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Brief communication

The granulocyte-associated transcription factor Krüppel-like factor 5 is silenced by hypermethylation in acute myeloid leukemia

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ABSTRACT

Krüppel-like factor 5 (*KLF5*) has been implicated as a tumor suppressor in various solid tumors such as breast and prostate, and recent studies have demonstrated a role for this protein in neutrophil differentiation of acute promyelocytic leukemia cells in response to ATRA. Here, we show that *KLF5* expression increases during primary granulocyte differentiation and that expression of KLF5 is a requirement for granulocyte differentiation of 32D cells. In AML, we show that *KLF5* mRNA expression levels are reduced in multiple French–American–British subtypes compared to normal controls, and also in leukemic stem cells relative to normal hematopoietic stem cells. We demonstrate that in selected AML cases, reduced expression is associated with hypermethylation of the *KLF5* locus in the proximal promoter and/or intron 1, suggesting that this may represent a Class II genetic lesion in the development of AML.

1. Introduction

Krüppel-like factor 5 (*KLF5*) belongs to the family of KLF transcription factors of which 17 members have been identified to date. The KLF proteins share a highly homologous DNA binding domain consisting of three cysteine-2/histidine-2 zinc finger motifs separated by 7 conserved amino acids at the carboxy terminal of the proteins. Members of this family have been implicated in a wide variety of cellular processes including proliferation, self-renewal, differentiation, and apoptosis, and accordingly deregulation of these factors can contribute to the pathogenesis of human diseases such as cancer [1].

Like other KLF family members, *KLF5* can either activate or repress target genes depending on factors such as post-translational modification or recruitment of specific coactivators/corepressors. *In vivo* models have demonstrated roles for *KLF5* in biological processes such as embryonic development, cardiovascular remodelling, adipogenesis, inflammatory stress responses, and intestinal development [2]. The cellular function of *KLF5* appears to be context-dependent; *KLF5* has a pro-proliferative function in a number of epithelial cell types, vascular smooth muscle cells, keratinocytes, and fibroblasts, and it plays a role in maintenance of embryonic stem cell self-renewal. Conversely, it functions as an inducer of differentiation in adipocytes [2]. Recently, Humbert et al. demonstrated a role for *KLF5* in granulocyte differentiation of acute promyelocytic leukemia (APL) cells [3]. We had previously identified *KLF5* as a gene for which mRNA expression increased during GM-CSF-induced granulocyte–macrophage differentiation of the murine FDB1 cell line [4], consistent with a role in myeloid differentiation. Our results are in agreement with those of Humbert and colleagues, and consistent with reduced expression of *KLF5* attenuating granulocyte differentiation in response to G-CSF signaling.

Class II mutations in AML contribute to the block in myeloid differentiation and cooperate with lesions that drive proliferation and survival (Class I mutations). These lesions commonly involve transcription factors which normally act to promote myeloid differentiation such as *RUNX1* and *CEBPA*, which are frequently down-regulated or disrupted by mutation in acute myeloid leukemia (AML). We performed extended analyses of

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KLF5 expression in primary human AML samples, and investigated hypermethylation of the *KLF5* gene locus as a potential mechanism for the observed down-regulation in AML. Collectively, our data are consistent with *KLF5* functioning as a tumor suppressor in the myeloid lineage, and suggest that hypermethylation of *KLF5* may act as an AML Class II abnormality.

2. Design and methods

2.1. Differentiation of 32D cells and shRNA knock-down of KLF5

32D cells and human leukemia cell lines were maintained as specified by the suppliers (for details see Supplementary Materials and Methods). A small hairpin RNA (shRNA) cassette targeting murine *KLF5* (target sequence 5′ GAACTGGCCTCTACAAATC 3′) and one non-targeting control cassette (target sequence 5′ GCACGACTTCTTCAAGTCCTT 3′) were cloned into the retroviral pMSCV-ΔLTR-H1 (pMΔH) construct (based on the pRetroSuper vector). 32D populations expressing the shRNA constructs were generated using a retroviral spinfection method which has been described previously [5]. Cells were seeded at 1×10^5/ml in culture medium supplemented with 50 ng/ml human G-CSF (Peprotech) for differentiation to granulocytes over 7 days.

2.2. Assessment of cell growth and differentiation

Live cell number was determined using Trypan Blue staining. To assess morphologic differentiation, cytospin preparations were stained with May-Grünwald Giemsa stain and photographed at an original magnification of 200×. For detection of myeloperoxidase enzyme activity, cytospin preparations were treated using the Sigma–Aldrich Leukocyte peroxidase (Myeloperoxidase) kit according to the manufacturer's instructions (Hanker–Yates method) and scored by counting a minimum of 200 cells per slide. Expression of the myeloid differentiation marker CD11B was assessed by flow cytometry using PE conjugated antibodies and matched isotype controls from eBiosciences. Detection of gene expression using quantitative reverse transcription PCR (Q-RT-PCR) is outlined in Supplementary Materials and Methods.

