Received: 31 May 2024 | Accepted: 7 October 2024

DOI: 10.1111/bjh.19836

BJHaem
BRITISH JOURNAL OF HAEMATOLOGY

SHORT REPORT

Haematopoiesis

CDK8/19 inhibition triggers a switch from mitosis to endomitosis in cord blood megakaryocytes

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Funding information
National Heart, Lung, and Blood Institute, Grant/Award Number: HL046925, HL156052 and HL69990

Summary
Neonatal and adult megakaryocytes differ in proliferative capacity and ploidy levels, and neonatal and adult platelets differ in function, gene expression, and protein content. The mechanisms underlying these differences are incompletely understood. CDK8 and CDK19 are transcriptional kinases part of the CDK-mediator complex, which regulates gene transcription in a cell-specific manner. We discovered that cortistatin A, a potent highly selective inhibitor of CDK8/CDK19, significantly reduced cell expansion and increased ploidy in cord blood-derived megakaryocytes. These phenotypic changes were associated with gene expression changes that partially overlapped developmentally regulated genes. These findings might have relevance for the management of developmental megakaryocyte disorders.

KEYWORDS
cord blood, megakaryocytes, neonatal haematology, transcription

INTRODUCTION

There are substantial differences between cord blood- (CB; neonatal) and adult peripheral blood-derived (PB) megakaryocytes (MKs). Neonatal MK progenitors proliferate at increased rates versus adult progenitors, generating 10-fold more MKs when cultured in thrombopoietin. Neonatal MKs also undergo cytoplasmic maturation without nuclear polyploidization, resulting in small, low-ploidy, yet fully mature MKs. In contrast, adult MKs undergo successive rounds of endomitosis to reach higher ploidy, with cytoplasmic maturation coupled to polyploidization. These phenotypic differences are associated with changes in the expression of cell cycle molecules

Zhi-Jian Liu and Christopher Thom contributed equally to this work.

and transcription factors (TFs), as well as differences in platelet function.[1]

In eukaryotes, transcription of all protein-coding genes is accomplished by polymerase (Pol) II. The mediator (MED)–protein complex links DNA-binding TFs and Pol II, regulating Pol II-dependent transcription in a cell type- and signal-specific manner. Cyclin-dependent kinase (CDK) 8 and CDK19 are transcriptional kinases that bind Cyclin C, MED12/MED12L, and MED13/MED13L to form 'CDK modules' that reversibly associate with MED. The CDK-mediator complex can activate or repress transcription, particularly at super enhancers, DNA regions that control the expression of master regulatory genes for cell identity.[2]

We investigated the effects of cortistatin A (CA), a potent and specific small molecule inhibitor of CDK8 and CDK19, on human megakaryopoiesis. CA can potently inhibit the proliferation of acute myeloid leukaemia (AML) cell lines with different oncogenic drivers, including a megakaryoblastic cell line (SET2) derived from a patient with a JAK2_V617F mutation.[3] We found that human CB-derived MKs treated with CA exhibited a switch from mitosis to endomitosis, accompanied by phenotypic changes that resembled adult MKs, as well as transcriptional changes reflecting altered cell cycle progression and key MK TF activities.

METHODS

$CD34^+$ cells, isolated by positive selection from full-term CB samples obtained with IRB approval, were cultured in serum-free medium (StemSpan) containing 50 ng/mL of rHuTPO for 11 or 14 days as described. CA or DMSO were added at indicated concentrations and times. RNA expression was measured using the Affymetrix Human Genome U133 Plus 2.0 Array. Differential gene expression and pathway analyses used publicly available software platforms. Methodologic details are provided in Supplemental Methods. Microarray data are available through NCBI (GSE264379, GSE264381, GSE264384).

