SUPPLEMENTARY APPENDIX

Erythropoietin inhibits osteoblast function in myelodysplastic syndromes via the canonical Wnt pathway

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Materials and methods

Culture of human bone marrow stromal cells

Cells were cultured in DMEM (Invitrogen) with 10% FCS (Invitrogen) and 1% penicillin/streptomycin (PAA) and were used in passages 1-5. To generate osteogenic cells, 70% confluent cells were switched to the medium supplemented with 100 µM ascorbate phosphate, 5 mM β-glycerol phosphate, and 10 nM dexamethasone (all from Sigma-Aldrich). In most experiments, differentiation medium has been applied for 10 days in order to determine the anabolic effect of Epo.

Alizarin red staining

Osteoblast cultures were fixed in 10% paraformaldehyde for 30 min and stained with 1% alizarin red S (pH 5.5, Sigma-Aldrich) for 20 min at room temperature. Excess dye was removed by repeatedly washing the plates with distilled water. The amount of incorporated calcium was eluted with 100 mM cetylpyridinium chloride (Sigma-Aldrich) for 10 min at room temperature. Aliquots were taken and measured photometrically at 540 nm in duplicates.

Primer sequences for RT-PCR

β-actin s: ccaaccgcgagaagatga, *β-actin* as: ccagaggcgtacagggatag;

ALP s: caaccctggggaggagac, *ALP* as: gcattggtgttgtacgtcttg;

OPG s: gaagggcgctaccttgagat, *OPG* as: gcaaactgtatttcgctctgg;

col1a1 s: aaggtattgctggacagcgt, *col1a1* as: tgtttgccaggttcaccaga;

CCND1 s: agaggcggaggagaacaaac, *CCND1* as: agcgtgtgaggcggtagtag;

FOSL1 s: accacaccctccctaactcc, *FOSL1* as: ctgctgctactcttgcgatg;

JUN s: agagcatgaccctgaacctg, *JUN* as: ccgttgctggactggattat;

FZD4 s: ccaggattccttccaagtca, *FZD4* as: ccatgtccttgtggcctact;

EPOR s: tcctgacgctctccctcatc, *EPOR* as: gctggaagttacccttgtgg.

PCR conditions

PCR conditions were 95 °C for 10 min followed by 40 cycles with 95 °C for 10 s, 56 °C for 10 sec and 72 °C for 30 s. The melting curve as assessed in the following program: 60 °C for 1 min and 95 °C continuous. The results were calculated using the ΔΔCT method and are presented in x-fold increase relative to *β-actin* mRNA levels.

TCF/LEF-reporter assay

A TCF/LEF-reporter assay (Qiagen) was done using the murine myoblast C2C12 cell line, which is commonly used to study BMP and Wnt signaling. These cells were seeded at a concentration of 1.5 × 10^4 cells per well in 48-well plates and were cultured in DMEM-F12 medium with 10% fetal bovine serum, and transfected with the Signal TCF/LEF Reporter (CCS-018L) (Qiagen, Hilden, Germany) to assess the activation of the TCF/LCF Wnt promotor. Briefly, 123 ng/cm^2 of the promotor construct was transfected using the FuGENE HD Transfection Reagent (Promega, Madison, WI, USA) according to the manufacturer's protocols. After 24 h, C2C12 cells were treated with Wnt3a-containing L-cell medium and Epo (10-500 IU/ml). Luciferase activity was assayed 24 h post treatment using the Dual Luciferase Reporter Assay kit (Promega) as instructed by the manufacturer.

