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Dear Editor,

Myelodysplastic syndromes (MDS) are malignant disorders of myeloid progenitors, characterised by bone marrow failure, peripheral cytopenias and progression to acute myeloid leukaemia (AML) [1]. Currently, the DNA methyltransferase 1 (DNMT1) depleting drugs 5-azacytidine and decitabine are the only drugs approved in the USA to treat all subtypes of MDS. Unfortunately, only 40–50% of patients achieve some response with these drugs, and these are not typically durable beyond some months or years. Whilst it is known, these drugs repressive epigenetic modifications of chromatin caused by DNA methylation, to presumably reactivate tumour suppressor genes [1], the specific gene/s targeted are unknown. PU.1 is a master transcription factor driving granulocyte and monocyte lineage fates, and partial loss-of-function of PU.1 has been shown to induce AML onset in mice and humans [2, 3]. The most frequent acquired mutation in de novo AMLs, is in nucleophosmin (NPM1). The NPM1 protein is a cofactor for PU.1 and mutated NPM1 has recently been shown to cause partial loss-of-function of PU.1 by relocating NPM1/PU.1 from the nucleus into the cytoplasm resulting in suppression of monocytic/granulocytic terminal differentiation [4]. In addition, within a PU.1 overexpressing cell line model, microarray analysis revealed that Jun Dimerization Protein 2 (JDP2), a downstream target of PU.1 which represses acetylation of core histones in vitro and in vivo, was significantly suppressed [5]. JDP2 mediates broader effects on regulation of lineage-differentiation programs [6, 7] and has also been found downregulated in AML patients [6] but its role in MDS has not been explored. In this study, we measured the gene expression of PU.1 and JDP2 in total bone marrow and selected CD34+ cells from 12 newly diagnosed MDS patients stratified according to IPSS-R score (6-low, 3-intermediate, 3-high risk), 2 AML patient and 10 normal controls. Results obtained were also compared with a larger cohort of patients from Bloodspot data [8].

Both PU.1 and JDP2 were down regulated in our MDS patients compared to normal controls. In addition, we found an inverse correlation between PU.1/JDP2 expression and disease status, with expression of these genes declining with more aggressive disease per IPSS-R classification ($F = 2.95$, $p < .04$ and $F = 3.5$, $p < .03$ respectively), and with lowest levels in AML (Fig. 1a). To extend the results, we examined PU.1 and JDP2 expression data in MDS vs normal samples in the Bloodspot expression database, and again, PU.1/JDP2 were significantly downregulated in MDS vs normal controls ($p < 0.01$; $p < 0.05$, respectively) (Fig. 1b). A positive correlation of PU.1 and JDP2 expression

(R = 0.9333, s = 0.0004) was also consistent with a regulatory link between these two genes. To confirm that JDP2 suppression is a direct result of reduced PU.1, we initially performed PU.1-knockdown in K562 cells stably expressing PU.1 short interfering RNAs versus control cells (Fig. 1c) and successively re-expressed PU.1 by transfection. Interestingly, these analyses reveal only a partial reduction in JDP2 expression when analysed by RT-PCR and Western blot (Fig. 1d) and when PU.1 is fully re-expressed, this does not coincide with JDP2 re-gain (Fig. 1e), suggesting a more complex regulatory mechanism. PU.1 and JDP2 expression correlate and are concurrently reduced with the extent of differentiation arrest and aggression/prognosis in MDS/AML. Furthermore, in patients achieving a clinically significant response to Azacitidine, we demonstrated a significant upregulation in PU.1 and JDP2 expression compared with non-responders (Fig. 1f). This suggests that PU.1/JDP2 could be prognostic and potentially a prediction biomarkers for 5-azacytidine and/or decitabine therapy in MDS. Further studies on a larger cohort of patient are undergoing to establish the impact of PU.1/JDP2 expression in MDS evolution.

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Figure Legends

Fig. 1

Samples were enriched for the mononuclear fraction by Ficoll separation and CD34+ cells collected by microbead kit (miltenyi). RNA extraction was performed (RNAeasy Mini Kit (Qiagen, UK)) and cDNA was then synthesised and quantified using iScript cDNA Synthesis Kit (Bio-Rad, UK). PU.1 and JDP2 RT-qPCR was performed on a Fast Real-Time PCR System using StepOne Plus software (Applied Biosystems, UK). RT-qPCR data was analysed using the 2 ^–ΔΔCT method and results expressed (n = 3) as fold change in target gene expression ± standard error of the mean relative to housekeeping gene GAPDH. Statistical analysis was performed using unpaired t tests and ANOVA, with $p < 0.05$ considered significant. Total protein was obtained via lysis with mRIPA buffer + protease inhibitors and run on a SDS-PAGE gel. Proteins were then transferred to nitrocellulose membrane and probed with primary antibodies for PU.1, JDP2 and GAPDH (control) before visualisation with an HRP-linked anti-rabbit IgG secondary antibody and detection using chemiluminescent reagent (Thermo ECL, UK). PU.1-knockdown was performed in K562 cells using PU.1 short interfering RNAs (Thermo FisherR). PU.1 re-expression was performed by transient transfection using pCMV6-XL4-PU.1 (OriGene). a PU.1 and JDP2 expression in MDS graded according to IPSS-R low, intermediate, high risk and AML. b BloodPool database (http://servers.binf.ku.dk/bloodspot/) analysis reveals downregulation of PU.1 (SPI1) and JDP2 in bone marrow samples from patients with MDS comparing with normal controls ($p < 0.01$; $p < 0.05$ respectively). c Optimising PU.1 knockout with different si-RNA concentrations in K562. d PU.1 knockout (left panel: 2, 3, 4) results in only partial reduction of JDP2 expression (right panel). e PU.1 re-expression: optimising PU.1 plasmid concentration (left panel) results in different levels of JDP2 re-expression. When PU.1 is fully re-expressed (left panel *), there is no concomitant JDP2 re-expression (right panel *). f Low PU.1 and JDP2 expression in untreated patients. PU.1 and JDP2 upregulate only in patients responding to AZA comparing with no responders