RESULTS

CA induces a phenotypic switch from neonatal to adult-like megakaryocytes

To interrogate the effects of CDK8/19 inhibition on CB-derived (neonatal) megakaryopoiesis, we added CA or DMSO to the MK culture medium at each bi-weekly media change for 14 days. CA treatment resulted in a dose-dependent decrease in cell expansion and an increase in MK ploidy (Figure 1A). CA treatment restricted to the first 4 days of culture, prior to MK differentiation, had no effect on the ultimate cell number

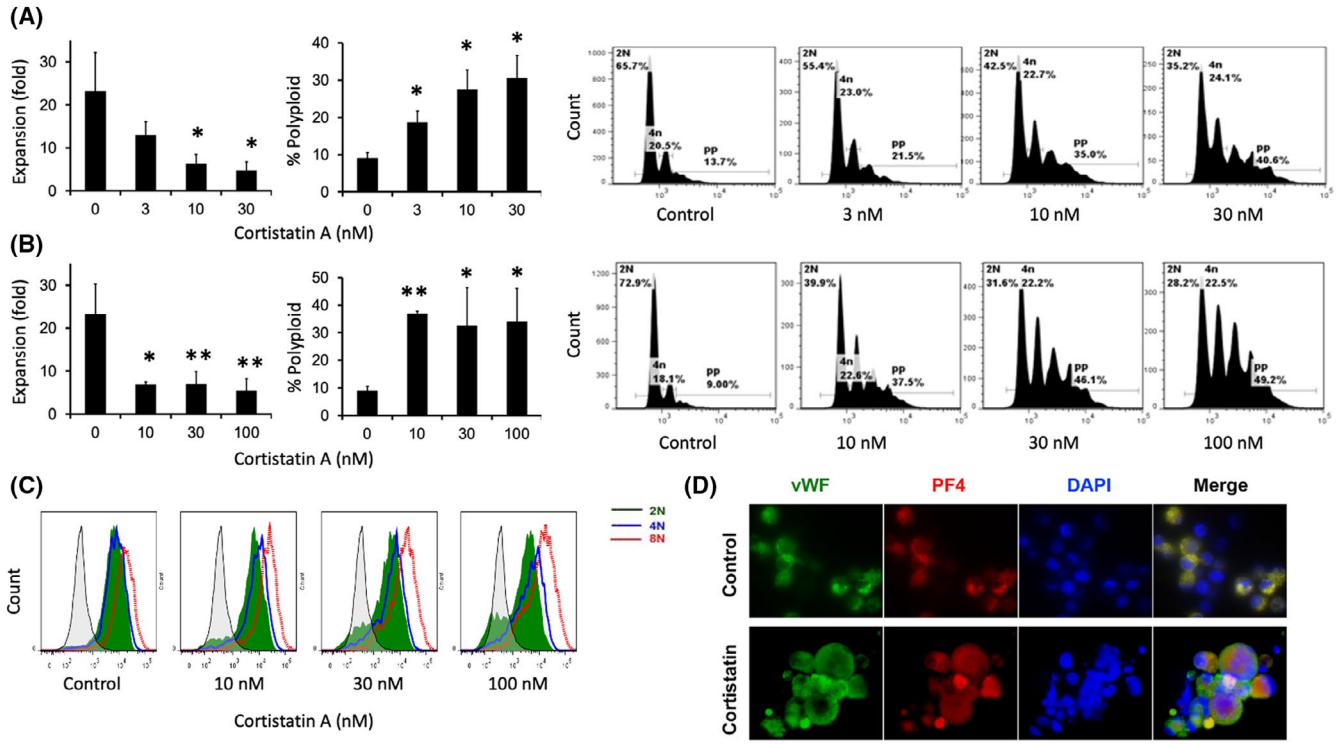

FIGURE 1 CB-MKs treated with cortistatin A (CA) develop features of adult MKs. (A) CB $CD34^+$ cells were cultured in a serum-free medium with rHuTPO for 14 days. Treatment with CA for the entire culture period resulted in a dose-dependent reduction in cell expansion (4.7 ± 2.2 vs. 23.1 ± 9.6-fold expansion in CA 30 nM vs. DMSO control; $p = 0.045$) with a concomitant increase in MK ploidy level. (B) Treatment with CA only during the second half of the culture (days 7–14) induced similar dose-dependent changes in cell expansion and ploidy. (C) Treatment with CA also resulted in an increased correlation between ploidy level and surface CD42b expression. (D) CA and DMSO-treated (control) MKs both exhibited vWF and PF4 protein expression, typical of MKs. Figures reflect averages or representative findings from $n = 4$ experiments. $^*p < 0.05$, $^{**}p < 0.01$.