Co-culture of CD34+ cells with primary human MSC

Primary MSC were plated at a density of 1–2 x 10^4/cm^2 in DMEM with 10% FCS and pre-treated with Epo 50 IU/ml and/or LiCl 25 mM for seven days. Freshly isolated CD34+ cells were seeded on the confluent layer of primary MSC in CellGro SCGM medium (CellGenix, Freiberg, Germany) supplemented with 10% FCS, 10 ng/ml SCF (Biosource, Camarillo, CA, USA), 10 ng/ml FLT3-L (Miltenyi Biotec), and 10 ng/ml IL-3 (Biosource) at a density 1 x 10^4/cm^2. CD34+ cells under stroma-free conditions (plasma treated polystyrene tissue plastic, PTP) were plated at the same density in the same medium. After seven days, the co-culture supernatant was gently mixed, and the non-adherent HSPC fraction was collected. The MSC layers were washed twice with phosphate-buffered saline to collect the remaining non-adherent cells. Cells were counted using a hemocytometer. The HSPC phenotype was analyzed using CD34-FITC (Miltenyi Biotec), CD38-PE (R&D Systems) as well as CD90-APC (eBioscience) antibodies at a FACS Calibur (BD).

Moreover, a 4 weeks cobblestone area forming-cell (CAF-C) assay was performed with or without pre-treatment of the MSC layer. Therefore, 1x10^3 magnetically isolated CD34+ cells were co-cultured using LTC-IC media (Stem Cell Technologies, Cologne, Germany) supplemented with 1x10^{-6} M Hydrocortisone (Sigma Aldrich, Munich, Germany).
After 4 weeks cells were harvested and 1x10^4 cells were plated in enriched methylcellulose medium with recombinant cytokines (MethoCult H4435, Stem Cell Technology) to perform a colony forming unit (CFU) assay. After 2 weeks, colonies were counted and classified under a microscope.

Table S1

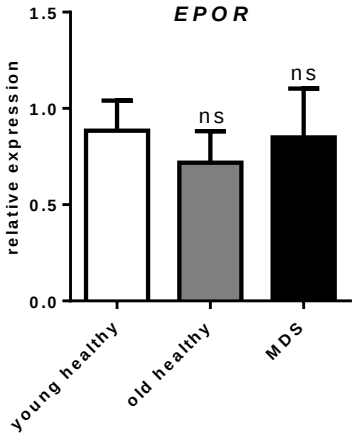
Patients' characteristics

Mean age [years], (range)	69 (51-90)
Gender: Male / Female	19 / 11
Diagnosis (WHO 2008)	
RCUD	1
RARS	2
RCMD	10
MDS RAEB-1	4
MDS RAEB-2	6
MDS RAEBt	1
CMML-1	3
CMML-2	2
AML	1
Cytogenetics	
Normal caryotype	16
Isolated del(5q)	5
Other*	7
No data	2
Therapy at bone marrow sampling	
No therapy	25
Azacythidine	2
Lenalidomide	1
Azacythidine plus Lenalidomide	1
Erythropoietin	1

* del(20q), -Y, trisomy 8, monosomy 7, trisomy 21, del(11q)

Figure S1

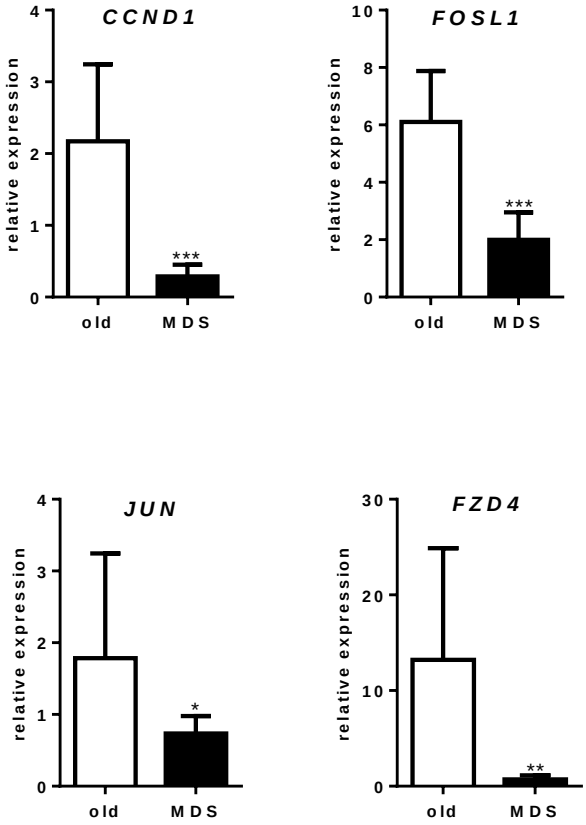
Expression of *EPOR* in young healthy, old healthy and MDS MSC



Gene expression analysis of Epo receptor *(EPOR)* using real-time polymerase chain reaction (PCR) in MSC from young and old healthy donors and MDS patients. *ns* – not significant *vs.* young healthy donors.

