Suppressive effects of anagrelide on cell cycle progression and the maturation of megakaryocyte progenitor cell lines in human induced pluripotent stem cells

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Supplementary data

Materials and Methods

Anagrelide

Anagrelide hydrochloride was purchased from Abcam (Cambridge, UK). Before being used to treat imMKCL cells, it was dissolved in dimethyl sulfoxide and diluted with distilled water.

Maintenance of the cells

The imMKCL cells were cultured and maintained in Iscove's modified Dulbecco's medium (Sigma-Aldrich, MO, USA) with 10% fetal bovine serum (Access Biologicals LLC, CA, USA) and 1% penicillin–streptomycin (ThermoFisher, Basingstoke, UK) at 37 □ in a 5% CO_2 atmosphere by adding 50 ng/ml of thrombopoietin (BioLegend, CA, USA), 50 ng/ml of stem cell factor (BioLegend), and 100 ng/ml of doxycycline (Clontech, CA, USA) under feeder-free conditions.

MTS assays

To evaluate the effect of anagrelide treatment on cell proliferation, imMKCL cells and cells from other cell lines were seeded into 96-well plates with various concentrations of anagrelide (0, 1, and 10 μM) in undifferentiated and differentiated states. After 48 h and 96 h of treatment with Cell Titer 96® Aqueous One Solution Reagent (Promega, Madison, WI, USA), absorbance at 490 nm was measured. The assay was performed according to the manufacturer's instructions.

BrdU cell proliferation assay

For the cell proliferation assays, imMKCLs were seeded into 96-well plates in differentiated states with various concentrations of anagrelide (0–10 μM). After 48 h of co-culture with anagrelide (24 h after labeling with BrdU), the proliferation rate in imMKCLs was measured using a BrdU Cell Proliferation ELISA Kit (Abcam), according to the manufacturer's instructions.

Quantitative RT-PCR

Gene expression related to megakaryopoiesis and platelet generation was analyzed using quantitative RT-PCR. The total RNA was extracted using an RNeasy Mini kit (Qiagen, Venlo, Netherlands), and the reverse transcription step was performed with a Prime-Script RT Reagent Kit (Takara Bio, Shiga, Japan), according to the manufacturer's instructions. RT-PCR was performed using a StepOnePlus Real-Time PCR System (Applied Biosystems, Norwalk, CT, USA). The data are presented as relative expression levels normalized to *GAPDH* expression. The primer sequences are shown in Supplementary Table S1.

Flow cytometry and antibodies

The monoclonal antibody used in the flow cytometry analyses were fluorescein isothiocyanate (FITC)-conjugated anti-human CD41 (integrin αIIb, *ITGA2B*) and phycoerythrin (PE)-conjugated anti-human CD42b (gpIbα, *ITGB3*), obtained from BioLegend (CA, USA). To analyze the number of generated platelets, flow cytometry analyses were performed as previously described.[1-3] ImMKCLs were cultured without doxycycline for seven days, and the generated platelets in the supernatants were stained

and combined with antibodies at room temperature. The platelet samples were washed, diluted with staining medium (phosphate-buffered saline containing 3% fetal bovine serum), and analyzed using flow cytometry. Trucount Tubes (BD Biosciences, UK) were used to quantify and count the generated platelets. The flow cytometry analyses were performed using a FACS Canto II (BD Biosciences). The data were analyzed using FlowJo software (TreeStar, Ashland, OR, USA).

RNA sequencing and the subsequent analyses

We performed RNA sequencing of imMKCLs either untreated or treated with 1 μM of anagrelide for two days in both undifferentiated and differentiated states. A total of 500 ng of RNA was used to prepare the library with a NEBNext® UltraTM RNA Library Prep Kit for Illumina (New England BioLabs, Ipswich, MA, USA). All the samples used for RNA sequencing were assigned to a single unique index. The quality of the libraries was evaluated using a High Sensitivity DNA Kit (Agilent, Santa Clara, CA, USA). The sequencing was performed using HiSeq1500 (Illumina) with a single-read sequencing length of 60 bp. TopHat version 2.0.13 (with the default parameters) was used to map the reads to the reference genome (UCSC/hg19) with annotation data from iGenomes (Illumina). Gene expression levels were quantified using Cuffdiff (Cufflinks version 2.2.1, with the default parameters). A gene ontology analysis was conducted using the Database for Annotation, Visualization, and Integrated Discovery.[4] Six annotated gene ontology terms were summarized by REVIGO,[5] and a gene set enrichment analysis was performed, as described.[6]

Cell cycle analysis

ImMKCLs were incubated with various concentrations of anagrelide (0, 1, and 10 μM) in undifferentiated and differentiated states for 48 h and stained with BrdU and 7AAD using a FITC BrdU Flow Kit (BD Biosciences, UK). A flow cytometry analysis was performed.