or MK ploidy (Figure S1A). In contrast, CB-derived MK progenitors treated with CA from culture days 7–14 exhibited a dose-dependent reduction in cell expansion (6.2 ± 1.7 vs. 24.9 ± 2.2-fold expansion in 100 nM CA vs. control, $p = 0.003$) and increase in ploidy (34.0 ± 12% vs. 8.9 ± 1.7% MKs ≥8 N in 100 nM CA vs. control; $p = 0.014$), similar to cells treated for the entire culture period (Figure 1B). CA treatment did not affect MK differentiation (94 ± 2.3% $CD41^+$ cells in cultures treated with 100 nM CA vs. 93.4 ± 2.7% in controls). This suggested that CA impacts committed ($CD41^+$) MK progenitor expansion and ploidy.

To assess how CA affected MK maturation, we measured surface CD42b (a mature MK marker) by flow cytometry in CA-treated MKs of different ploidy levels. Untreated CB-MKs had similarly high CD42b mean fluorescent intensity (MFI) in low-ploidy (2–4 N) and high-ploidy (≥8 N) MKs, as previously described. In contrast, CA-treated MKs showed a correlation between ploidy and CD42b MFI, typical of adult MKs (Figure 1C).[1] Both control and CA-treated MKs displayed high vWF and PF4 intensity by immunofluorescence (Figure 1D) and formed pro-platelets in vitro (Figure S1B), features of mature neonatal and adult MKs. These findings suggested that CDK8/19 inhibition in CB-derived MK progenitors induced a phenotypic change towards adult-like MKs.

CA impacts relevant kinase pathways in CB-MKs

To ascertain how CA impacted CB-MK biology, we profiled CB-MK gene expression over the first 8 h of treatment ($n = 3$) and separately after 96 h differentiation ($n = 3$), when CB-MKs had transitioned to an adult-like MK phenotype. In the first 8 h, 460 genes were differentially regulated in CA versus DMSO controls (Figure S2A; Table S1). At 96 h, 3004 genes were differentially expressed (Figure S2B; Table S2).

Using the Enrichr platform,[4,5] we found that downregulated genes at 8 and 96 h matched gene expression signatures in cells with stable CDK8 or CDK19 knockdown[6] (Figure S2C). We also found an upregulated gene signature that matched Aurora Kinase A (AurKA) inhibition (Figure S2C).[7]

To determine whether CA treatment directly and selectively affected CDK8/19 versus other kinase DNA-binding activities, we performed kinase capture assays. CA inhibited CDK8/19 association with DNA, but not DNA interactions with other kinases, including AurKA (Figure S2D). This suggested that in addition to direct effects on CDK8/19 function, CA treatment indirectly impacted AurKA regulation and/or downstream pathways.

CA affects pathways in CB-MKs that are also developmentally regulated

We next interrogated if CA-induced changes in CB-MK gene expression resembled differences between neonatal and adult MKs. An historical dataset comparing the gene expression profile of primary CB- ($n = 5$) and adult-derived MKs ($n = 4$) identified 632 differentially regulated genes ($p < 0.05$, ≥1.5 log[FC]), although fewer than half (177) matched differentially expressed genes in CB-MKs following 96 h CA treatment (Figure 2A; Table S3).

Since a limited number of genes were concordantly altered in CA- versus untreated CB-MKs and adult versus CB-MK comparisons (Figure 2B), we reasoned that pathway-level changes would better elucidate processes responsible for the phenotypic effects of CA. Thus, we investigated which biological pathways were altered by CA treatment in CB-MKs, and differentially regulated between neonatal and adult MKs. Genes associated with AurKA inhibition were altered in adult versus CB-MKs, similar to effects seen in CB-MKs after CA treatment (Figure S2C). Other pathways affected by CA that were also differentially regulated in adult versus neonatal MKs included the regulation of haemostasis and platelet activation, signalling, and aggregation (Figure 2C; Figure S3), inflammatory response pathways, specifically TNFα signalling via NFκB (Figure 2D), and important signalling pathways, including PI3K-Akt, mTOR, and phospholipase D (Figure 2E). Interestingly, several cell cycle pathways were downregulated 8 h after CA treatment, but were upregulated after 96 h treatment and in adult versus CB-MKs (Figure S4).

