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BRIEF COMMUNICATION

HUWE1 cooperates with RAS activation to control leukemia cell proliferation and human hematopoietic stem cells differentiation fate

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Abstract

Acute myeloid leukemia (AML) is a poor prognosis hematopoietic malignance characterized by abnormal proliferation and differentiation of hematopoietic stem cells (HSCs). Although advances in treatment have greatly improved survival rates in young patients, in the elderly population, ~70% of patients present poor prognosis. A pan-cancer analysis on the TCGA cohort showed that AML has the second higher *HUWE1* expression in tumor samples among all cancer types. In addition, pathway enrichment analysis pointed to RAS signaling cascade as one of the most important pathways associated to *HUWE1* expression in this particular AML cohort. In silico analysis for biological processes enrichment also revealed that *HUWE1* expression is correlated with 13 genes involved in myeloid differentiation. Therefore, to understand the role of HUWE1 in human hematopoietic stem and progenitor cells (HSPC) we constitutively expressed $KRAS^{G12V}$ oncogene concomitantly to *HUWE1* knockdown in stromal co-cultures. The results showed that, in the context of $KRAS^{G12V}$, HUWE1 significantly reduces cell cumulative growth and changes myeloid differentiation profile of HSPCs. Overall, these observations suggest that HUWE1 might contribute to leukemic cell proliferation and impact myeloid differentiation of human HSCs, thus providing new venues for RAS-driven leukemia targeted therapy approach.

Acute myeloid leukemia, HUWE1, and RAS pathway

Acute myeloid leukemia (AML) is a poor prognosis hematopoietic malignance characterized by uncontrolled proliferation and abnormal differentiation of hematopoietic stem cell (HSC). Differentiation impairment leads to accumulation of immature myeloid cells and consequently impacts the production of red blood cells, platelets, and white blood cells [1, 2]. Since the incidence increases with age, as global life expectancy increases, there is a high probability that more people will develop AML [3]. Although advances in treatment have greatly improved survival rates in young patients, in the elderly population, ~70% of patients present poor prognosis and frequently experience death within 1 year after diagnosis [4]. Next-generation sequencing has extensively shown that AML is a complex disease, presenting a vast number of mutations cooperating for tumorigenesis, besides the mutational background heterogeneity among patients [1]. These studies have also identified many somatic mutations in epigenetic regulators involved in processes such as DNA methylation and post-translational histone modifications [5, 6]. HUWE1 is an E3 ubiquitin ligase that targets multiple substrates, such as transcription factors and histones, participating in a variety of cellular processes like differentiation, proliferation, apoptosis, DNA repair, and response to stress [7]. Owing to this characteristic, loss of its function results in the development of many diseases, including cancer [7]. A pan-cancer analysis on the TCGA data set showed that AML has the second higher *HUWE1* expression in tumor

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samples among all cancer types (Fig. S1). HUWE1 has been related to lymphoid commitment and lymphocytes homeostasis, as well as the maintenance of HSPCs stemness, although the extension of its function on leukemic cells remains largely unknown [8–10].

Considering this scenario, the present study was conducted to investigate the role of HUWE1 in human hematopoietic stem and progenitor cells (HSPCs) in the context of KRAS activation.