Apoptosis assay

Two and seven days after anagrelide treatment, imMKCLs were stained with annexin V-FITC and propidium iodide using a FITC Annexin V Apoptosis Detection Kit 1 (BD Pharmingen, UK) and analyzed using flow cytometry.

Statistical analysis

Three independent in vitro experiments were performed as described in the corresponding figure legends. For each experiment, three biological replicates were generated, unless stated otherwise. The statistical analysis used the mean value for each experiment. The variances were similar between the groups. Statistical analyses were performed using Microsoft Excel version 14.6.2 (Microsoft Corporation, Redmond, WA, USA) and GraphPad Prism version 7 (GraphPad Software, San Diego, CA, USA). Differences in continuous variables were evaluated using Student's *t*-test for two-group comparisons or one-way ANOVA with post-hoc Bonferroni test for multiple group comparisons. The data are presented as the mean values of independent experiments or samples ± standard error of mean. Statistical significance was considered at levels of * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

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Supplementary Figure Legends

Supplementary Figure S1. MTS assays of non-megakaryocytic lineage cell lines

MTS assays were performed to analyze cells treated with anagrelide at concentrations of 0, 1, and 10 μM for t 0, 48, and 96 h. (A) HL-60 (AML M3) cells. (B) THP-1 (AML M5) cells. (C) K562 (CML) cells. Abbreviations: AML, acute myeloblastic leukemia; CML, chronic myeloid leukemia. Data are expressed as the mean ± SD from three independent experiments by the one-way ANOVA with post-hoc Bonferroni test.

Supplementary Figure S2. Suppressive effects of anagrelide on the cell cycle of imMKCLs in the differentiated state

ImMKCLs were treated with 0, 1, or 10 μM anagrelide for two days, after which cell cycle analysis was performed by labeling with 7AAD stained for DNA content and BrdU stained for cells in the S phase. The scatter plot histograms show 7AAD (*x*-axis) and BrdU (*y*-axis) in imMKCLs in the differentiated state. The right-hand bar graph shows the percentage of cells in each phase.

Supplementary Figure S3. Apoptosis assay

After 48 h of treatment with anagrelide, imMKCLs were stained with Annexin V-FITC and propidium iodide (PI) using a FITC Annexin V Apoptosis Detection Kit 1 (BD Pharmingen, UK) and analyzed using flow cytometry at day 2 (A) and day 7 (B). The flow cytometry showed immunofluorescence. The plots surrounded in the upper panels show the megakaryocytes (imMKCLs); these have been expanded in the lower panels and sorted according to Annexin V and PI levels. In the lower panels, the lower left

