STIMULUS REPORT

blood advances

Inhibition of EZH2 and immune signaling exerts synergistic antitumor effects in chronic lymphocytic leukemia

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Key Points

- Microenvironmental stimuli affect EZH2 expression and function in CLL.
- Combined B-cell signaling and EZH2 inhibition showed synergistic effects on primary CLL cells.

Introduction

Enhancer of zeste homolog 2 (EZH2) is the catalytic subunit of the polycomb repressive complex 2 (PRC2), which induces gene repression through trimethylation of histone H3 at lysine 27 (H3K27me3).[1] Evidence suggests that EZH2 overexpression is associated with clinical aggressiveness in chronic lymphocytic leukemia (CLL), conferring a survival advantage to the malignant cells.[2-4] Interestingly, ex vivo inhibition of EZH2 enzymatic activity in primary CLL cells by drugs[5,6] induced downregulation of H3K27me3 levels, leading to increased cell apoptosis.[2]

Microenvironmental interactions of CLL cells with bystander cells mediated by the B-cell receptor (BcR), Toll-like receptors (TLRs), and CD40, among others,[7,8] are also critical for CLL development and progression.[9] This scenario is reinforced by the remarkable therapeutic efficacy of the Bruton tyrosine kinase inhibitor ibrutinib and the phosphatidylinositol 3-kinase δ inhibitor idelalisib in CLL.[10-12] However, despite their overall high efficacy, these drugs only rarely induce complete clinical responses when used as monotherapy, underscoring the existence of additional relevant processes that may underlie resistance and suboptimal outcomes.[13-16] Considering the significant role of external triggering in promoting CLL cell survival and proliferation,[17] and the emerging role of EZH2 in these processes,[18] the current article explored potential links between microenvironmental stimuli and EZH2 expression and whether synergism may exist between EZH2 and signaling inhibitors ex vivo in primary CLL cells.

Methods

Blood samples were collected under informed consent from patients diagnosed with CLL according to the International Workshop on Chronic Lymphocytic Leukemia/National Cancer Institute guidelines.[19] The study was approved by the local ethics committee of CERTH (decision on 18 August 2014) and conducted in accordance with the Declaration of Helsinki.

Clinicobiological data for the patient cohort are given in supplemental Table 1. $CD19^+$ B cells were negatively selected from whole blood and cultured in the presence or absence of specific ligands for certain time points, depending on the assay. Quantification of EZH2 messenger RNA (mRNA) levels was achieved by real-time quantitative polymerase chain reaction. Protein expression of p-PLCγ2, EZH2, and H3K27me3 was assessed by using western blotting and/or flow cytometry. Cell viability, Bcl-2, Bcl-xl, Mcl-1, cleaved poly-ADP ribose polymerase (PARP), and cleaved caspase-3 were assessed by using flow cytometry. Purified CLL cells were cultured in the presence of the EZH2 inhibitors GSK126 and GSK343, and/or ibrutinib, idelalisib, and venetoclax, and the combination index, cell viability, and H3K27me3 levels were measured. Coculture system experiments of purified CLL cells with stromal HS-5 cells were performed in the presence of GSK126. Detailed information about the methodology is provided in the supplemental Methods.