Since CDK8/19 regulates the function of the mediator complex, which links DNA-binding TFs and Pol II to modulate Pol II-dependent transcription, we also investigated whether the genes upregulated by CA were targets of known MK TFs. As seen in Figure S5, genes differentially regulated by short (8 h) and long (96 h) CA treatment reflected transcriptional regulation from GATA1, GATA2, and RUNX1, without consistent elevations of the mRNA levels of the TFs themselves. Adult MKs, compared with CB-MKs, showed gene expression changes consistent with GATA1 and GATA2 activities, but not RUNX1.

CA treatment affects STAT1 activation in CB-MKs

JAK/STAT signalling is critical for MK and platelet homeostasis.[8] JAK/STAT-related genes were also amongst those differentially expressed after CA treatment and in adult versus neonatal MKs (Figure 2F). STAT1 phosphorylation at S727 is a known CDK8 substrate, and can promote neoplastic growth via JAK–STAT activation.[3] To confirm effective CDK8 inhibition in CB-MKs, we evaluated CA effects on STAT1-S727 and Y701 phosphorylation following IFNγ and TPO stimulation. As expected, CA decreased STAT1-S727 phosphorylation in CB-MKs stimulated with TPO or IFNγ but had no effect on STAT1-Y701 phosphorylation (Figure 2G). We also confirmed by gene set enrichment analysis that genes induced in CA-treated CB-MKs matched genes induced by CA treatment in SET2 megakaryoblastic cells (Figure S6A) as well as a custom gene set associated

