

Comparative proteomic analysis of nuclear and cytoplasmic compartments in human cardiac progenitor cells. Functional evaluation of IL1A and IMP3

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Expanded METHODS

Cells and culture conditions

hCPC were grown in a combination of DMEM/F12 and Neurobasal medium (1:1) supplemented with 10% fetal bovine serum (FBS; ESCq, Invitrogen, Madrid, Spain and Saint-Aubin, France), 2 mM L-glutamine (Lonza, Verviers, Belgium), penicillin-streptomycin (100 and 1000 U/mL, both from Lonza), B27 (1×), N2 (1×), β -mercaptoethanol (50 μ M) and ITS (all from Invitrogen, Carlsbad, CA), and with the growth factors bFGF (10 ng/mL; Invitrogen), IGF-II (30 ng/mL) and EGF (20 ng/mL) (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 ($5\text{--}8 \times 10^7$) 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 [1]. 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 [2] (Martínez-Bartolomé et al., 2008) with an additional filtering for a precursor mass tolerance of 15 ppm [3]. False discovery rate (FDR) was calculated using inverted databases and a refined method for peptide identification using decoy databases [4].

References expanded Methods

1. 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).
2. Martínez-Bartolomé, S., et al. Properties of average score distributions of SEQUEST: the probability ratio method. *Mol. Cell Proteomics* **7**, 1135-1145 (2008).
3. 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).
4. 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'
<i>IGF1</i>	Fw: AGGAAGTACATTTGAAGAACGCAAGT Rw: CCTGCCGTGGCATGTCA
<i>IGF1R</i>	Fw: AAAATGCTGACCTCTGTTACCTC Rv: GCTTATTCCCCACAATGTAGTT
<i>IGF2</i>	Fw: CCGAAACAGGCTACTCTCCT Rv: AGGGTGTTTAAAGCCAATCG
<i>IGF2R</i>	Fw: GAAGGTGAAGGTCCGAGT Rv: GAAGATGGTGATGGGATTTT
<i>IMP2 (IGF2BP2)</i>	Fw: GTTCCCGCATCATCACTCTTAT Rv: GAATCTCGCCAGCTGTTTGAO
<i>IMP3(IGF2BP3)</i>	Fw: GTCAAGTGCAGAAAGTTGTGTC Rv: GCAATCTGCTTTGGTTTGGC
<i>IL1A</i>	Fw: GAATGACGCCCTCAATCAAAGT Rv: TCATCTTGGGCAGTCACATACA
<i>IL1B</i>	Fw: AAACAGATGAAGTGCTCCTCCAGG Rv: TGGAGAACACCACTTGTGCTCCA
<i>IL1RI</i>	Fw: GTGATTGTGAGCCAGCTA Rv: TGTTTGCAGGATTTTCCACA
<i>IL1RA</i>	Fw:GGCACTTGGAGACTTGATGAAAGAT Rv: TCGCTGAGTACCTGCCAAGA
<i>WT1</i>	Fw: GACCAGCTCAAAGACACCAAAGGA Rv: ACAGCTGAAGGCTTTTCACTTGT
<i>GATA4</i>	Fw:AGAAAACGGAAGCCCAAGAACC Rv: TGCTGTGCCGTAGTGAGATGA
<i>SOX17</i>	Fw: CGCACGGAATTTGAACAGTA Rv: GGATCAGGGACCTGTCACAC
<i>TBX3</i>	Fw: TGGGGACCTCTGATGAGTCCT Rv: CCATGCTCCTCTTTGCTCTC
<i>HOXA10</i>	Fw: GCCCTTCCGAGAGCAGCAAAG Rv: AGGTGGACGCTGCGGCTAATCTCTA
<i>HOXD8</i>	Fw:GATAACTTACAGAGACAGCCGATTTTAC Rv: CCAATATTACCACTGGACGATTACA
<i>P4HA1</i>	Fw: CCCTGAGACTGGAATTTGACCACAGC Rv: GGGGTTTCACTGTCTCCCACTCCA
<i>ASPH</i>	Fw: TTTCTCAAAGTGCAGCCTCC Rv: TGGGCACTTCTACAGTTCC
<i>ASPHD1</i>	Fw: TCGAAGCTTTATGAGTGCCA Rv: CAGCCAGGAGGATCTTTAG
<i>PTRF</i>	Fw: GACTCAAAGCCAAAGTGCCC Rv: CAAAAGCTGCTTTCACGTCTT
<i>GUSB</i>	Fw: CAACGAGCCTGCGTCCCAACC Rv: ACGGAGCCCCCTTGTCTGCT
<i>GAPDH</i>	Fw: AACTGCTTGGCACCCTGGC Rv: CTGGAGAGCCCTCGGCCAT
<i>cMYC</i>	Fw: CTTGTACCTGCAGGATCTGA Rv: GTCGAGGAGAGCAGAGAATC
<i>CDK6</i>	Fw: GTGCCCTGTCTCACCCTATC Rv: GACCCATAAGCCACCAAGG
<i>HMGA2</i>	Fw: AAGTTGTTTCAAGAAGCCTGCTCA Rv: TGGAAAGACCATGGCAATACAGAAT
<i>PTPRF</i>	Fw: ACCATGCTATGTCCGCAG Rv: CCTTGGTCGGATTCCTCACT
<i>CD9</i>	Fw: GAGGCACCAAGTGCAATCA Rv: AGCCATAGTCCAATGGCAAG
<i>NEMO</i>	Fw: GGTGGAGCACCTGAAGAGAT Rv: CAGAGCCTGGCATTCTTAG
<i>ICAM3</i>	Fw: GTAAGTCCCGTCCGTTG Rv: ACTTTGTCCCGTCTTCGT
<i>GATA2</i>	Fw: AGCCGGCACCTGTTGTGCAA Rv: TGAATTCTCTGATGCACT
<i>OCT4</i>	Fw: CCCCAGGGCCCCATTTTGGTACC Rv: GGCACAACTCCAGTTTTC
<i>MDB3</i>	Fw: GGTGACCAAGATTACCAACCAC Rv: GCTCCTCAGAATGTGGAAGG
<i>DIDO3</i>	Fw: ACTCTTGCCCTTTGAGGGACCA Rv: TCGGGTCCGCTTTTCGTCCA