HUWE1 is required for the proliferation of KRAS activated-expressing cells

To gain insights into the molecular mechanisms underlying HUWE1's role in leukemogenesis, we performed an in silico analysis on TCGA LAML data set. The pathway enrichment analysis of genes correlated with *HUWE1* expression pointed to RAS signaling as one of the most important pathways associated to *HUWE1* expression (Fig. S2). To functionally investigate the link between HUWE1 expression and RAS activation, we selected leukemic cell lines harboring either RAS mutations or *BCR-ABL* fusion gene (which also activates RAS signal transduction) [11] and subjected them to *HUWE1* knockdown. Analysis of cumulative growth was then performed as it is one of the hallmarks of RAS activation [11]. As shown in Fig. S3, HUWE1 absence decreased the proliferative capacity of all cell lines (Nalm-6, K562, HL-60, and THP-1), suggesting that HUWE1 is important to maintain neoplastic cell proliferation. Next, to address whether HUWE1 would have the same role in human leukemogenesis we constitutively expressed $KRAS^{G12V}$ oncogene in HSPCs stromal co-cultures (with MS5) either alone or associated with *HUWE1* knockdown. After 3 weeks in co-culture, active $KRAS^{G12V}$ alone provoked an 8.5-fold increase in HSPCs cumulative growth in comparison with non-transduced controls, as previously reported [12]. In contrast, in the absence of HUWE1, HSPCs expressing $KRAS^{G12V}$ had a clear reduction (36%) of cumulative growth ($p = 0.001$ and $p = 0.003$ in shHUWE1 #1 and #3, respectively), suggesting that the ubiquitin ligase HUWE1 has an important role in HSPCs proliferation capacity upon $KRAS^{G12V}$ expression (Fig. 1a).

To address if the aforementioned reduction of cumulative growth caused by HUWE1 downregulation would also impact the presence of immature HSPCs on stromal co-culture, we evaluated the cobblestone areas, which are generated by cobblestone area forming cells (CAFCs) [12]. In cultures harboring only $KRAS^{G12V}$ the CAFCs were largely increased, whereas upon combination with *HUWE1* knockdown the number of CAFCs were dramatically reduced, as observed in Fig. 1b. These results corroborate the cumulative growth profile of HSPCs co-cultures, and suggest that indeed, oncogenic $KRAS^{G12V}$-mediated cell proliferation strongly depends on the presence of HUWE1. Altogether, these findings indicate that HUWE1 has a role in the cell proliferation induced by KRAS activation in the leukemogenesis process.

HUWE1 has a role in myeloid differentiation

To further investigate if HUWE1 could also impact the HSPCs self-renewal capability, we performed a clonogenic capacity assay. To that aim, HSPCs cells were transduced, sorted, and plated in methylcellulose to enumerate the presence of progenitor cells. As shown in Fig. S4 cells expressing $KRAS^{G12V}$ showed a significant reduction of the total number of colonies compared with control ($p = 0.003$). The same reduction was observed for both bursting-forming unit-erythrocyte (BFU-E) colonies and colony-forming unit–granulocyte/macrophage (CFU-GM) colonies (92.8% and 40.8% reduction, respectively) in comparison with control ($p = 0.001$ and $p = 0.042$, respectively) (Fig. 1c). Notably, when $KRAS^{G12V}$-expressing cells were subjected to *HUWE1* knockdown, the observed phenotype was greatly improved. Compared with $KRAS^{G12V}$ alone, *HUWE1* inhibition provoked a massive reduction of BFU-E colonies impairing the colony formation in ~88.9% ($p = 0.004$). In CFU-GM colonies, the phenotype was also apparent and showed a significant reduction in the same context ($p = 0.037$) (Fig. 1c). Altogether, these results suggest that HUWE1 might be relevant to HSPCs self-renewal capacity in the context of oncogenic KRAS activation.

Considering that HUWE1 knockdown potentiates $KRAS^{G12V}$-induced impairment of CFU-GM colony formation, we wondered if it would also impact myeloid commitment profile. To address whether HUWE1 has a role on the differentiation profile of HSPCs, the cells were evaluated for myeloid lineage commitment upon *HUWE1* knockdown. As observed in Fig. 1d, normal HSPC co-cultures predominantly differentiated towards $CD15^+$ granulocytes as compared with $CD14^+$ monocytes. However, *HUWE1* knockdown (#1 and #3) interestingly resulted in a differentiation profile towards the monocytic lineage ($CD14^+$) ($p = 0.016$) (Fig. S5), suggesting that HUWE1 might have a role in the myeloid lineage commitment.

