acetonitrile gradient consisting of B (90% acetonitrile, 0.5% formic acid), in 180 min, 30–43% in 5 min and 43–90% in 2 min. A ~200 nL/min flow rate
was used to elute peptides from the nanocolumn to an emitter nanospray needle for real time ionization and peptide fragmentation onto an ion trap-orbitrap hybrid mass spectrometer (Orbitrap Elite, Thermo-Fisher). To increase proteome coverage, tryptic peptides were fractionated by cation exchange chromatography (Oasis HLB-MCX column; Waters Corp., Milford, MA), desalted and analyzed as above.

**Bioinformatics identification and analyses.**

For peptide identification, MS/MS spectra were searched with the SEQUEST HT algorithm implemented in Proteome Discoverer 1.4.0.29 (Thermo Scientific). For database searching at the Uniprot database containing all sequences from human genome (March 6, 2013; 70024 entries; including common contaminants), search parameters were selected as follows: trypsin digestion with 2 maximum missed cleavage sites, precursor and fragment mass tolerances of 800 ppm and 1.2 Da, respectively, for the orbitrap Elite and 2 Da and 0.02 Da, respectively, for the QExactive, carbamidomethyl cysteine as fixed modification and methionine oxidation as dynamic modification. Peptide identification was validated using the probability ratio method [3] Martinez-Bartolomé et al., 2008) with an additional filtering for a precursor mass tolerance of 15 ppm [4]. False discovery rate (FDR) was calculated using inverted databases and a refined method for peptide identification using decoy databases [5].

**References expanded Methods**
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2. Torán, J.L., et al. Definition of a cell surface signature for human cardiac progenitor cells after comprehensive comparative transcriptomic and proteomic characterization. *Sci Rep.* **9**, 4647-4663 (2019).

3. Martínez-Bartolomé, S., et al. Properties of average score distributions of SEQUEST: the probability ratio method. *Mol. Cell Proteomics* **7**, 1135-1145 (2008).

4. Bonzon-Kulichenko, E., García-Marques, F., Trevisan-Herraz, M., Vázquez, J. Revisiting peptide identification by high-accuracy mass spectrometry: problems associated with the use of narrow mass precursor windows. *J. Proteome Res.* **14**, 700-710 (2015).