Figure S2

Validation of Wnt pathway array



Gene expression analysis of target genes of canonical Wnt pathway *JUN, FOSL1* and *CCND1* and a receptor of canonical Wnt pathway *FZD4* using real-time polymerase chain reaction (PCR) in MSC from old healthy donors compared to MSC from MDS patients. N≥3. *P<0.05, **P<0.01, ***P<0.001.

Table S2

Wnt RT-PCR array comparing old healthy MSC versus MDS MSC

Gene Symbol	Fold Change	p value	Gene Symbol	Fold Change	p value
AES	1,45	0,939489	*LEF1*	1,83	0,481211
APC	-1,74	0,507632	*LRP5*	1,48	0,42143
AXIN1	-1,1	0,616254	*LRP6*	1,44	0,364346
AXIN2	-1,08	0,985166	*MAPK8*	-1,75	0,695049
BCL9	1,2	0,88372	*MMP7*	-2,83	0,286548
BTRC	1,16	0,980036	*MYC*	-1,39	0,517012
CCND1	-4,39	0,331062	*NFATC1*	1,83	0,437689
CCND2	2,24	0,434063	*NKD1*	-1,1	0,979433
CSNK1A1	-1,38	0,503379	*NLK*	-2,77	0,058
CSNK2A1	-1,09	0,6178	*PITX2*	3,04	0,160823
CTBP1	1,15	0,815141	*PORCN*	1,45	0,658716
CTNNB1	2,88	0,026574	*PPARD*	-1,38	0,210573
CTNNBIP1	-1,1	0,979433	*PRICKLE1*	1,12	0,491051
CXXC4	-1,1	0,979433	*PYGO1*	-1,1	0,767336
DAAM1	1,44	0,521415	*RHOA*	-1,38	0,566291
DAB2	1,15	0,825009	*RHOU*	1,15	0,965801
DIXDC1	-1,08	0,827649	*RUVBL1*	1,15	0,720457
DKK1	-4,32	0,298738	*SFRP1*	1,45	0,442
DKK3	1,49	0,38337	*SFRP4*	4,79	0,28414
DVL1	1,14	0,98187	*SOX17*	-1,1	0,979433
DVL2	1,15	0,617804	*TCF7*	-2,82	0,134905
EP300	-1,1	0,837535	*TCF7L1*	-2,14	0,543132
FBXW11	1,45	0,92175	*TLE1*	-2,77	0,59791
FBXW4	1,85	0,560941	*VANGL2*	-1,07	0,994388
FGF4	-1,1	0,979433	*WIF1*	-1,1	0,979433
FOSL1	-5,55	0,142565	*WISP1*	3,71	0,095112
FOXN1	-1,1	0,979433	*WNT1*	-1,1	0,979433
FRAT1	-1,1	0,979433	*WNT10A*	-1,1	0,979433
FRZB	-1,1	0,950729	*WNT11*	-1,1	0,979433
FZD1	2,97	0,309061	*WNT16*	-1,1	0,979433
FZD2	-1,75	0,570363	*WNT2*	-1,1	0,979433
FZD3	1,45	0,580445	*WNT2B*	-1,1	0,979433
FZD4	-4,49	0,199761	*WNT3*	-1,1	0,979433
FZD5	1,87	0,377975	*WNT3A*	-1,1	0,979433
FZD6	-1,7	0,521139	*WNT4*	-1,04	0,988171
FZD7	-1,7	0,424526	*WNT5A*	-2,13	0,259013
FZD8	1,15	0,904233	*WNT5B*	-2,68	0,492557
FZD9	-1,1	0,979433	*WNT6*	-1,1	0,979433
GSK3A	-1,39	0,726464	*WNT7A*	-1,1	0,979433
GSK3B	-1,1	0,963367	*WNT7B*	-1,1	0,979433
JUN	-7,08	0,046897	*WNT8A*	-1,1	0,979433
KREMEN1	1,15	0,542232	*WNT9A*	-1,08	0,984477

