Inhibition of BCR::ABL1 tyrosine kinase activity Aids in the Generation of Stable Chronic Myeloid Leukemia Induced Pluripotent Stem Cells

Esther Sathya Bama Benjamin[1, 2], Dinesh Babu[3*], Gaurav Joshi[1,2*], Bharathi M Rajamani[1], Krittika Nandy[3], Sonam Rani[3], Senthamizhselvi Anandan[1], Chitra Premkumar[3], Madhavi Maddali[1], Aby Abraham[1], Shaji R Velayudhan[1,3 #], Poonkuzhali Balasubramanian[1#]

[1] Department of Haematology, Christian Medical College- Vellore
[2]Sree Chitra Tirunal Institute for Medical Sciences & Technology (SCTIMST), Thiruvananthapuram
[3]Centre for Stem Cell Research (a unit of INSTEM, Bengaluru), Christian Medical College, Vellore
Corresponding authors
* Equal contribution

Address for correspondence
Dr. Shaji R Velayudhan
rvshaji@cmcvellore.ac.in
and
Dr. Poonkuzhali Balasubramanian
bpoonkuzhali@cmcvellore.ac.in

Department of Haematology Christian Medical College Vellore-632004, and
Centre for Stem Cell Research (a unit of INSTEM, Bengaluru)

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Abstract

Induced pluripotent stem cells (iPSCs) generated from patients with chronic myeloid leukemia (CML) have the potential for disease modeling to study disease pathogenesis and screening therapeutic interventions. In this study, we aimed to generate iPSCs from $CD34^+$ hematopoietic progenitors of CML patients with varying responses to tyrosine kinase inhibitor (TKI) therapy. The generated CML-CD34-iPSC colonies displayed atypical "dome-shaped" morphology and underwent spontaneous differentiation in a few days. However, supplementation with imatinib (IM), the most widely used TKI to treat CML patients, in the culture medium improved the stability and maintenance of all isolated CML-CD34-iPSC colonies, allowing them to be maintained for more than 20 passages without significant differentiation. In contrast to previous studies, our results indicate that suppressing the BCR::ABL1 oncogenic pathway is essential for efficiently generating stable CML-iPSC colonies. Furthermore, we successfully differentiated these iPSCs to $CD34^+$ hematopoietic progenitors both in the presence and absence of IM. This robust protocol for generating CML-iPSCs provides a valuable resource for disease modelling. The generated iPSCs will be a valuable tool for investigating CML pathophysiology, drug resistance mechanisms, and drug screening to identify novel and effective therapies for this disease.

Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm that arises from hematopoietic stem cells (HSCs) transformed by BCR::ABL1 oncogenic fusion protein with constitutive serine/threonine kinase activity expressed in these cells. BCR::ABL1 triggers various downstream signaling pathways, including RAS/RAF/MEK/ERK, JAK2/STAT, and PI3K/AKT/mTOR pathways, leading to the accumulation of granulocytes at various stages of myeloid maturation (1,2). Although targeted therapy with tyrosine kinase inhibitors (TKIs), such as imatinib (IM), dasatinib (DA), and nilotinib (NIL), has dramatically improved the long-term survival of CML patients, only around 50% of patients achieve treatment-free remission (TFR) (3). The persistence of leukemic stem cells (LSCs) is one of the primary causes of disease recurrence (4). Due to the rarity of the LSC population, it is challenging to obtain

78 sufficient cells for research to understand the mechanisms of TKI resistance of these
79 cells and screen drugs to eradicate them (4,5).

80 Induced pluripotent stem cells (iPSCs) derived from patients with genetic
81 diseases or cells with genetic alterations can be differentiated into scalable quantities
82 of disease-relevant differentiated cells. Thus, they are a powerful tool for studying
83 disease pathogenesis and drug screening (5). iPSCs generated from patients with
84 leukemias help understand how oncogenes and patient-specific chromosomal
85 abnormalities influence the development of leukemia-like phenotypes *in-vitro* (6).
86 CML-iPSCs generated from the same patient at various stages of the disease and from
87 patients with different responses to TKI therapy are valuable resources for
88 investigating molecular and epigenetic mechanisms of CML progression and drug
89 resistance, leading to the identification of novel therapeutic targets and drug
90 candidates for the treatment of CML (7,8). Moreover, iPSCs can be efficiently
91 genetically manipulated by gene editing methods to identify specific genes involved in
92 disease pathogenesis (9).