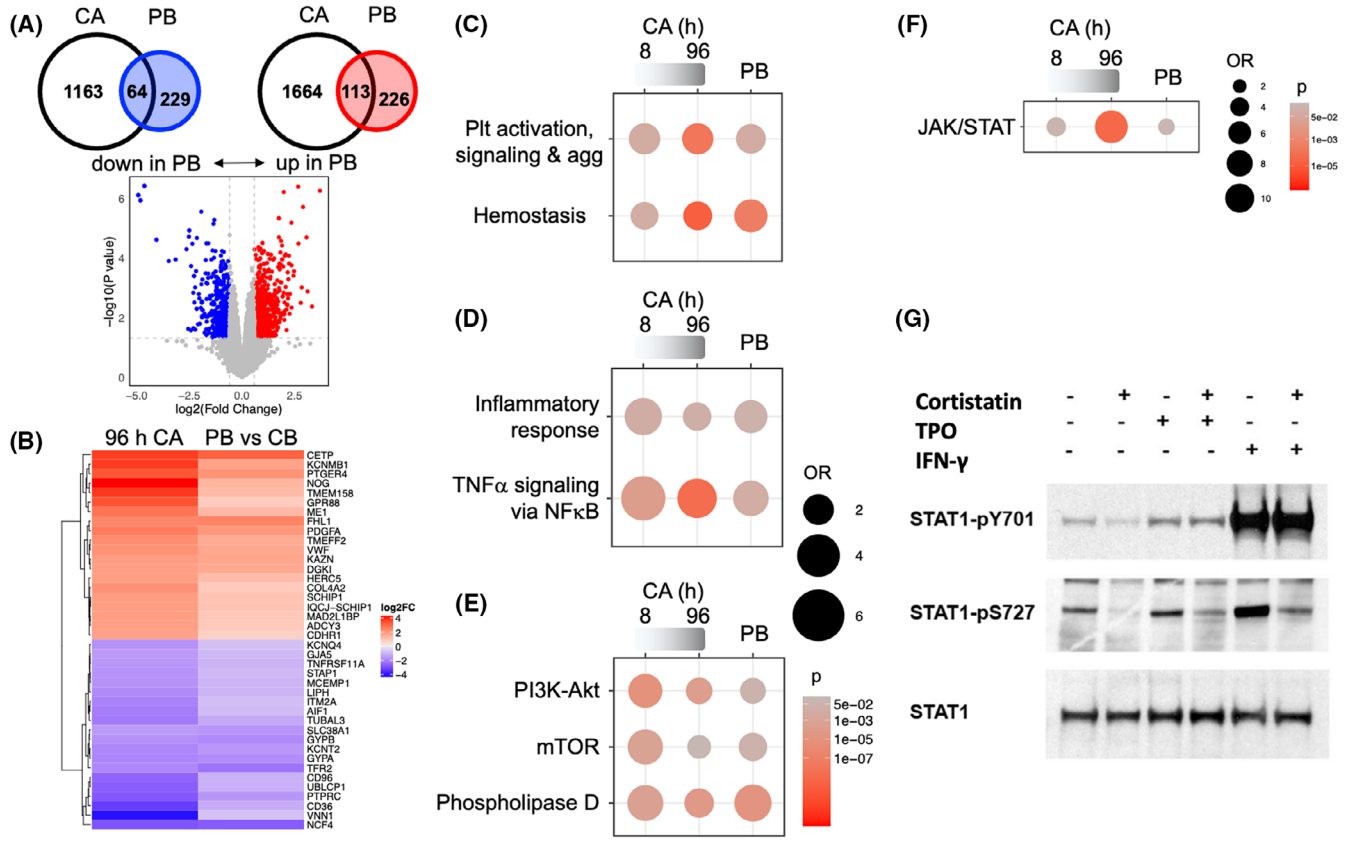

FIGURE 2 CA induces signalling pathway changes in CB-MKs that resemble some of the differences between adult (PB) and neonatal (CB) MKs. (A) Volcano plot depicts gene expression differences between adult PB- and neonatal CB-derived MKs in microarray analysis. Venn diagrams show overlap in genes differentially regulated in PB versus CB-MKs and/or in CB-MKs following 96 h CA versus DMSO treatment. (B) Heat map depicting top gene expression changes that were directionally consistent in PB versus CB and in 96 h CA treatment versus controls. (C) Platelet activation and haemostasis-related pathways were significantly enriched amongst differentially expressed genes in CB-MKs after short (0–8 h) or long (96 h) CA treatments. These pathways were also differentially regulated in PB versus CB-MKs. (D) Inflammatory response pathway genes were significantly altered following CA treatment in CB-MKs, and also in PB versus CB-MKs. (E) Genes in the PI3K-Akt, mTOR, and phospholipase D pathways were altered by CA treatment, and in PB versus CB-MKs. (F) JAK/STAT signalling pathway genes were altered in CB-MKs after short (0–8 h) or long (96 h) CA treatment. These pathways were also significantly altered in PB versus CB-MKs. (G) CB-MKs at day 11 of culture were depleted of growth factors for 3 hours, followed by treatment with DMSO or 100 nM CA for 1 h. Cells were subsequently stimulated with 50 ng/mL TPO or 10 ng/mL IFNγ for 20 min. STAT1 phosphorylation at S727 (a known CDK8 substrate) and Y701 (not a substrate) were then analysed by WB. CA treatment inhibited STAT1 phosphorylation at S727, but not Y701, indicating that CA specifically inhibited CDK8/19 kinase activity in CB-MKs. Database identifiers are shown in Table S4.

with the top 500 enhancer-bound peaks of STAT1 pS727 previously reported in SET2 cells (Figure S6B,C).[3] Many of these genes were also enriched in adult versus CB-MKs (Figure S6B,C).[3]

DISCUSSION

This study revealed that CDK8/19 inhibition induces a phenotypic change in CB-MKs to adult-like MKs, characterized by reduced cell expansion, increased polyploidization, and a coupling of ploidy and cytoplasmic maturation. Several other agents increase MK polyploidization, including nicotinamide, SRC inhibitors, and inhibitors of Rho kinase (ROCK) and AurKA.[7,9,10] Whilst we demonstrated that CA does not directly inhibit ROCK or AurKA, CA treatment induced genes similar to those seen following AurKA inhibition. This suggests that CDK8/19 and AurKA inhibition might affect similar downstream programmes, with both inducing polyploidization of committed, but not uncommitted, MK progenitors.[7] Future studies are needed to determine the extent to which kinase activities and downstream cellular responses to CDK8/19 and AurKA overlap.