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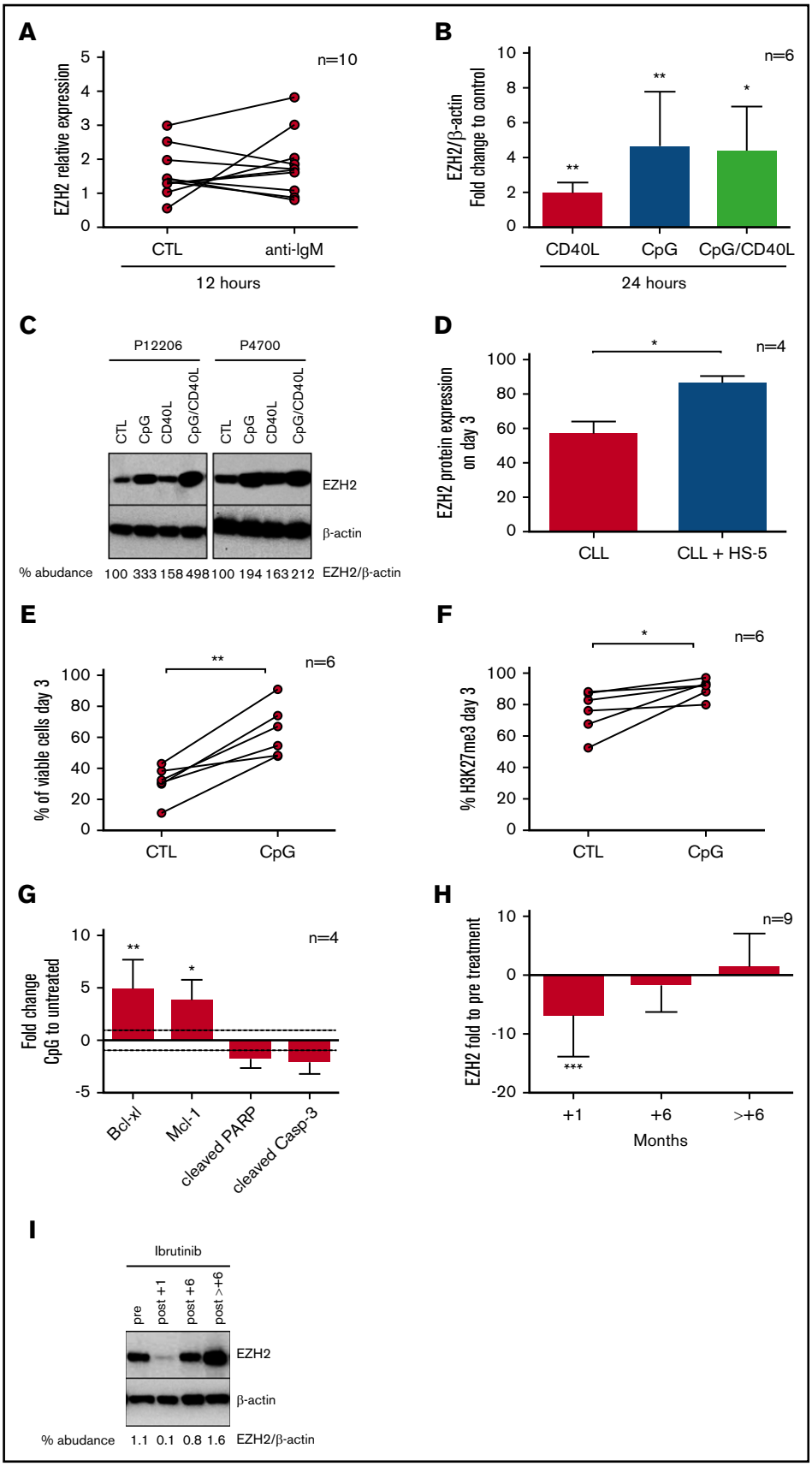

Figure 1. External stimuli and ibrutinib treatment modulate EZH2 expression. (A) EZH2 mRNA analysis using real-time quantitative polymerase chain reaction of 10 CLL cases after BcR stimulation for 12 hours. In 5 of 10 cases, EZH2 was upregulated (FC = 1.7; $P < .05$), whereas the remaining were downregulated (FC = 1.4; $P < .01$). In the graph, 2 connected points represent EZH2 relative expression in 2 different conditions for each patient. (B) Each bar in the graph shows the mean values of the FC of EZH2 protein expression (as analyzed by using western blotting) in cells stimulated with CD40L and/or CpG for 12 hours, normalized to unstimulated control cells. Asterisks indicate significant differences compared with the unstimulated control. (C) Immunoblotting analysis of EZH2 protein expression and β-actin of 2 representative cases. (D) Each bar in the graph shows the mean values with standard deviation of EZH2 expression in CLL cells alone or CLL cells cocultured with HS-5 cells for 3 days (n = 4, FC = 1.5; $P < .05$). CLL cell viability analysis (E) and H3K27me3 levels (F) at day 3 after TLR9 stimulation, using flow cytometry. In the graph, connected points represent the percentage of viable cells or H3K27me3 levels in 2 conditions: unstimulated control (CTL) and the cells stimulated with CpG (n = 6; FC = 2.1, $P < .01$; FC = 1.2, $P < .05$). (G) Each bar in the graph shows the mean values of FC of Bcl-xl, Mcl-1, cleaved PARP, and cleaved caspase-3 of stimulated CLL cells with CpG for 3 days, compared with unstimulated control cells. Asterisks indicate significant differences compared with the unstimulated control (n = 4, FC = 4.8, $P < .01$; FC = 3.7, $P < .05$; FC = −1.7 and FC = −2, respectively). (H) $CD19^+$ cells from patients before treatment initiation with ibrutinib and at 1, 6, and >6 months under treatment were lysed for western blotting analysis of EZH2 protein expression. Each bar in the graph represents the median values with range of EZH2/β-actin protein expression normalized to the pretreatment sample (n = 9, FC = 4.3, $P < .001$). (I) Immunoblotting analysis of EZH2/β-actin levels for 1 representative case. *$P < .05$, **$P < .01$, ***$P < .001$. IgM, immunoglobulin M.