93 Previous studies have demonstrated the successful generation of CML-iPSCs
94 from peripheral blood mononuclear cells (10) and $CD34^+$ hematopoietic stem and
95 progenitor cells (HSPCs) isolated from CML patients (7,11–13). CML-iPSCs exhibit
96 resistance to IM like CML-LSCs, so they are considered suitable for investigating the
97 cellular mechanisms underlying IM resistance (12,14,15). CML-iPSCs could be
98 differentiated into hematopoietic progenitors that recapitulated some of the
99 pathophysiologic features of the disease (7,10,12–14). However, it remains unclear
100 how *BCR::ABL1* expression and TKI treatment impact the efficiency and quality of iPSC
101 generation from CML cells.

102 In this study, we describe the generation of iPSCs from the $CD34^+$ cells of CML
103 patients who exhibited varying responses to TKI therapy for disease modeling and
104 investigating the effect of BCR::ABL1 oncoprotein expression on the maintenance of
105 CML-iPSCs. In contrast to previous studies, we found that the expression and activity
106 of BCR::ABL1 affect the maintenance of CML-CD34-iPSCs, and it was essential to
107 suppress the TKI activity of BCR::ABL1 fusion protein by TKI supplementation for their
108 survival and maintenance. The CML-iPSCs generated using our protocol could be
109 differentiated into BCR::ABL1 positive hematopoietic progenitors. The patient-specific

110 CML-iPSCs and the differentiated hematopoietic cells generated by this method have
111 a wide range of applications, including high throughput CRISPR-Cas9 screening, to
112 identify novel pathways involved in CML pathogenesis and drug resistance.
113

114 Materials and methods

115 **Ethical statement:** This study was approved by the institutional review board and the
116 institutional bio-safety committee of Christian Medical College, Vellore.
117 **Isolation and expansion of CD34⁺ cells:** Peripheral blood samples were obtained
118 from a healthy donor (N=1), who underwent mobilization of CD34⁺ cells for
119 hematopoietic transplantation, and newly diagnosed chronic phase-CML (CP-CML)
120 patients (N=4) after obtaining written informed consents. Peripheral blood
121 mononuclear cells (PBMNCs) were isolated using Ficol density gradient centrifugation.
122 The CD34⁺ cells were purified from PBMNCs by the magnetic separation method
123 (EasySep, Stem Cell Technologies) and were cryopreserved. The cryopreserved
124 CD34⁺ cells of the CML patients and the normal donor were thawed and cultured in
125 CD34⁺ cell expansion medium constituting StemSpan™ SFEM II medium (Stem Cell
126 Technologies) supplemented with CD34⁺ Expansion Supplement and UM729 (Stem
127 Cell Technologies). The cells were cultured for 6-7 days with half medium change
128 every alternative day to assess the cell proliferation.
129 **Reprogramming of CML and normal CD34⁺ cells:** The CD34⁺ cells were cultured in
130 the expansion medium for 3-days and then nucleofected with episomal
131 reprogramming plasmids (pCXLE-hOCT3/4-shp53 (Addgene: 27077), pCXLE-hSK
132 (Addgene 27078) and pCXLE-hUL (Addgene: 27080) (kind gift from Shinya
133 Yamanaka), as described previously (16). Electroporation was carried out using Neon
134 Transfection System (ThermoFisher) at 1600V, 10ms, and 3 pulses. After five days of
135 culture, the nucleofected cells were transferred to Matrigel-coated plates containing
136 Essential-8 (E8) medium (ThermoFisher Scientific). Individual colonies were hand-
137 picked between day 21 and day 32 and were passaged and maintained in mTeSR Plus
138 medium (StemCell Technologies) supplemented with 10μM IM (Sigma Aldrich) to
139 generate stable iPSC clones.
140 **Western Blot:** The iPSCs were dissociated using 1mM EDTA (ThermoFisher
141 Scientific), and the protein lysates were prepared in radioimmunoprecipitation assay