5. Navarro, P., et al. General statistical framework for quantitative proteomics by stable isotope labeling. *J. Proteome Res.* **13**, 1234-1247 (2014).
| Gene         | Sequence 5´-3´                     |
|--------------|------------------------------------|
| IGF1         | Fw: AGGAAGTACATTGAGAAGACGCAGT     |
|              | Rw: CTCGCGGTTGGCCGATGCA           |
| IGF1R        | Fw: AAAATGCTGACCTCTGTTAACCCT     |
|              | Rw: GCGTTATCCCCACAAATATGAGT      |
| IGF2         | Fw: CGGAAAGGCTACTCTCCTTCC        |
|              | Rw: AGGGTGTTTAAAGCCAATCGG        |
| IGF2R        | Fw: GAAAGGGGACTGGTGGACTGAGT      |
|              | Rw: GAGAGGTCGATGCGGAAGATTTCC     |
| IGF2BP2 (IMP2) | Fw: GTTCGCGCATCTACTCTTAT          |
|              | Rw: GAATCTCGCCACCTGGTGGTGAOL     |
| IGF2BP3 (IMP3) | Fw: GTCAAGTGCAAGATGTTTGTC       |
|              | Rw: GCAATCTGTTCTTTGTTTGGGCC      |
| IL1A         | Fw: GAATTGACCGCCCTTCAATCAAAGT   |
|              | Rw: TCATCTTGAGGAGTCATACCA        |
| IL1B         | Fw: AAACAGATGAGTGCTACTTCTCCAGG  |
|              | Rw: TGGAGAAACACCTGTTGGCTCCA      |
| IL1RI        | Fw: GTGATTGTGAGGCCGAGTTTCCCA     |
|              | Rw: TGGTTGAGAGTTTCCCCACAGA       |
| IL1RA        | Fw: GGCACCTGGGACTCTGATGAAAGAT   |
|              | Rw: TCTGCTGAGTACCTCGGGAAGAGA    |
| IL6          | Fw: GGCACCTGGCAGAAAACCACCAGAGA |
|              | Rw: GCAAGTCTCCTCTATTGAATACCA     |
| IL8          | Fw: CTGGCGGTGGTCTCTTGAC          |
|              | Rw: CCTTGGGAAAAACCTGACCCCTT     |
| CXCL12       | Fw: GAACGCGCAAGGGTGTGGTGAGT     |
|              | Rw: TCTGAGTCGACGGCTAGGCGG       |
| BNIP3        | Fw: TGAAGCTGGAGGAGTAGCTCAGCAG   |
|              | Rw: CCGCTTGGATATTTGCTGCTGGTT    |
| BNIP3L       | Fw: GTGGAATGACGACACCAACCAACAG  |
|              | Rw: CCTGGGGGATATTTTCCCCACAGA    |
| ICAM3        | Fw: GTAACCTGGGCTCTCTGCTGAGT    |
|              | Rw: ACTTTGCTCCCCCGCTCTTGGGT     |
| NEMO         | Fw: GTGGAGCACTGGAGGAGAT         |
|              | Rw: CAGAGCCTGCGACCTTCTTACAG     |
| CDK6         | Fw: GTGCGGTCTCCTTCACCCCATAC    |
|              | Rw: GACCCATAAGGACCCCAACAGG      |
| CASP8        | Fw: CTGCGGTGGGGGACCCGAGCTGAG   |
|              | Rw: TCACCGTGGGACACCTGCAGACCCTCTC |
| CASP10       | Fw: TGCCAGGTGATGAGGAGATACAGAC |
|              | Rw: CCAAGGGCCAGCCCAAGAAAATACA   |
| WT1          | Fw: GACGCGTCTAAAAAGACACCAAAAGGA|
|              | Rw: ACAGCTGAGGGGTCTTTTCACCTG    |
| GATA4        | Fw: AGAAAAAGGACGCCCAAGACAAGC  |
|              | Rw: TGCTGCTGCCGCTAGTGAGAATGCTG |
| SOX17        | Fw: GCAGACGGATTTTGAAGGATGA     |
|              | Rw: GGATCGAGGACCTGCGACCCACAGC  |
| TBX3         | Fw: TGGGAGCTCTGCTAGTGAGTCTCT   |
|              | Rw: CCAATGTCCTTCCTTCCTTCCGCT    |
| HoxA10       | Fw: GCCGCTTCGAGAGCAAGAACAGAAG |
|              | Rw: AGGTGGAGCCTGGGGGCTATCTCTA  |
| HoxD8        | Fw: GATAAATTCAGAGAGACGCCGTATTTTAC|
|              | Rw: CCAATATTACATGGAGAGAATTTG    |
| P4HA1        | Fw: CCGTGAGACTGGGAAATTTGACCCAGGACG|
|              | Rw: GGGGTTGAATGCTGGTCCAATGCTCCTCA |
| ASPH         | Fw: TTCTCACAATGCGACGCTCCT      |
|              | Rw: TGGGACACTTCCTACAGTCCCG      |
| ASPHD1       | Fw: TCGAAGCTGTATTGAGTGCCCA     |
|              | Rw: CACCCAGAGGGATCTTTTAG        |
| PTRF         | Fw: GACTCAAAGGCCAAACACTGCC     |
|              | Rw: AAAAAGTCTGTTTACAGCTTCC      |
| GUSB         | Fw: CAAAGGCCGCTGCTGCCACACC    |
|              | Rw: AGCGAGGACCGCCTTCTCTCCTGCT  |
| GAPDH        | Fw: AACCTGCTGGCACCCTTGCC      |
|              | Rw: CTGGAGAGCCCTCGGACCAT       |
| cMYC         | Fw: CTGGTACCTGAGAGATCTGAG     |
|              | Rw: GTGGAAGAGAGCAAGAAATAC       |
| CDK6         | Fw: GTGCGGTCTCCTACCCCAACAG     |
|              | Rw: GACCCAAAGGACCCCAAGAGG      |
| Gene  | Fw       | Rev       |
|-------|----------|-----------|
| HMGA2 | AAGTTGTTTCAGAAGAAGCCTGCTCA | TGGAAAGACCATGGCAATACAGAAT |
| PTPRF | ACCATGCTATGTCCGCAGACCATGGCAATACAGAAT | TGGAAAGACCATGGCAATACAGAAT |
| CD9   | GAGGCACCAAGTGCATCAAGCAGGAAGAGAAG | AGCCATAGTCATGAATGGGAAAG |
| NEMO  | GGTCGAGCCACTGGAAGAGAT | CAGAGCCTGGCATTCCTTAG |
| ICAM3 | GTAAGTCGCCCTCCGGTTG | ACTTTGTCCCCGCTTTGGT |
| GATA2 | AGCCGGCACACGTGTGGCAAGCATGACTTCCTGTCCGACT | TGACTTCTCCTGCATGCAGCT |
| OCT4  | CCCAGGGCCCCTTTTGTTACC | GCCACAAAACCTCAGGTTTTC |
| MDB3  | GGTCGGCCAGATCACTGGAAG | GCTCTAGAATGTCGAAGG |
| DIDO3 | ACTCTTGGGCTTTTGGGAGG | TCGGTTCCGTTTTTCTGCGA |
Table 2: List of all primary and secondary antibodies used in the study.