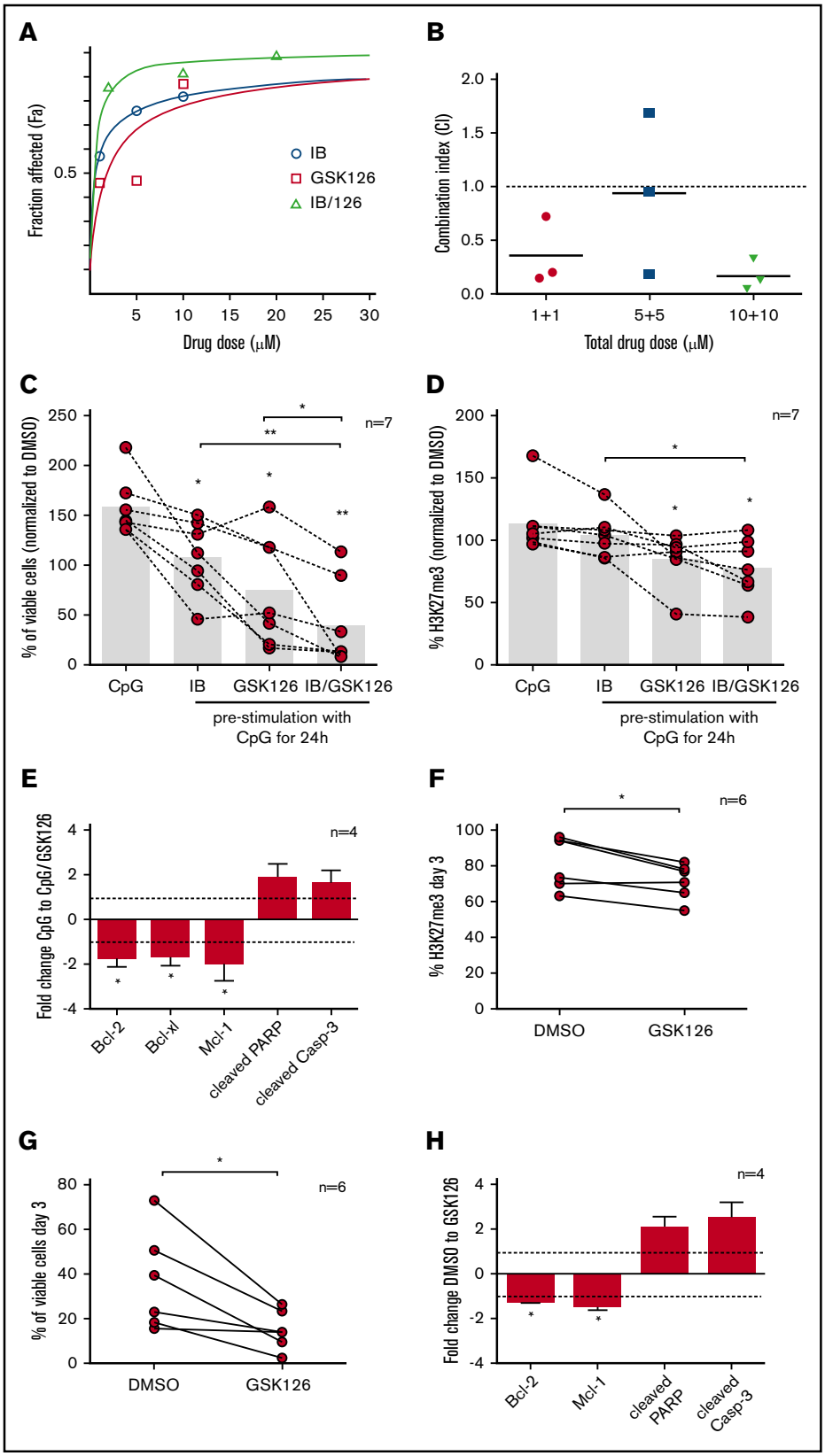

Figure 2. Synergistic effects of ibrutinib (IB) plus GSK126 in primary CLL cells. (A) Negatively selected CD19^{+} cells from 3 CLL cases were assessed for cell viability after prestimulation with CpG for 24 hours, followed by single IB or GSK126 treatment (1, 5, and 10 μM) or combined treatment with these drugs in a 1:1 ratio at the aforementioned doses for a total of 72 hours. In the graph, the y-axis depicts the fraction affected values and the x-axis depicts either the single drug dose treatment (blue and