142 buffer supplemented with a protease inhibitor mixture (Roche Applied Science, IN,
143 USA) and 2mM phenylmethylsulfonyl fluoride (Sigma-Aldrich). About 30µg of whole
144 cell lysate was loaded in 10% SDS-polyacrylamide gel, and the proteins were
145 transferred to a polyvinylidene difluoride membrane. The membrane was blocked with
146 10% non-fat dry milk powder in tris-buffered saline containing 0.1% Tween 20 (TBST)
147 for two hours and then incubated with an anti-phosphoCRKL antibody (Cell Signaling
148 Technology) and Anti β –Actin antibody (ThermoFisher Scientific). The proteins were
149 detected using the ECL chemiluminescence kit (Thermo Scientific, Pierce), and the
150 images were captured using the FluorChem E system (Protein Simple).
151 **Analysis of *BCR::ABL1* fusion transcripts:** RNA was extracted from patients'
152 PBMNCs and iPSCs using TRI Reagent (Sigma-Aldrich). cDNAs were prepared from
153 2 µg RNA with random hexamers using the High-Capacity cDNA Synthesis Kit (Thermo
154 Scientific), followed by RT-PCR to identify the *BCR::ABL1* fusion transcripts using a
155 previously described protocol (17). The *BCR::ABL1* mRNA copies were assessed
156 using an in-house ddPCR assay (Datari et. al., manuscript under preparation). iPSC
157 colonies were screened for BCR::ABL1 kinase domain mutations using a previously
158 described protocol (18).
159 **Alkaline phosphatase staining:** Alkaline Phosphatase staining was performed using
160 the Alkaline Phosphatase Detection Kit (Sigma-Aldrich) following the manufacturer's
161 instructions.
162 **Flow cytometry**: To analyze the expression of TRA-1-60 in iPSCs, the colonies were
163 dissociated using TrypLE (Thermo Fisher Scientific) and then stained using a PE-
164 conjugated TRA-1-60 antibody (Thermo Fisher Scientific) for 20 mins. The cells were
165 washed with mTeSR Plus (Stem Cell Technologies) containing Revitacell
166 (Thermo Fisher Scientific), and the expression was analyzed using Navios (Beckman
167 Coulter) or FACS Aria (BD Biosciences) flow cytometers. To measure the purity of
168 CD34+ cells after their magnetic separation from PBMNCs, the cells were stained with
169 an APC-conjugated anti-CD34 antibody (BioLegend).
170 **Immunofluorescence:** Immunofluorescence for pluripotency markers, such as SOX2,
171 TRA-1-81, OCT4A, SSEA-4, NANOG and TRA-1-60, was performed as previously
172 described (19).

173 **Trilineage differentiation:** Trilineage differentiation of iPSCs to ectoderm, endoderm
174 and mesoderm lineages was carried out using STEMdiff tri-lineage differentiation Kit
175 (Stem Cell Technologies) as per the manufacturer's protocols.
176 **Hematopoietic differentiation of iPSCs:** For the hematopoietic differentiation of
177 iPSCs, we used the STEMdiff™ Hematopoietic Kit (Stem Cell Technologies). Briefly,
178 iPSCs were either single-cell sorted or seeded as small aggregates in mTeSR Plus
179 (Stem Cell Technologies). Subsequently, the culture medium was changed to
180 STEMdiff™ A medium, with or without IM. After three days, 70,000 cells were reseeded
181 in STEMdiff™ A medium and maintained for another three days. Following this, the
182 medium was switched to STEMdiff™ B. After 12 days of culture, the cells present in
183 suspension were harvested and analyzed by flow cytometry for the expression of
184 hematopoietic surface markers.
185 **Colony-Forming Assay:** In each experiment, 7,000 hematopoietic progenitors
186 generated from iPSCs were plated onto MethoCult GF H4636 medium (Stem
187 Cell Technologies). The number of colonies in each experiment was scored on day 14
188 following the manufacturer's instructions.
189