| Primary antibodies          | Reference | Commercial provider                      |
|-----------------------------|-----------|------------------------------------------|
| Anti-human IGF2R            | AF2447    | R&D Systems, Minneapolis, MN             |
| Anti-human ASPHD1           | Ab19731   | Abcam, Cambridge, MA                     |
| Anti-human P4HA1            | HPA026593 | Sigma-Aldrich (St Louis, MO).            |
| Anti-human IL1A/IL1-1F1     | MAB200    | R&D Systems, Minneapolis, MN             |
| Anti-human IL1B              | sc-7884   | Santa Cruz Biotechnology, Inc            |
| Anti-human IL1RI             | AF269     | R&D Systems, Minneapolis, MN             |
| Anti-human IGF2BP2 (IMP2)   | ab128175  | Abcam, Cambridge, MA                     |
| Anti-human IGF2BP3 (IMP3)   | ab177942  | Abcam, Cambridge, MA                     |
| Anti-hnRNP U antibody       | ab10297   | Abcam, Cambridge, MA                     |
| Anti-α-tubulin               | CP06      | Calbiochem, La Jolla, CA, USA            |
| Anti-GAPDH antibody         | Ab9485    | Abcam, Cambridge, MA                     |

| Secondary antibodies        | Reference | Commercial provider                      |
|-----------------------------|-----------|------------------------------------------|
| Goat Anti-rabbit HRP        | P0448     | Dako, Glostrup Denmark                   |
| Rabbit Anti-Mouse-HRP       | P0260     | Dako, Glostrup Denmark                   |
| Rabbit Anti-Goat HRP        | P0449     | Dako, Glostrup Denmark                   |
| Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor® 546 conjugate | A-11035 | Invitrogen, Carlsbad, CA, USA            |
| Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor® 546 conjugate | A-11030 | Invitrogen, Carlsbad, CA, USA            |
| Donkey anti-Goat IgG (H+L) Secondary Antibody, Alexa Fluor® 546 conjugate | A-11056 | Invitrogen, Carlsbad, CA, USA            |
| DAPI                        | 32670     | Sigma-Aldrich (St Louis, MO).            |
Identification (IPA); nuclear and cytosol fractions from hCPC and hMSC

| IPA location          | IPA location | hCPC    | hMSC    | Total |
|-----------------------|--------------|---------|---------|-------|
|                       |              | Cytopl  | Nucl    | SubT  | Cytopl | Nucl    | SubT  |       |
| Cytoplasam            |              | 748     | 523     | 1271  | 707    | 469     | 1176  | 2447  |
| Extracellular Space   |              | 44      | 47      | 91    | 28     | 40      | 68    | 159   |
| Nucleus               |              | 223     | 369     | 592   | 166    | 348     | 514   | 1106  |
| Plasma Membrane       |              | 84      | 103     | 187   | 70     | 109     | 179   | 366   |
| unknown               |              | 91      | 60      | 151   | 98     | 73      | 171   | 322   |
| Total general         |              | 1190    | 1102    | 2292  | 1069   | 1039    | 2108  | 4400  |

Over represented PANTHER Pathways

- Cytoskeletal regulation by Rho GTPase
- Axon guidance mediated by semaphorins
- Pyridoxal phosphate salvage pathway
- Vitamin B6 metabolism
- EGF receptor signaling pathway