Results and discussion

To explore the impact of BcR stimulation on EZH2 expression, we stimulated $CD19^+$ B cells from 10 selected BcR-responsive CLL cases (supplemental Figure 1A-D) with anti–immunoglobulin M for 12 hours (supplemental Figure 1E) and observed that EZH2 mRNA expression was variably affected (Figure 1A). We next investigated the potential impact of other microenvironmental signals and found that in CLL cases responsive to TLR9/CD40 activation (supplemental Figure 1F), EZH2 mRNA expression did not change significantly (fold change [FC] = 1.2) with soluble CD40L (CD40L), whereas TLR9 stimulation with cytosine-phosphorothioate-guanine oligodeoxynucleotides (CpG) or costimulation with CpG/CD40L caused pronounced upregulation of EZH2 mRNA levels (FC = 8.9 [$P < .05$] and FC = 10.5 [$P < .05$], respectively) (supplemental Figure 1G). Similar results were obtained at the protein level in cells treated with CD40L (FC = 1.9; $P < .01$), CpG (FC = 4.6; $P < .01$), and CpG/CD40L (FC = 4.3; $P < .05$); the strongest effect was observed after TLR9 stimulation (Figure 1B-C). Moreover, coculture of CLL cells with the HS-5 stromal cell line for 3 days induced EZH2 protein expression (FC = 1.5; $P < .05$) (Figure 1D). Overall, these findings support the theory that EZH2 expression can be regulated by signals emanating from the tumor milieu. This outcome is in line with observations showing that EZH2 expression is significantly upregulated in the proliferation centers of CLL/SLL lymph nodes[18] in which malignant cells receive multiple signals from bystander cells.[17]

Focusing on TLR9, we observed that TLR9-induced EZH2 upregulation was accompanied by a significant increase in H3K27me3 levels (FC = 1.2; $P < .05$), cell viability (FC = 2.1; $P < .01$), and the expression of the antiapoptotic Bcl-xl (FC = 4.8; $P < .01$) and Mcl-1 (FC = 3.7; $P < .05$), along with a concomitant decrease in the levels of the pro-apoptotic cleaved PARP (FC = −1.7) and cleaved caspase-3 (FC = −2) (Figure 1E-G). Moreover, coculture of CLL cells with HS-5 cells induced CLL viability (FC= 1.6; $P < .05$) and H3K27me3 levels (FC = 1.8; $P < .05$) (supplemental Figure 2A-D). Hence, EZH2 seems to be implicated in the microenvironmentally induced antiapoptotic response in CLL that involves regulation of apoptotic regulators, in line with the literature.[20,21]

We then studied the effects of ibrutinib on EZH2 protein expression in longitudinal CLL samples from patients under treatment and found significantly reduced EZH2 levels (FC = 3; $P < .001$) at 1 month; however, at later time points, residual CLL cells expressed EZH2 at levels similar to baseline (Figure 1H-I; supplemental Figure 3A), likely reflecting the fact that they retain the ability to proliferate, as already shown by us and others.[2,18] Relative to this, it was recently shown that during ibrutinib therapy, TLR can cooperate with BcR signaling to enhance CLL proliferation and survival, particularly in microenvironmental niches.[22] On these grounds, we argue that targeting both immune signaling and EZH2 might prove a potentially beneficial treatment strategy for CLL.

To explore potential synergism of signaling and EZH2 inhibitors, cells were pre-stimulated through TLR9 for 24 hours ex vivo and then exposed to single or combined treatment with ibrutinib + GSK126 (supplemental Figures 3 and 4), revealing synergistic effects (Figure 2A-B). We then explored synergistic effects in an extended group of patients and found that treatment of TLR9-stimulated CLL cells with ibrutinib + GSK126 induced a significant decrease in leukemic cell viability compared with isolated inhibition (FC < 1.2 and $P < .05$ for all comparisons); concordant results were obtained regarding H3K27me3 levels (Figure 2C-D). This finding was also accompanied by a significant reduction in the antiapoptotic proteins Bcl-2, Bcl-xl, and Mcl-1 (FC = −1.7, FC = −1.6, FC = −1.9; $P < .05$) and an increase in the levels of the apoptosis mediators, cleaved PARP and cleaved caspase-3 (FC = 1.8, FC = 1.6) (Figure 2E).