2.3. AML samples and controls

A panel of 29 *de novo* AML patient samples (Supplementary Table 1) was assembled using bone marrow mononuclear cell (MNC) samples taken at diagnosis with consent from the Queen Elizabeth Hospital, Adelaide, AUSTRALIA (Ethics approval number 2007072) and the Royal Adelaide Hospital/Institute of Medical and Veterinary Science, Adelaide, AUSTRALIA (Ethics approval number 070604), and normal bone marrow samples were obtained using an approved collection process through the Royal Adelaide Hospital, Adelaide AUSTRALIA (5 $CD34^+$ and 4 MNC controls).

2.4. Methylation analysis

Genomic DNA samples were assessed for methylation by the Australian Genome Research Facility (AGRF), Brisbane AUSTRALIA. Amplicons were designed with the aid of Sequenom EpiDesigner Software to assess methylation of *KLF5* over genomic coordinates chr13:72521648-72526071 of the March 2006 (NCBI36/hg18) assembly (International Human Genome Sequencing Consortium). Quantitative methylation analysis was performed using a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry-based approach (Sequenom EpiTYPER using the MassARRAY system). For details regarding assay design and identification of differentially methylated regions see Supplementary Fig. 1.

For further experimental details refer to Supplementary Materials and Methods.

3. Results

3.1. KLF5 expression is up-regulated in the granulocyte lineage and is necessary for granulocyte differentiation in response to G-CSF

Bioinformatic analysis of published microarray data sets to evaluate the expression of *KLF5* and selected other members of the *KLF* family showed that *KLF5* expression was specifically up-regulated in the granulocyte lineage compared to mouse stem cell [6] or human $CD34^+$ progenitor [7] populations respectively (Fig. 1A). The expression patterns observed for several other *KLF* genes are consistent with reported function, for example KLF1 has a known role in erythroid differentiation, and KLF4 is involved in monocyte differentiation [8]. The granulocyte-restricted pattern of expression for *KLF5* is consistent with a lineage-specific role in granulocyte development.

To test the functional role of KLF5 during granulocyte differentiation, we knocked down *KLF5* expression in 32D cells which normally differentiate to granulocytes in response to G-CSF, during which time *KLF5* expression increases (Fig. 1B). Effective knock-down of *KLF5* expression was observed using Q-RT-PCR and Western blotting when compared to cells expressing a non-targeting control (Fig. 1C). Reduced *KLF5* expression was associated with a 1.8-fold increase in cell expansion (Fig. 1D) and attenuated granulocyte differentiation (Fig. 1E–F) in response to G-CSF. This was evident morphologically (as seen by the presence of immature myelocytes in the *KLF5* shRNA population on Day 7 in G-CSF – Fig. 1E), and also on analysis of markers of granulocyte differentiation (Fig. 1F). Expression of the myeloid differentiation marker CD11B decreased, as did expression of the neutrophilic granule markers Myeloperoxidase (MPO) and *Lactotransferrin* (*Ltf*). These changes may be mediated by reduced expression of the late-acting granulocytic transcription factor *Cebpe*, which showed a 2.1-fold reduction in expression in cells transduced with the *KLF5* shRNA. Taken together these results are consistent with a requirement for *KLF5* in granulocyte development in response to cytokine stimulation.

3.2. Reduced KLF5 expression is common in AML and not restricted to a particular FAB subtype

We next determined if the relative expression of *KLF5* is modified in AML compared with normal hematopoietic populations. Q-RT-PCR analysis demonstrated that average *KLF5* expression was significantly (2.3-fold) lower in a local cohort of *de novo* AML patient samples than in $CD34^+$ control samples (Fig. 2A). Expression was also lower in a panel of human leukemia cell lines (Fig. 2A). Although sample numbers were limited, we observed significantly reduced expression in French–American–British (FAB) subtype M3 (Fig. 2B), which is consistent with the data presented by Humbert et al. demonstrating a functional role for *KLF5* in differentiation of APL cells. Additionally, we found that *KLF5* expression was also significantly reduced in the immature M1 subtype and monocytic M4 subtype compared to $CD34^+$ controls. To validate our findings in a larger cohort of patient samples we accessed raw data from a previously published microarray study performed by Valk et al. [9]. Analysis of this data set showed that *KLF5* gene