Whilst there was limited overlap between transcriptomic changes induced by CA treatment and the genes differentially regulated in adult versus CB-MKs, CA treatment impacted some pathways that are differentially regulated in adult versus CB-MKs (e.g., those associated with haemostasis, platelet activation, and signalling). These findings were consistent with known developmental differences in platelet function.[11,12] CA effects on PI3K/AKT and mTOR pathways were also consistent with reports comparing neonatal versus adult MKs and platelets.[1,13] We further identified significant enrichment of the phospholipase D pathway from differentially regulated genes

in CA-treated CB-MKs as well as in adult versus neonatal MKs. Phospholipase D supports platelet formation via actin modulation in vivo. Its activities as a guanine exchange factor (GEF) impact metabolic and GTPase signalling.[14] This may be a novel pathway contributing to differences between adult and neonatal MKs and platelets. CA treatment also significantly affected genes that regulate inflammatory responses, including TNFα signalling via NFκB. This was consistent with our recent report detailing developmental differences in immune/inflammatory protein content in adult versus neonatal platelets,[13] and another showing that CDK8/19 inhibition suppresses NFκB-mediated gene induction in response to TNFα.[15]

Mechanisms underlying the transition from mitosis to endomitosis in CA-treated CB-MKs are likely complex. CA treatment of CB-MKs induced an initial (8 h) downregulation of cell cycle pathways, followed by an upregulation of the same pathways after 96 h similar to that observed in adults compared with CB-MKs (Figure S4). The reasons for these opposite time-dependent effects are unknown. In SET-2 cells, STAT1-S727 phosphorylation by CDK8 enhances cell expansion by restraining the expression of a subset of cell identity genes.[3] Blocking STAT1-S727 phosphorylation in CB-MKs increased the expression of the same set of STAT1 targets as in SET-2 cells. However, other CA targets also likely contribute to the phenotype changes. Consistent with the effects of CDK8/19 regulating mediator activity and thus TF binding and transcription of Pol II genes, we observed that CA-induced genes were enriched for targets of major MK transcription factors including RUNX1 and GATA1, which are known to be involved in the regulation of MK polyploidization.

In summary, we report a novel effect for CDK8/19 inhibition in CB-MKs inducing a phenotypic change into polyploid MKs and altering the expression of genes involved in cell cycle regulation and of MK TF targets. Whilst some of these transcriptional changes match developmental differences between PB and CB-MKs, CA does not fully trigger an ontogenic switch at the molecular level. Nevertheless, features of neonatal MKs contribute to the pathogenesis of developmental MK/platelet disorders that uniquely or more severely affect neonates and infants (i.e. Down syndrome transient myeloproliferative disorder and thrombocytopenia-absent radius syndrome), and to delayed platelet engraftment following CB-derived stem cell transplantation.[1] Further studies are needed to determine whether CDK8/19 represents a new target for the management of these disorders.

AUTHOR CONTRIBUTIONS

Z-JL, CT, IIN, HEP, RT-M, and FF-M performed the research and analysed the data. Z-JL, LMR, FF-M, MDS, and MS-V designed the research study and contributed essential reagents or tools. CT and MS-V wrote the article.

FUNDING INFORMATION

This study was supported by the NIH (K99-HL156052 to CST, R01-HL69990, and P01-HL046925 to MSV).

CONFLICT OF INTEREST STATEMENT

MDS is a founder and paid advisor to Nuvalent Inc. HEP was an employee of Harvard University at the time of this research.

DATA AVAILABILITY STATEMENT

Microarray data are available through NCBI (GSE264379, GSE264381, and GSE264384).

ETHICS APPROVAL STATEMENT

Cord blood samples were obtained during healthy full-term scheduled caesarean section deliveries at Beth Israel Deaconess Hospital, Boston, with Institutional Review Board (IRB) approval and in accordance with the Declaration of Helsinki. Adult $CD34^+$ cells were purchased from the Fred Hutchinson Cancer Centre.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Liu Z-J, Thom C, Nitulescu II, Pelish HE, Rimsza LM, Teruel-Montoya R, et al. CDK8/19 inhibition triggers a switch from mitosis to endomitosis in cord blood megakaryocytes. Br J Haematol. 2024;205(6):2481–2486. https://doi.org/10.1111/bjh.19836