Similar experiments were also performed with ibrutinib, idelalisib, and/or another EZH2 inhibitor (GSK343) and with ibrutinib, GSK126, and/or the BCL-2 inhibitor venetoclax. We found that all combinations induced a comparable significant viability reduction compared with the control cells that were not exposed to any drug but also to cells exposed to single inhibitors. Moreover, synergism was confirmed between GSK126 and venetoclax (supplemental Figure 5). Interestingly, cases with low EZH2 levels that were unresponsive to TLR9 stimulation showed no significant differences after single vs combined inhibition (supplemental Figure 6). Additional experiments found that EZH2 inhibition is also effective in the stromal coculture system (supplemental Figure 7). Our results indicate that the identified synergism is likely linked to a particular functional status shaped by immune activation and the resultant EZH2 induction, further emphasizing the value of targeting distinct albeit interconnected mechanisms and pathways contributing prosurvival and drug escape signals to the CLL clone.

Finally, using the EZH2 inhibitors (GSK126 and GSK343), we treated primary cells from samples collected at the time of lack of

Figure 2. (continued) red labels) or the total drug dose after combinational treatment (green label). All dose–response curves plotted in the graph concern 1 representative case. (B) The interaction between IB and GSK126 at a 1:1 ratio in graded concentrations (1-10 μM) was synergistic (combination index [CI] values <1) in primary CLL cells. Scatter plots in the graph present the mean values of the CI of 3 CLL cases. Effects of incubation with CpG for 24 hours after single or combined treatment of 5 μM IB and/or 10 μM GSK126 for 3 days on CLL cell viability (C) and H3K27me3 levels (D) as measured by using flow cytometry. Connected points in the graphs represent the percentage of viable cells or H3K27me3 levels for each case in all conditions described, normalized to control cells (dimethyl sulfoxide [DMSO]–treated). The bars in the graphs show the mean values. Asterisks above bars indicate significant differences compared with CpG-treated cells (n = 7). (E) Each bar in the graph shows the mean values of FC of Bcl-2, Bcl-xl, Mcl-1, cleaved PARP, and cleaved caspase-3 (Casp-3) of stimulated cells with CpG for 24 hours after treatment with 10 μM GSK126 for 3 days, compared with CpG-stimulated control cells. Asterisks indicate significant differences compared with the CpG-stimulated control cells (n = 4; FC = −1.7, $P < .05$; FC = −1.6, $P < .05$; FC = −1.9, $P < .05$; FC = 1.8; and FC = 1.6, respectively). Effects of incubation with 10 μM of the EZH2 inhibitor GSK126 for 3 days on levels of H3K27me3 (F) and on CLL cell viability (G) using flow cytometry. Connected points in the graphs represent the percentage of H3K27me3 or viable cells treated with GSK126 or DMSO-treated control cells (n = 6). (H) Each bar in the graph shows the mean values of FC of Bcl-2, Mcl-1, cleaved PARP, and cleaved caspase-3 (Casp-3) of cells treated with 10 μM GSK126 for 3 days compared with DMSO-treated cells. Asterisks indicate significant differences compared with control cells (DMSO-treated) (n = 4; FC = −1.2, $P < .05$; FC = −1.4, $P < .05$; FC = 2; and FC = 2.5). *$P < .05$, **$P < .01$.

response in CLL cases resistant to ibrutinib or idelalisib (supplemental Table 1). We found a statistically significant ($P < .05$) decrease in H3K27me3 levels and CLL cell viability (Figure 2F-G; supplemental Figure 8) accompanied by a significant decrease in the antiapoptotic regulators Bcl-2 and Mcl-1 (FC = −1.2, FC = −1.4; $P < .05$) and an increase in the proapoptotic proteins cleaved PARP and cleaved caspase-3 (FC = 2, FC = 2.5) (Figure 2H).

In conclusion, EZH2 expression seems to be regulated by signals from the CLL microenvironment, whereas the combination of EZH2 and immune signaling inhibitors has synergistic antitumor effects. Moreover, EZH2 inhibitors are effective in primary CLL cells from cases with suboptimal response to immune signaling inhibitors, further highlighting that EZH2 might represent a rational choice for combination treatment of CLL, capable of improving the efficacy/quality of responses to signaling inhibitors.

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Authorship

Contribution: E.C., S.N., N.P., and K.S. were responsible for conception and design; E.C., S.N., K.K., A.R., M.A.A., D.B., and E.F. developed the methods; N.S., A.A., P.G., R.R., and K.S. acquired the data (ie, provided patient samples, provided facilities); E.C., S.N., and N.P. analyzed and interpreted the data (eg, statistical analysis); E.C., S.N., P.G., R.R., T.T., N.P., and K.S. wrote, reviewed, and/or revised the manuscript; and N.P. and K.S. supervised the study.

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