

Congenital erythrocytosis associated with gain-of-function *HIF2A* gene mutations and erythropoietin levels in the normal range

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SUPPLEMENTARY METHODS

Patients

All patients had an elevated hematocrit (Hct), with normal leukocyte and platelets counts, and serum EPO level normal or inappropriately high for their Hct.

Materials

Human VEGF Quantikine ELISA Kits TFR were obtained by R&D Systems (Minneapolis, MN 55413, USA). sTfR Human ELISA (soluble Transferrin Receptor) was from Biovendor (Brno, The Czech Republic). All the peptides employed were synthesized by GenScript Corporation (Scotch Plain, NJ 07076.0855, USA).

Mutation screening

All patients were screened for *VHL*, *EPOR*, *PHD1*, *PHD2*, *PHD3*, *HIF1A*, *HIF2A* gene mutations.¹

Erythroid precursor cultures

The details of preparation procedure, culture conditions and cell characterization are reported in ref 1.

Plasmids

pGRE5xE1 plasmid is a GAL4-responsive reporter plasmid containing the firefly *luciferase* gene under control of E1b promoter and five GAL4 response elements (kind gift of D. Peet, Adelaide, Australia).

In vitro prolyl 4-hydroxylation assays

Briefly, biotinylated human wild-type or mutant HIF-1 α - or HIF-2 α -derived peptides (HIF-1 α , amino acids 555–573; HIF-2 α , amino acids 523–542) were bound to NeutrAvidin-coated 96-well plates (Thermo Scientific, Rockford, IL USA 61101).

Recombinant GST-PDH1, GST-PHD2 or GST-PHD3 enzymatic assays were carried out for 1 h at room temperature. Bound VBC complex was detected by rabbit anti-thioredoxin antibodies and secondary horseradish peroxidase-coupled anti-rabbit antibodies (Sigma) using the 3,3',5,5'-tetramethylbenzidine substrate kit (Thermo Scientific). The peroxidase reaction was stopped by adding H₂SO₄, and absorbance was determined at 450 nm in a microplate reader. Inter-assay comparability was guaranteed by calibration of each experiment to an internal standard curve using hydroxyproline-containing peptides.^{2, 3}

Luciferase assays

Subconfluent 6-wells of HEK293 cell cultures were co-transfected with 125 ng of pGRE5xEluc, 250 ng of wild-type or mutated pM3-HIF2 α (404-569, wild-type or mutated sequence) and 200 ng of the respective PHD expression construct or empty expression vector. Mastermixes contained 3.5 ng pRL-SV40 to normalize for transfection efficiency. 24 hours post transfection, cultures were equally distributed onto 12-well plates and grown for additional 24 hours at 20% oxygen. Cells were subjected to dual luciferase assay as recommended by the manufacturer.^{2, 3}

References

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