190 ## Results

191

192 **Generation of iPSCs from normal and CML CD34$^+$ cells:**

193 As CD34$^+$ cells of CML patients are known to constitute the leukemia
194 stem/progenitor cells (LSPCs) that are associated with disease recurrence (4,20), we
195 aimed to reprogram CML-CD34$^+$ cells from two patients who achieved complete
196 cytogenetic response and one who achieved suboptimal response (>1% *BCR::ABL1*
197 transcripts detected in peripheral blood cells after 12months) after TKI therapy (**Suppl**
198 **Table 1**) and a healthy volunteer as described (**Figure 1A**). CD34$^+$ cells purified from
199 CML patients and those from a healthy donor were cultured in CD34$^+$ expansion
200 medium. The number of CD34$^+$ cells increased by 15 to 20-fold (Mean: 20.2±:3.34)
201 without reducing the percentage of CD34$^+$ cells (**Figure S1A** and **S1B**).

202 We nucleofected proliferating CD34$^+$ cells from day 3 of the culture with
203 episomal reprogramming plasmids (13). The normal CD34$^+$ cells gave rise to iPSC
204 colonies with typical morphology between day 14 and day 18 (**Figure 1B**). These

205 colonies were hand-picked between day 20 and day 24 to generate stable iPSC
206 clones. The normal iPSC clones maintained typical iPSC morphology without
207 differentiation for 12 passages and expressed high levels of pluripotency markers
208 (**Figures 1C** and **1D**). In contrast, $CD34^+$ cells from TKI-responsive CML patients did
209 not form typical iPSCs but formed several "dome-shaped" colonies after 30 days
210 (**Figures 1B and C**). These colonies were surrounded by adherent cells (**Figure 1C)**
211 that resembled those formed by the spontaneous differentiation of iPSCs. We isolated
212 11 “dome-shaped” colonies from 3 patients and cultured them for 4 to 5 passages.
213 They also expressed TRA-1-60 in the initial passages (**Figure 1D**). During subsequent
214 passages, the "dome-shaped" colonies peeled off from the bottom of the plates and
215 floated in the medium, leaving the differentiated cells attached to the plate.
216

217 **IM supplementation aids in the maintenance, stability, and pluripotency of CML**
218 **iPSCs**

219 Previous studies showed that CML-iPSCs express *BCR::ABL1* transcripts
220 present in the donor cells used for reprogramming (10–12,14). In our study, the
221 "dome-shaped" CML-CD34-iPSC colonies also expressed the same types of
222 *BCR::ABL*1 fusion transcripts (e13a2 and e14a2) as those present in the respective
223 patients' peripheral blood cells used for reprogramming (**Figure S2A** and **S2B**).
224 Additionally, karyotyping confirmed the presence of t(9;22) in all iPSC clones (**Figure**
225 **S2C**), and none of the clones had mutations in the *BCR::ABL1* kinase domain.

226 As it has been reported that TKI treatment enhances pluripotency marker
227 expression in BCR::ABL1+ iPSC clones (15), we hypothesized that the atypical
228 morphology and high rate of spontaneous differentiation observed in our CML-CD34-
229 iPSC colonies were due to the constitutive expression of BCR::ABL1 fusion protein
230 and its tyrosine kinase (TK) activity. To inhibit the TK activity of the BCR::ABL1 fusion
231 protein, we supplemented the medium with 10μM IM and monitored the colonies for
232 changes in their morphology characteristics. We observed that within two days of IM
233 supplementation, the colonies gained the typical iPSC morphology (**Figure 2A**) with
234 an increase in the TRA-1-60+ cell population (**Figures 2B** and **2C**). All the clones were
235 positive for pluripotency marker expression (**Figure 2D**). They also could be
236 differentiated into three germ layers, ectoderm, endoderm, and mesoderm (**Figure**

