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

Ekaterina Balaian,1,* Manja Wobus,1,* Heike Weidner,1 Ulrike Baschant,2,3 Maik Stiehler,4 Gerhard Ehninger,4 Martin Bornhäuser,1,5 Lorenz C Hofbauer,2,3,5 Martina Rauner2,3,** and Uwe Platzbecker1,3,5***

1Medical Clinic I, University Hospital Carl Gustav Carus Dresden; 2Medical Clinic III, University Hospital Carl Gustav Carus Dresden; 3Center for Healthy Aging, University Hospital Carl Gustav Carus Dresden; 4University Centre for Orthopaedics & Trauma Surgery and Centre for Translational Bone, Joint and Soft Tissue Research, University Hospital Carl Gustav Carus Dresden and 5German Cancer Consortium (DKTK), partner site Dresden and German Cancer Research Center (DKFZ), Heidelberg, Germany

*EB and MW contributed equally to this work.
**MR and UP contributed equally to this work.

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Correspondence: uwe.platzbecker@uniklinikum-dresden.de
**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** forward: ccaaccgcgagaagatga, reverse: ccagaggcgtacagggtag;
- **ALP** forward: caaccctggggaggagac, reverse: gcattggttgtacgtcttg;
- **OPG** forward: gaagggcgctaccttgagat, reverse: gcaaactgtatttcgctctgg;
- **col1a1** forward: aaggtattgctggacagcgt, reverse: tgtttgccaggttcaccaga;
- **CCND1** forward: agagggcggagagaacaaac, reverse: agcgtgtgaggcggtagtag;
- **FOSL1** forward: accacaccctcccttaacctcc, reverse: cttgtgtgactggattat;
- **JUN** forward: agagcatgacccctgaacctg, reverse: cctgtgtggtggccctact;
- **FZD4** forward: ccaaggattccttcagctacag, reverse: ccaggattccttcagctacag;
- **EPOR** forward: tcctgacgctctcctcctcatc, reverse: gcgggaagttcccttgcggtagtag;

**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 $1.5 \times 10^4$ 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 $1 \times 10^4$/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, $1 \times 10^3$ magnetically isolated CD34+ cells were co-cultured using LTC-IC media (Stem Cell Technologies, Cologne, Germany) supplemented with $1 \times 10^{-8}$ M Hydrocortisone (Sigma Aldrich, Munich, Germany). After 4 weeks cells were harvested and $1 \times 10^5$ 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)
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.
Gene expression analysis of target genes of canonical Wnt pathway \textit{JUN}, \textit{FOSL1} and \textit{CCND1} and a receptor of canonical Wnt pathway \textit{FZD4} using real-time polymerase chain reaction (PCR) in MSC from old healthy donors compared to MSC from MDS patients. N$\geq$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        | 0.939499| LRF1        | 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         | 0.883722| 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| NFKB1       | -1.1        | 0.979433|
| CSNK1A1     | -3.28       | 0.160823| 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        | 0.225734| PPARD       | -1.38       | 0.210573|
| CTNNB1P1    | -1.1        | 0.979433| PRICKLE1    | 1.12        | 0.491051|
| CXXC4       | -1.1        | 0.979433| PYGO1       | -1.1        | 0.76336 |
| DAAM1       | 1.44        | 0.521413| RHOA        | -1.38       | 0.566291|
| DAB2        | 1.15        | 0.825009| ROU         | 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        | 0.96187 | 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       | 0.99438 |
| FG4         | -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       | 0.425903|
| FZD8        | 1.15        | 0.904233| WNT5B       | -2.68       | 0.42557 |
| 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         | -0.08       | 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| NDK1        | -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|
| CTNNB1P1    | -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        | 0.315566|
| 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|