Table S3

Wnt RT-PCR array comparing control MDS-OB versus MDS-OB treated with 50IU/ml Epo

Gene Symbol	Fold Change	p value	Gene Symbol	Fold Change	p value
AES	2,43	0,304949	*LEF1*	1,2	0,888323
APC	3,06	0,621037	*LRP5*	-1,05	0,838012
AXIN1	-1,05	0,516011	*LRP6*	2,43	0,10382
AXIN2	-1,02	0,589588	*MAPK8*	1,21	0,797935
BCL9	-1,7	0,583195	*MMP7*	-3,34	0,190109
BTRC	-1,66	0,529272	*MYC*	-2,09	0,338621
CCND1	-6,64	0,227573	*NFATC1*	-1,32	0,495706
CCND2	-1,32	0,619024	*NKD1*	-1,64	0,663126
CSNK1A1	-2,65	0,232593	*NLK*	-1,05	0,915245
CSNK2A1	-1,03	0,566058	*PITX2*	-1,65	0,261395
CTBP1	-1,05	0,656213	*PORCN*	1,52	0,46961
CTNNB1	-1,33	0,647739	*PPARD*	1,21	0,725888
CTNNBIP1	-1,66	0,751085	*PRICKLE1*	1,19	0,826624
CXXC4	-1,66	0,393192	*PYGO1*	2,39	0,006422
DAAM1	-1,32	0,494376	*RHOA*	9,85	0,480058
DAB2	-1,65	0,430239	*RHOU*	1,61	0,41697
DIXDC1	-1,33	0,556827	*RUVBL1*	2,4	0,133194
DKK1	1,21	0,68962	*SFRP1*	1,19	0,623284
DKK3	1,52	0,331566	*SFRP4*	1,52	0,331556
DVL1	1,21	0,513332	*SOX17*	-1,66	0,393192
DVL2	1,2	0,738157	*TCF7*	1,2	0,899603
EP300	1,51	0,429128	*TCF7L1*	1,52	0,415797
FBXW11	-1,05	0,65117	*TLE1*	1,9	0,294725
FBXW4	-2,1	0,04423	*VANGL2*	-1,29	0,464344
FGF4	-1,66	0,393192	*WIF1*	-1,66	0,393192
FOSL1	1,91	0,188223	*WISP1*	1,92	0,596867
FOXN1	-1,66	0,393192	*WNT1*	-1,66	0,393192
FRAT1	1,23	0,534275	*WNT10A*	-1,66	0,393192
FRZB	-2,09	0,138903	*WNT11*	-1,66	0,393192
FZD1	-1,04	0,93904	*WNT16*	-1,66	0,393192
FZD2	2,39	0,113645	*WNT2*	-1,66	0,393192
FZD3	-1,05	0,89145	*WNT2B*	2,41	0,260037
FZD4	1,2	0,419314	*WNT3*	-1,33	0,434398
FZD5	1,2	0,996399	*WNT3A*	-1,66	0,393192
FZD6	-2,65	0,226772	*WNT4*	-1,3	0,485021
FZD7	-1,66	0,408788	*WNT5A*	1,92	0,10214
FZD8	-2,09	0,045485	*WNT5B*	-1,05	0,983899
FZD9	-1,66	0,393192	*WNT6*	-1,66	0,393192
GSK3A	-1,04	0,706973	*WNT7A*	-1,66	0,393192
GSK3B	12,45	0,031833	*WNT7B*	-1,66	0,393192
JUN	-1,32	0,48817	*WNT8A*	-1,66	0,393192
KREMEN1	-1,32	0,480324	*WNT9A*	-1,7	0,345276