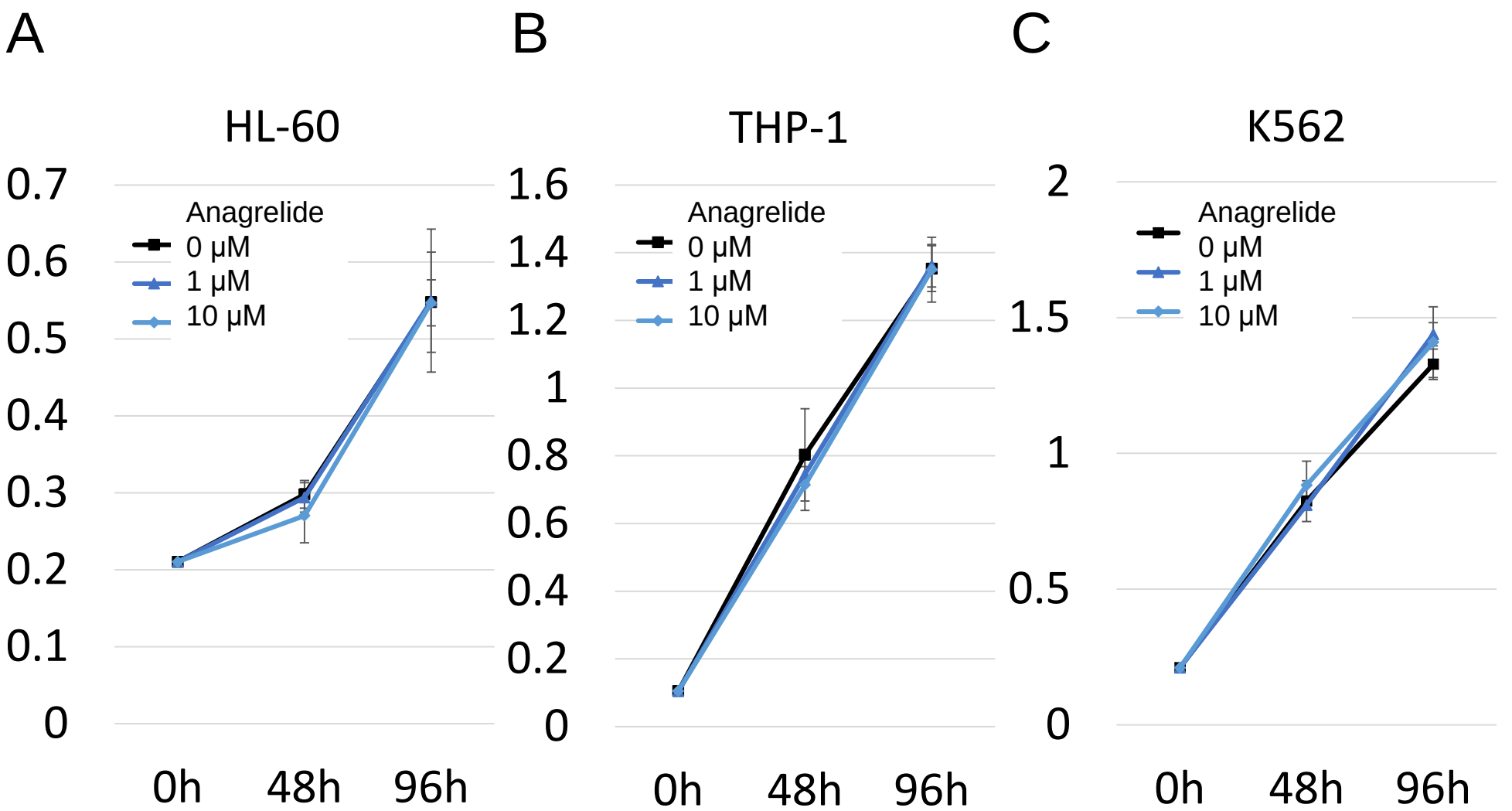
represents live cells, the lower right represents cells in early apoptosis, and the upper right represents cells in late apoptosis.

Supplementary Table S1.

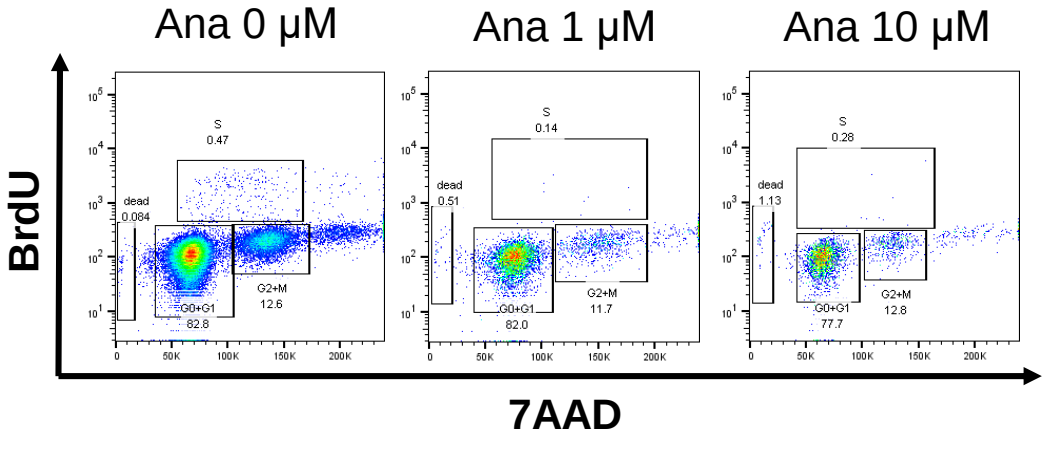
The primers used for qPCR were as follows:

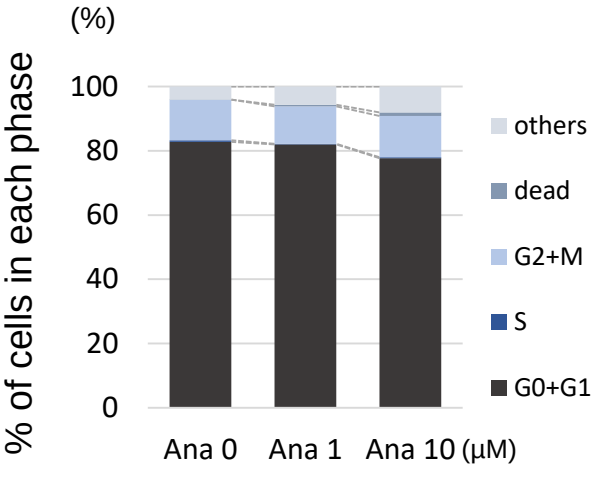
Gene Name		Sequence (5’ to 3’)
ITGA2B (CD41)	Fw	ATAGGAATCGCGATGTTGGTGA
	Re	ATCTTGCTGTTTGGATTCTGGC
ITGB3 (CD61)	Fw	AGCCTGTGTCACCATACATGT
	Re	GTCAGCACGTGTTTGTAGCC
TRIB3	Fw	CAAGCTGTGTCGCTTTGTCTT
	Re	GAGTATCTCAGGTGCCACGTAG
PF4	Fw	GCTGCTGTTCCTGGGTT
	Re	GGTCCCCATCTTCTTCAGCTT
GAPDH	Fw	CTGACTTCAACAGCGACACC
	Re	TAGCCAAATTCGTTGTCATACC

Supplementary Figure S1.



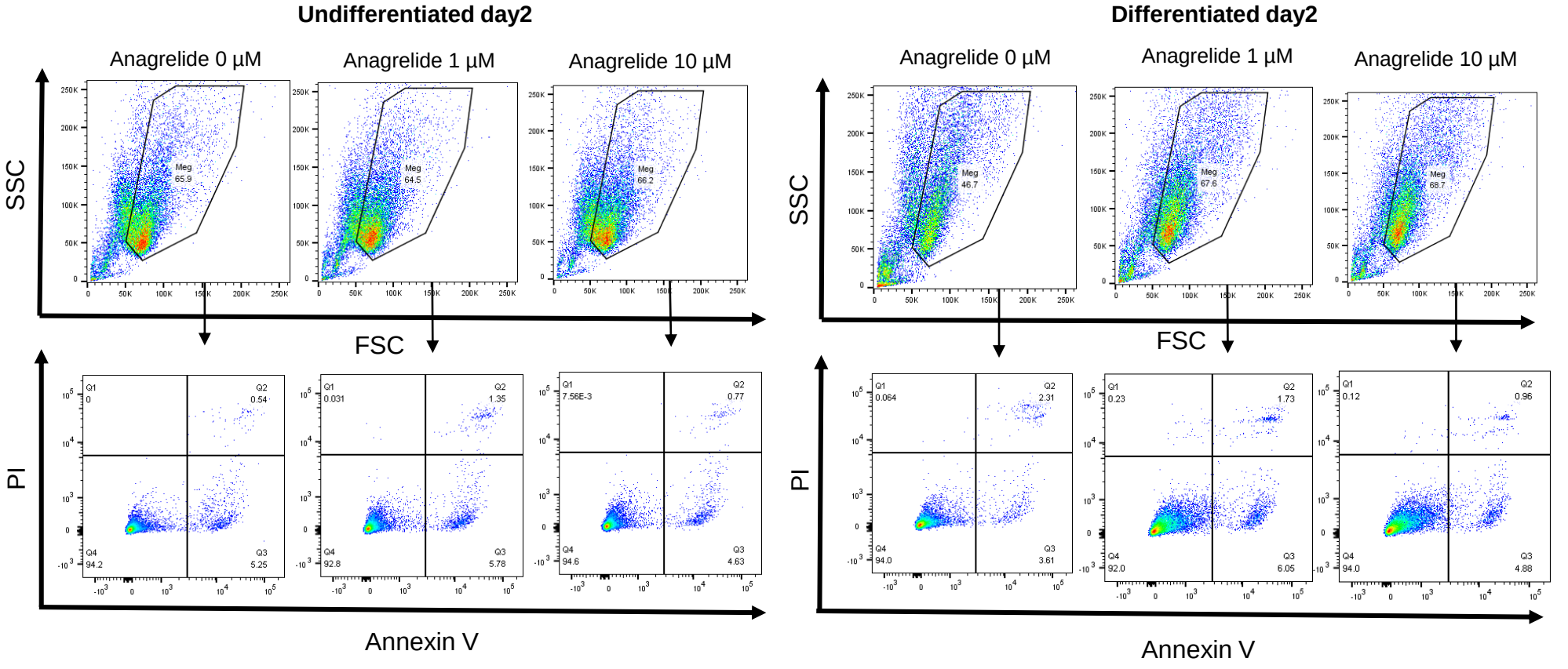
Supplementary Figure S2.





Supplementary Figure S3.

A



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