Validation of cytoplasmic overexpressed proteins in hCPC. a) Protein identification (IPA) in subcellular fractions prepared from hCPC (hCPC3) and hMSC (hMSC19); b) PANTHER Pathway analysis of cytoplasmic overexpressed proteins in hCPC; c) Comparative RT-qPCR expression analysis of ASPH in the three independent isolates of hCPC (hCPC 1–3), two human fibroblasts (HDF1 and F3) and two hMSC isolates (MSC19 and MSC45). Assays were performed three times and data are expressed as mean ± SD; black lines summarize p-values (**<0.002) for hCPC vs. fibroblasts or hMSC (one-way ANOVA analysis of variance followed by the Bonferroni correction for multiple comparison); d) RT-qPCR comparative analysis of some of the identified cytoplasmic overexpressed proteins in hCPC, with other functions previously described as highly differentially overexpressed by hCPC. Expression in hCPC (hCPC1) was compared with hMSC (hMSC19) and whole human heart (hH) tissue; relative expression values to hCPC.
Validation of nuclear overexpressed proteins in hCPC. a) PANTHER Pathway analysis of nuclear overexpressed proteins in hCPC. b) Comparative RT-qPCR expression analysis of PTRF in the three independent isolates of hCPC (hCPC 1–3), two human fibroblasts (HDF1 and F3) and two hMSC isolates (MSC19 and MSC45). Assays were performed three times, normalized against GAPDH and data expressed as mean ± SD; black lines summarize p-values (one-way ANOVA analysis of variance followed by the Bonferroni correction for multiple comparison; ns, non-significant. c) RT-qPCR confirmation of some transcriptional factors defined by RNAseq (Toran et al., 2019) as significantly overexpressed (GATA4, SOX17, WT1, GATA2, TBX3 and MEF) or downregulated (HOXD8 and HOXA10) in hCPC vs. hMSC. Two hCPC isolates (1 and 2) were compared with two hMSC isolates (MSC19 and MSC45). Assays were performed three times and data are expressed as mean ± SD; black lines summarize p-values (**<0.02; *<0.05; one-way ANOVA analysis of variance followed by the Bonferroni correction for multiple comparison).
IL1A response to apoptosis/necrosis upon oxidative damage and evaluation of a potential role in immunoregulation. a) Comparative evaluation of IL1A, IL1B and IL1R1 expression by RT-qPCR in hCPC1, in homeostasis, and upon induction of apoptosis or necrosis, and relative to GusB. b) Densitometric analysis of the representative western blot shown in Figure 3d; nuclear/cytoplasmic ratio for IL1R, IL1A and IL1B are compared in in hCPC3 in homeostasis of after apoptosis induction. c, d) Evaluation of the potential role of IL1A in hCPC immunoregulation capacity. c) Confirmation of downregulation of IL1A (>70%) in hCPC (1,3) transfected with siIL1A (10 nM) compared with a negative control (siNeg) and untransfected control cells (control), by RT-qPCR relative to the expression of GusB; Assays were performed three times and data expressed as mean ± SD of the results relative to GusB; black lines sumarize p-values (**<0.02; *<0.05; one-way ANOVA analysis of variance followed by the Bonferroni correction for multiple comparison). d) Phytohemagglutinin-stimulated human CD3 T cells, labeled with CFSE, were co-cultivated with native hCPC cells (hCPC3), CPC-IL1A downregulated cells (hCPC siIL1A) or negative control transfected cells (hCPC siNeg). All three samples (hCPC, hCPC siIL1A and hCPC siNeg) demonstrated similar immunoregulatory capacity (% of proliferating CD3+ cells) at the higher cell doses analyzed (1:10–1:20);
**Evaluation of IMP3 and IL1A functional interaction and response to apoptosis/necrosis upon oxidative damage.**

**a)** Analysis of the effects of IMP3 downregulation on hCPC1 or hCPC3 response to oxidative damage induced by H$_2$O$_2$. hCPC control, siIMP3- or siNeg-transfected cells were exposed to H$_2$O$_2$ (500 μM) during 48 h; cultures were stained with the AnnexinV/ Propidium iodide (Anex.V / PI) and homeostatic viable (H3: Anex.V/- / PI-), apoptotic (H4: Anex.V+/ PI-), late apoptotic (H2: Anex.V+/ PI+) or necrotic (H1: Anex.V-/ PI+) cells were quantified by cytometry. Data correspond to a representative experiment; assays were performed three times and data expressed as mean ± SD are included in Figure 4E; **b)** Densitometric analysis of the representative western blot shown in Figure 5b; nuclear/cytoplasmic ratio for IGF2R, IMP2 and IMP3, in hCPC3 are compared in homeostasis of after apoptosis of induction. **C)** Evaluation of potential role of IMP3 in regulation of IL1A expression in hCPC. Using the hCPC3 isolate, hCPC control, siIMP3- or siNeg-transfected cells were evaluated, 48 h after transfection, by RT-qPCR. Assays were performed three times and data expressed as mean ± SD of the results relative to GusB; black lines summarize p-values (**<0.02; *<0.05; one-way ANOVA analysis of variance followed by the Bonferroni correction for multiple comparison).
Supplementary Fig. S5 online

full-length blots/gels
Figure 1f

- **P4HA1**
  - 65 kDa band

- **αTUB**
  - 60 kDa band
Figure 2f

**ASPHD1**

Note: same membrane used in Fig 1f, reused for anti-ASPHD1
Fig. 3e (Homeostasis)

**IL1R1**
- 80 KDa

**IL1A**
- 17 KDa

**IL1B**
- 18 KDa

Homeostasis
homeostasis

hnRNPU

120 KDa

GAPDH

Note: the same membrane used in Fig 5b was reused for these analysis. So the controls for hnRNPU and GAPDH are the same.
Figure 3e (apoptosis)

**IL1R1**
- apoptosis

**IL1A**
- apoptosis

**IL1B**
- apoptosis
Figure 4c

IMP3

69 kDa

αTUB

60 kDa