To support this hypothesis, we took advantage of the TCGA data set to perform another enrichment analysis, now focusing in Gene Ontology (GO) biological processes. This analysis revealed that *HUWE1* expression is correlated with 13 genes involved in myeloid differentiation (GO:0045637) (Fig. 1e). These data were validated in an independent

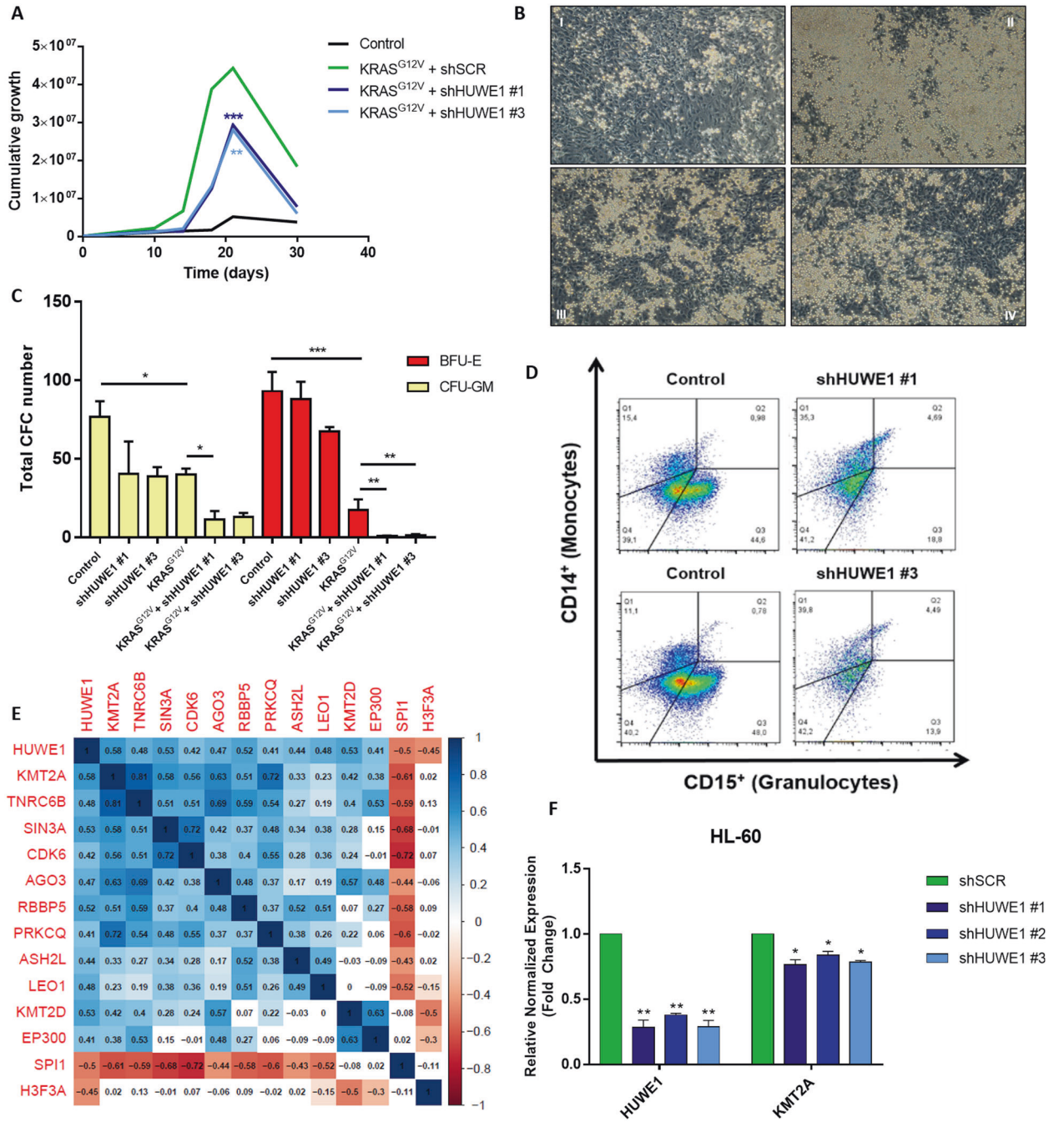

Fig. 1 Characterization of HUWE1's role in myeloid differentiation. a Co-cultures cumulative growth was monitored for 30 days and graph shows proliferative disadvantage of KRAS^{G12V}/shHUWE1 when compared with KRAS^{G12V} alone ($p = 0.001$ and $p = 0.003$ in shHUWE1 #1 and #3, respectively). **b** Images of co-cultures at week 3 showing reduced maintenance of long-term HSCs KRAS^{G12V}/shHUWE1 (III and IV) when compared with KRAS^{G12V} alone (II). Image I represents non-transduced HSPCs. **c** Colony-forming capacity (CFC) was assessed after 14 days. Statistics show reduction in the number of BFU-E colonies in KRAS^{G12V} compared with control ($p < 0.001$) and the exacerbated effect in KRAS^{G12V}/shHUWE1 compared with KRAS^{G12V} alone in both BFU-E ($p = 0.004$ and $p = 0.01$ in #1 and #3, respectively) and CFU-GM colonies ($p = 0.037$ and $p = 0.058$ in #1 and #3, respectively). **d** Cells were evaluated for myeloid lineage commitment and dot plots show a shift of granulocytic differentiation towards monocytic differentiation. All results are representative of two independent experiments in duplicates and statistical analysis was performed using one-way ANOVA with Bonferroni's post test. **e** Corrplot of Pearson's correlation between *HUWE1* and myeloid differentiation-related genes in TCGA LAML data set. Blank squares represent not significant correlations ($p > 0.05$). **f** Validation of *HUWE1* gene correlation with *KMT2A* in HL-60 using specific primers in RT-qPCR. Results are representative of three independent replicates and statistical analysis was performed using two-sided Student's *t* test. *$p \le 0.05$; **$p \le 0.01$; ***$p \le 0.001$.