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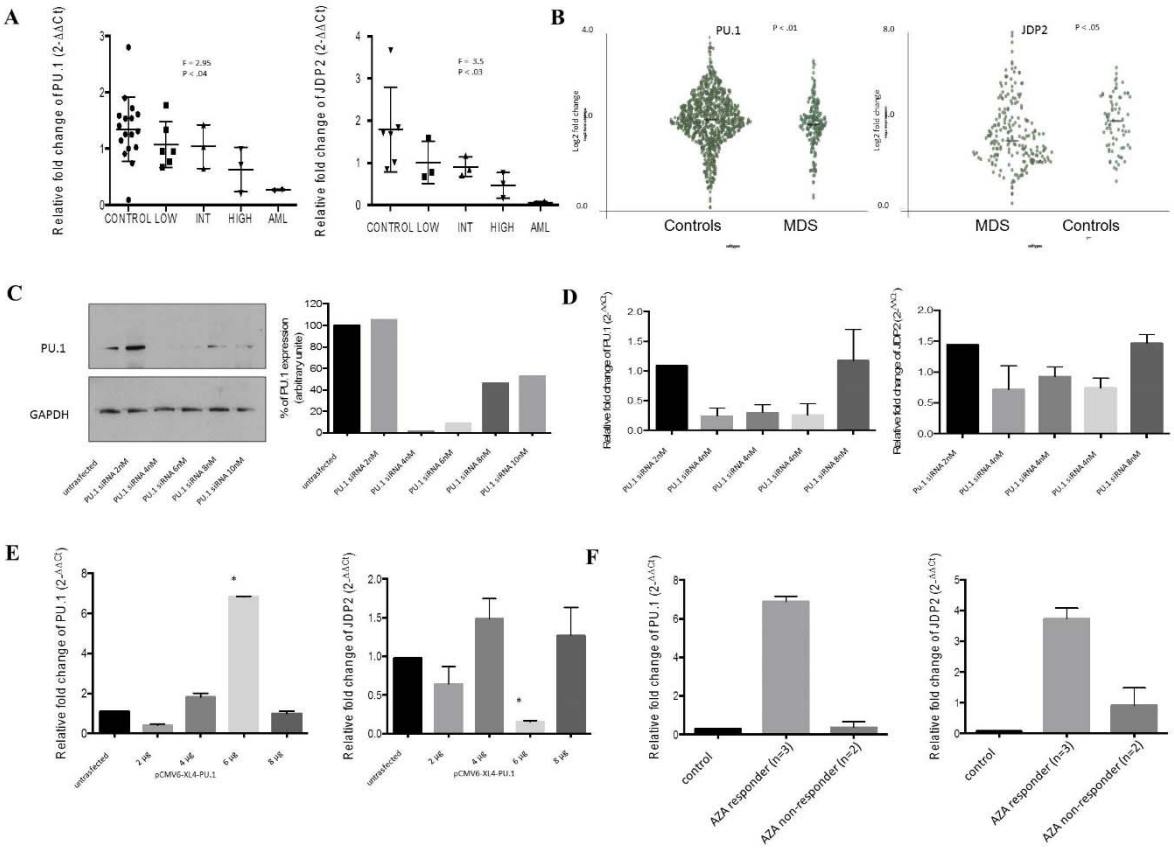
A
Relative fold change of PU.1 (2-ΔΔCt)
F = 2.95
P < .04
CONTROL LOW INT HIGH AML
Relative fold change of JDP2 (2-ΔΔCt)
F = 3.5
P < .03
CONTROL LOW INT HIGH AML
B
PU.1
P < .01
Log2 fold change
Controls MDS
JDP2
P < .05
Log2 fold change
MDS Controls
C
PU.1
GAPDH
% of PU.1 expression (arbitrary unite)
untransfected
PU.1 siRNA 2nM
PU.1 siRNA 4nM
PU.1 siRNA 6nM
PU.1 siRNA 8nM
PU.1 siRNA 10nM
D
Relative fold change of PU.1 (2-ΔΔCt)
Relative fold change of JDP2 (2-ΔΔCt)
E
Relative fold change of PU.1 (2-ΔΔCt)
untransfected
2 µg
4 µg
6 µg
8 µg
pCMV6-XL4-PU.1
Relative fold change of JDP2 (2-ΔΔCt)
F
Relative fold change of PU.1 (2-ΔΔCt)
control
AZA responder (n=3)
AZA non-responder (n=2)
Relative fold change of JDP2 (2-ΔΔCt)