237 **S3**). When IM was withdrawn, the IM-supplemented CML-CD34-iPSC colonies
238 reverted to the "dome-shaped" morphology within 2-4 days (**Figure 3A**), with a
239 reduction in cell viability (**Figure 3B**) and TRA-1-60 expression (**Figure 3C).** In
240 contrast, when the control iPSCs were treated with IM, there was a reduction in the
241 colony number, suggesting that IM supplementation specifically enhances the
242 maintenance of CML-CD34-iPSCs (**Figure S4**).
243 CRK-like proto-oncogene (CRKL) is a downstream target of BCR::ABL1 and is
244 phosphorylated by BCR::ABL1 fusion protein (20,21). As p-CRKL levels can be used
245 to determine the status of BCR::ABL1 kinase activity, the expression of p-CRKL was
246 analyzed in CML-CD34-iPSCs before and after the IM treatment (**Figure 3D**). It was
247 found that p-CRKL expression was decreased in CML-CD34-iPSCs after IM treatment,
248 suggesting that suppression of BCR::ABL1 mediated tyrosine phosphorylation resulted
249 in the stable maintenance of CML-CD34-iPSCs.
250
251 **The effect of IM in reprogramming of CML-CD34+ cells**
252 As IM supplementation inhibits BCR::ABL1 kinase activity and enhances the
253 stability and pluripotency of CML-iPSCs, we investigated the effect of IM on the
254 reprogramming of CML-CD34$^+$ cells. After nucleofection of CD34+ cells from two IM-
255 responsive patients, the cells were cultured in the reprogramming medium
256 supplemented with 10μM IM (**Figure 4A**). Reprogramming without IM supplementation
257 generated "dome-shaped" colonies after 3 weeks, and they gained typical iPSC
258 morphology after IM supplementation with increased TRA-1-60 expression (**Figure**
259 **4B**). However, continuous IM supplementation throughout reprogramming did not
260 generate "dome-shaped" colonies or colonies with typical morphology. These findings
261 suggest that while IM enhances the stability of CML-iPSCs by inhibiting *BCR::ABL1*
262 kinase activity, it impedes the reprogramming of CML-CD34$^+$ cells. Overall, our results
263 demonstrate that IM supplementation transforms the "dome-shaped" colonies formed
264 during the reprogramming of CML-CD34$^+$ cells into iPSCs with typical flat morphology.
265 Furthermore, IM improves the pluripotency of CML-CD34-iPSCs by inhibiting the
266 BCR::ABL1-mediated TK activity.
267
268 **Reprogramming of CD34$^+$ cells from an IM refractory CML patient**

269 The HPCs derived from the iPSCs generated from IM refractory CML patients
270 offer a valuable tool to investigate the mechanisms underlying IM therapy resistance
271 and develop a more effective treatment for those who do not respond well to IM.
272 Therefore, we reprogrammed $CD34^+$ cells from an IM refractory patient, with and
273 without IM supplementation, during the reprogramming process (**Figure 4A**). In
274 contrast to our observation in the IM responders' cells, we observed an early
275 emergence of iPSC colonies from the IM-refractory patient's cells (~3 weeks Vs. ~4
276 weeks). However, reprogramming of the cells from the IM refractory patient-generated
277 more colonies with continuous IM supplementation than without IM (**Figure 4C**). We
278 isolated 9 colonies, 8 of which exhibited "dome-shaped" morphology (**Figure 1C**),
279 while 1 showed typical iPSC morphology (**Figure S5A**). Karyotyping analysis of the
280 "dome-shaped" colonies generated from the IM refractory patient demonstrated the
281 presence of t(9;22), whereas the one clone with the typical iPSC morphology showed
282 a normal karyotype (**Figure S5A** and **S5B**). These results suggest that the "dome-
283 shaped" morphology of the CML-CD34-iPSCs is because of BCR::ABL1 expression in
284 these cells.
285
286 **Second and third generation TKIs also aid in the maintenance of CML iPSCs.**
287 Second generation TKIs, DA and NIL, and third generation TKI ponatinib were
288 approved for patients who develop resistance to or are intolerant to first or second-
289 generation TKIs, respectively. These TKIs target specific mutations in the BCR::ABL1
290 fusion protein, providing more effective and durable responses in CML patients. We
291 explored the effects of second and third generation TKIs, DA, NIL, and ponatinib, in
292 the maintenance of CML-CD34-iPSCs. We replaced IM with different concentrations
293 of DA, NIL, and ponatinib and evaluated their effects on CML-CD34-iPSCs. CML-
294 CD34-iPSCs treated with these TKIs generated stable the CML-CD34-iPSCs with 2nM
295 DA, 40nM NIL, and 2nM ponatinib, while at lower concentrations, resulted in "dome-
296 shaped" colonies (**Figure 5**). Together, our results suggest that the inhibition of
297 BCR::ABL1 TK activity promotes the pluripotency of CML-iPSCs.
298
299 **Hematopoietic differentiation of CML iPSCs**