List of RT-qPCR primers used in the study

<i>Primary antibodies</i>	<i>Reference</i>	<i>Commercial provider</i>
Anti-human IGF2R	AF2447	R&D Systems, Minneapolis, MN
Anti-human ASPHD1	Ab19731	Abcam, Cambridge, MA
Anti-human GPCR GPR9	Ab75850	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 IL1R1	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
<i>Secondary antibodies</i>	<i>Reference</i>	<i>Commercial provider</i>
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).

List of all primary and secondary antibodies used in the study

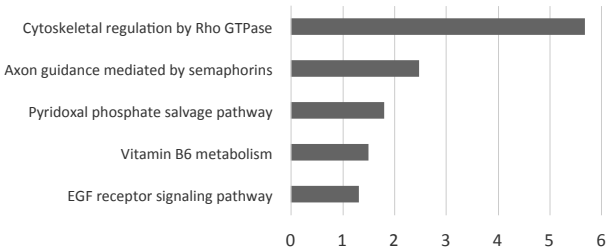
a

Identification (IPA) ; nuclear and cytosol fractions from hCPC and hMSC

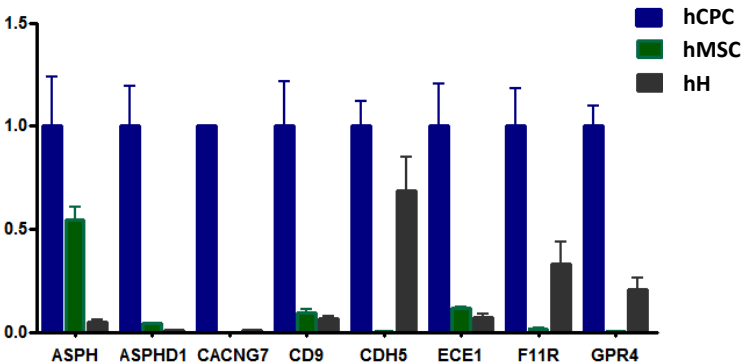
IPA location	hCPC			MSC			Total
	Cytopl	Nucl	SubT	Cytopl	Nucl	SubT	
Cytoplasm	748	523	1271	707	469	1176	2447
Extracellular Space	44	47	91	28	40	68	159
Nucleus	223	369	592	166	348	514	1106
Plasma Membrane	84	103	187	70	109	179	366
unknown	91	60	151	98	73	171	322
Total general	1190	1102	2292	1069	1039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