cohort of 194 AML patients from GEO database (GSE 114868) where the correlation between *HUWE1* and myeloid differentiation-related genes was concordant for 10 out of 13 genes (Fig. S6). In addition, within this gene set, seven genes were differentially expressed in AML patients when compared with health donors (Fig. S7), suggesting that *HUWE1* can have a role in myeloid differentiation towards malignant transformation. Of note, among this gene set we highlight *KMT2A* gene, which encodes the mixed-lineage leukemia 1 human protein, a histone methyltransferases widely described in gene fusions related to AML leukemogenesis (MLL-rearranged leukemias) [13].

To functionally validate these data, HL-60 cell line, a leukemic model of NRAS mutation, was used to evaluate the gene expression correlation between *HUWE1* and *KMT2A*. As expected, the positive correlation observed in in silico analysis was confirmed as *HUWE1* knockdown clearly reduced *KMT2A* mRNA transcripts levels ($p < 0.05$) (Fig. 1f; Table SI). Moreover, we took advantage of the Depmap Portal to evaluate 17 AML cell lines (Table SII) from the Cancer Cell Line Encyclopedia (CCLE) and Gene Dependency Dataset from Achilles RNAi data (DEMETER scores) [14]. As observed in Fig. S8, a Pearson correlation analysis between *HUWE1* and *KMT2A* gene expression strongly corroborates our finding ($R = 0.40$; $p = 0.03$). These results suggest that *HUWE1* can cooperate with *KMT2A* and impact myeloid differentiation. Although it is largely known that mutations in both *NRAS* and *KRAS* have been previously linked to AML proliferation and differentiation [15], this is the first report linking HUWE1 to this context.

Conclusions

This study provides valuable information to comprehend the underlying mechanisms of HUWE1 activity in hematopoietic cells with different RAS-driven contexts. Our data suggest that HUWE1 can modulate the myeloid differentiation profile upon RAS activation and also contributes to leukemic cell proliferation. These findings bring new insights to HUWE1 oncogenic role in the leukemogenesis process and provide additional information to support further investigations on HUWE1 inhibitors, which could have potential clinical translation in current leukemia therapy approaches.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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