300 Previous studies have demonstrated that CML-iPSCs can be successfully
301 differentiated into hematopoietic progenitor cells (HPCs), despite the expression of
302 BCR::ABL1 oncoprotein and its TK activity in these cells (7,14,22). However, the
303 efficiency of hematopoietic differentiation of CML-iPSCs has been reported to be lower
304 than normal iPSCs (7,10), and the resulting HPCs exhibited a partial recovery of TKI
305 sensitivity (13). Considering our finding that IM supplementation is necessary for the
306 maintenance of CML-CD34-iPSCs, we investigated whether IM supplementation is
307 also required for the differentiation of CML-iPSCs into HPCs.

308 To address this question, we initially performed single-cell sorting to obtain
309 iPSC colonies with an equal number of cells, which were subsequently subjected to
310 hematopoietic differentiation (**Figures 6A and B**). We incorporated IM
311 supplementation at different stages of the differentiation process (**Figure 6B**). We
312 differentiated CML-CD34-iPSCs using two different concentrations of IM (1μM and
313 2μM) as well as without any IM supplementation. We could obtain suspension cells
314 constituting of HPCs both in the presence and absence of IM (**Figure S6**). Interestingly,
315 CML-CD34-iPSCs differentiated without IM before and after mesoderm induction (E1)
316 (**Figure 6B**) showed a 5-fold increase in the yield of $CD34^+CD45^+$ HPCs and the total
317 number of suspension cells compared to those differentiated in the presence of IM
318 throughout the entire differentiation (E3 and E5) (**Figure 6D**). Flow cytometry analysis
319 of hematopoietic markers revealed that approximately 41% of the suspension cells
320 were $CD34^+CD45^+$ HPCs (**Figure 6C**). Utilizing single-cell sorting of iPSCs, we were
321 able to generate a sufficient number of HPCs for a drug screening experiment.

322 Based on the results of this experiment, we seeded iPSCs as small aggregates
323 and then subjected differentiation with and without IM supplementation after
324 mesoderm induction (**Figure 7A)**. Notably, the CML-CD34-iPSCs differentiated
325 without IM yielded approximately 30 times more HPCs **(Figure 7C**) compared to
326 differentiation with IM supplementation. The decline in HPC differentiation observed in
327 the presence of IM can be attributed to the sensitivity of CML-iPSC-HPCs to IM. Flow
328 cytometry analysis revealed that around 50% of the suspension cells constituted the
329 $CD34^+CD45^+$ population of HSPCs (**Figure 7B)**. Furthermore, hematopoietic colony
330 formation showed that the HPCs generated without IM had more myeloid progenitors
331 compared to those derived from a normal iPSC clone (**Figure 7D**). To further validate

these findings, we differentiated CML-CD34-iPSCs derived from two additional patients **(Figure 7E**). Flow cytometry analysis showed 40% to 70% $CD34^+CD45^+$ cells in the absence of IM from these two CML-CD34-iPSCs (**Figure 7G)**. Consistently, it was observed that differentiation in the absence of IM resulted in more HPCs compared to the other experimental groups (**Figure 7F**).