b

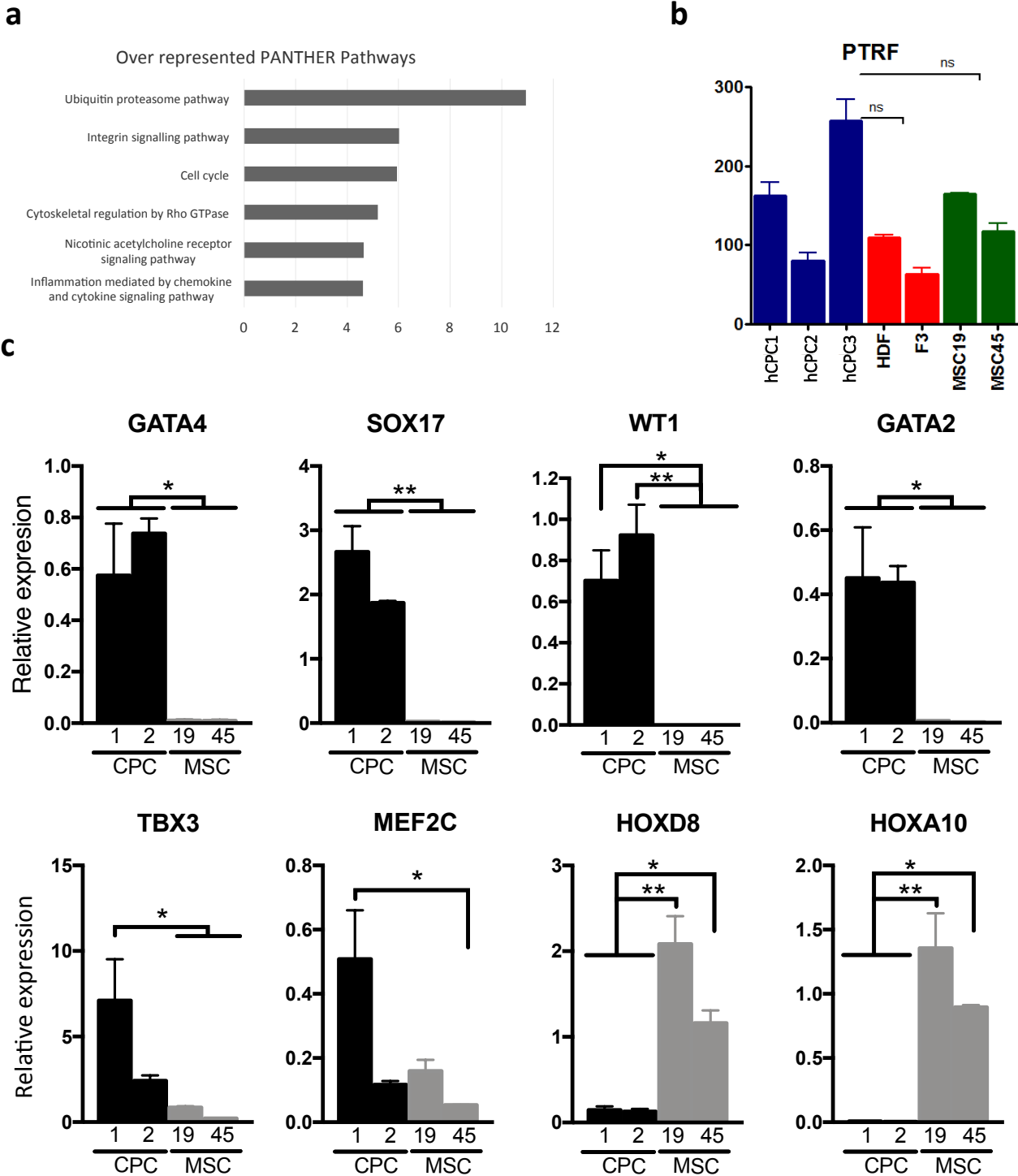
Over represented PANTHER Pathways



c

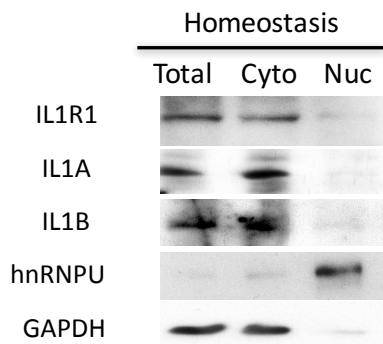


Validation of cytoplasmic overexpressed proteins in hCPC. **a)** Protein identification (IPA) in subcellular fractions prepared from hCPC (hCPC3) and hMSC (hMSC19). **b)** Protein Analysis through Evolutionary Relationships Pathway (PANTHER) analysis of cytoplasmic overexpressed proteins in hCPC. **c)** 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.

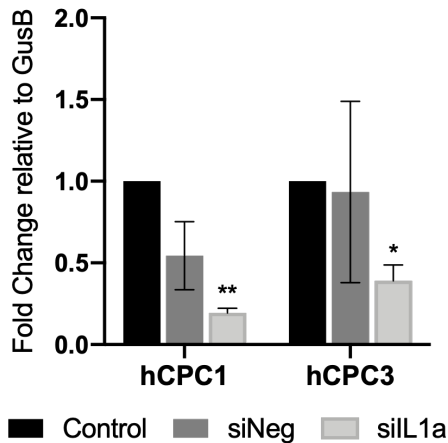


Validation of nuclear overexpressed proteins in hCPC. **a)** Protein Analysis through Evolutionary Relationships Pathway (PANTHER) analysis of nuclear overexpressed proteins in hCPC. **b)** Comparative RT-qPCR expression analysis of *PTRF* in the three independent isolates of hCPC (hCPC 1–3), two human fibroblasts (HDF1 and F3) and two hMSC isolates (MSC19 and MSC45). Assays were performed three times, normalized against *GAPDH* and data expressed as mean \pm 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 \pm SD; black lines summarize p-values (**<0.02; *<0.05; one-way ANOVA analysis of variance followed by the Bonferroni correction for multiple comparison).

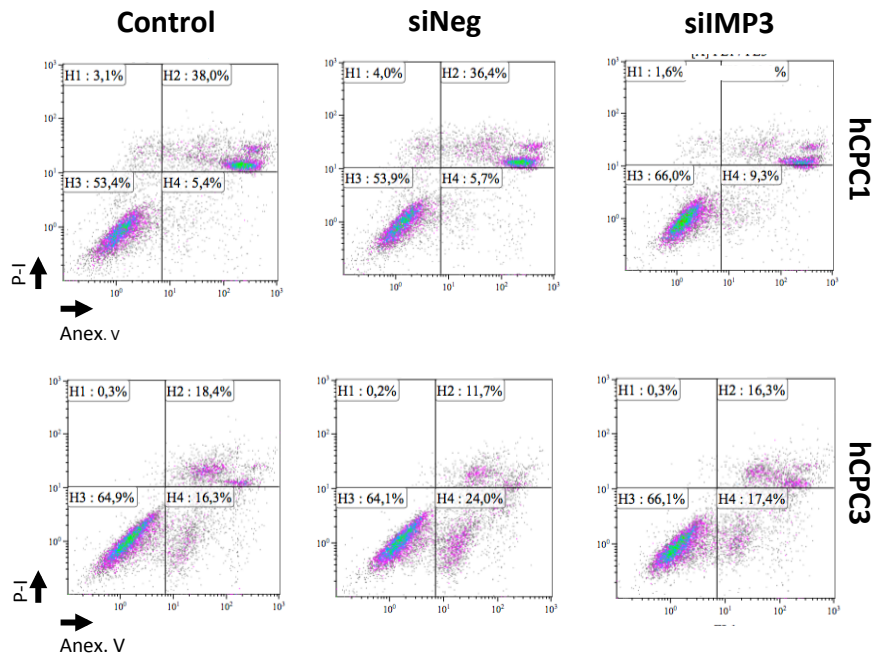
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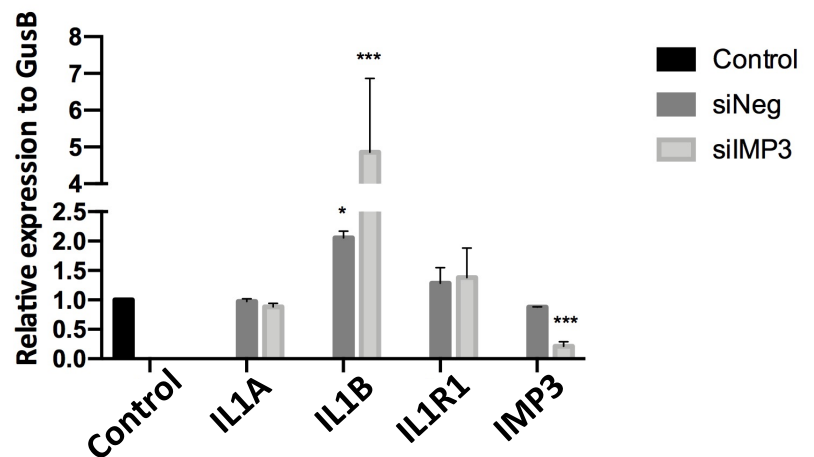
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c



d



Evaluation of IMP3 and IL1A functional interaction and response to apoptosis/necrosis upon oxidative damage. **a)** Western blot analysis of IL1R1, IL1A and IL1B expression in purified cytoplasmic (Cyto) and nuclear (Nuc) fractions of hCPC1 in homeostasis. GAPDH and hnRNPU were used as internal controls of cytoplasmic and nuclear fractions, respectively; **b)** Confirmation of downregulation of IL1A in hCPC (1,3) transfected with siIL1A compared with a negative control (siNeg) and untransfected control cells (control), by RT-qPCR relative to the expression of *GusB*; **c)** Analysis of the effects of IMP3 downregulation on hCPC1 or hCPC3 response to oxidative damage induced by H_2O_2 . hCPC control, siIMP3- or siNeg-transfected cells were exposed to H_2O_2 (500 μ M) 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 \pm SD are included in Figure 4E; **d)** 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 \pm 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).