Discussion

Eliminating CML-LSCs is a critical objective in the treatment of CML, as it can prevent disease recurrence, extend survival, improve quality of life, and enable curative treatments (3,4,23). However, the rarity of these cells poses a challenge in studying the molecular mechanisms that contribute to their survival. Generating iPSCs from CML $CD34^+$ cells can help overcome this hurdle. These cells can be expanded and redifferentiated into hematopoietic cells, providing a continuous supply of cells for extensive proteome, epigenome, transcriptome, and drug screening studies.

Several groups have generated CML iPSCs from PBMNCs (10) and $CD34^+$ cells (7,11,12,14) using lentiviral (14,15), retroviral (12,13), Sendai (11), or episomal vectors (7,10). However, these studies did not address the atypical morphology and low reprogramming efficiency of CML iPSCs. Additionally, the effect of BCR::ABL expression on the morphology and stabilization of CML-iPSC colonies (9) was not investigated.

We observed that the reprogramming efficiency of CML $CD34^+$ was approximately 50 times lower than the normal $CD34^+$ cells, as previously reported (7,11). One possible explanation for this low efficiency could be the presence of the BCR::ABL1 oncogene, which may disrupt the reprogramming process of CML $CD34^+$ cells (24). Furthermore, CML$CD34^+$ cells formed colonies later than normal $CD34^+$ cells, and they exhibited atypical colony morphology (14). Bedel et. al. reported that CML iPSCs had a distinct morphology resembling that of cell aggregates similar to mouse embryonic stem cells (12,14).

The instability and differentiation-prone nature of CML-CD34-iPSCs may be due to the presence of *BCR::ABL1* oncogene, which can interfere with the reprogramming factors required to generate stable iPSC colonies. Ito *et al*. demonstrated that the use

363 of molecularly targeted drugs such as IM, which can suppress the activity of the BCR-
364 ABL oncogene, can improve the transcriptional regulation of reprogramming factors
365 and enhance pluripotency marker expression (24). This study also demonstrated that
366 the expression of reprogramming factors in the BCR::ABL+ K562 cells increased
367 pluripotency marker expression after IM supplementation, confirming that oncogene
368 suppression improves the reprogramming of cancer cells. Our study confirmed these
369 findings and showed that IM supplementation was necessary to generate stable CML
370 iPSCs without spontaneous differentiation. IM supplementation improved the stability
371 and maintenance of all isolated CML-CD34-iPSC colonies, allowing them to be
372 cultured for over 20 passages without significant differentiation. The CML-CD34-iPSCs
373 cultured with IM showed increased proliferation, while IM withdrawal decreased the
374 proliferation and TRA-1-60 expression. These results were consistent with a previous
375 study that reported faster growth of CML-iPSCs in the presence of TKI (14,15).

376 The phosphorylation of CRKL is decreased in IM-treated primary $CD34^+$ HPCs
377 isolated from CML patients (25) and CML iPSCs (**Figure 3D**), suggesting that the BCR-
378 ABL TK activity in hematopoietic cells and CML iPSCs may involve similar pathways.
379 The fact that IM could stabilize the pluripotency of CML-iPSCs uncovers a novel
380 mechanism of pluripotency maintenance. Both CML-iPSCs and LSCs express BCR-
381 ABL fusion protein but are IM resistant. Therefore, CML-iPSCs created using our
382 strategy and the HPCs obtained from these iPSCs can be used for CRISPR-Cas9
383 screening to study the mechanisms of TKI resistance exhibited by these cells and
384 discover drugs that can effectively eliminate CML-LSCs.

385 CML-iPSCs generated in the presence of IM could be successfully
386 differentiated into HPCs. The iPSC-derived HPCs obtained from multiple patients will
387 help investigate the survival mechanisms of BCR-ABL+ hematopoietic cells in TKI-
388 resistant patients.

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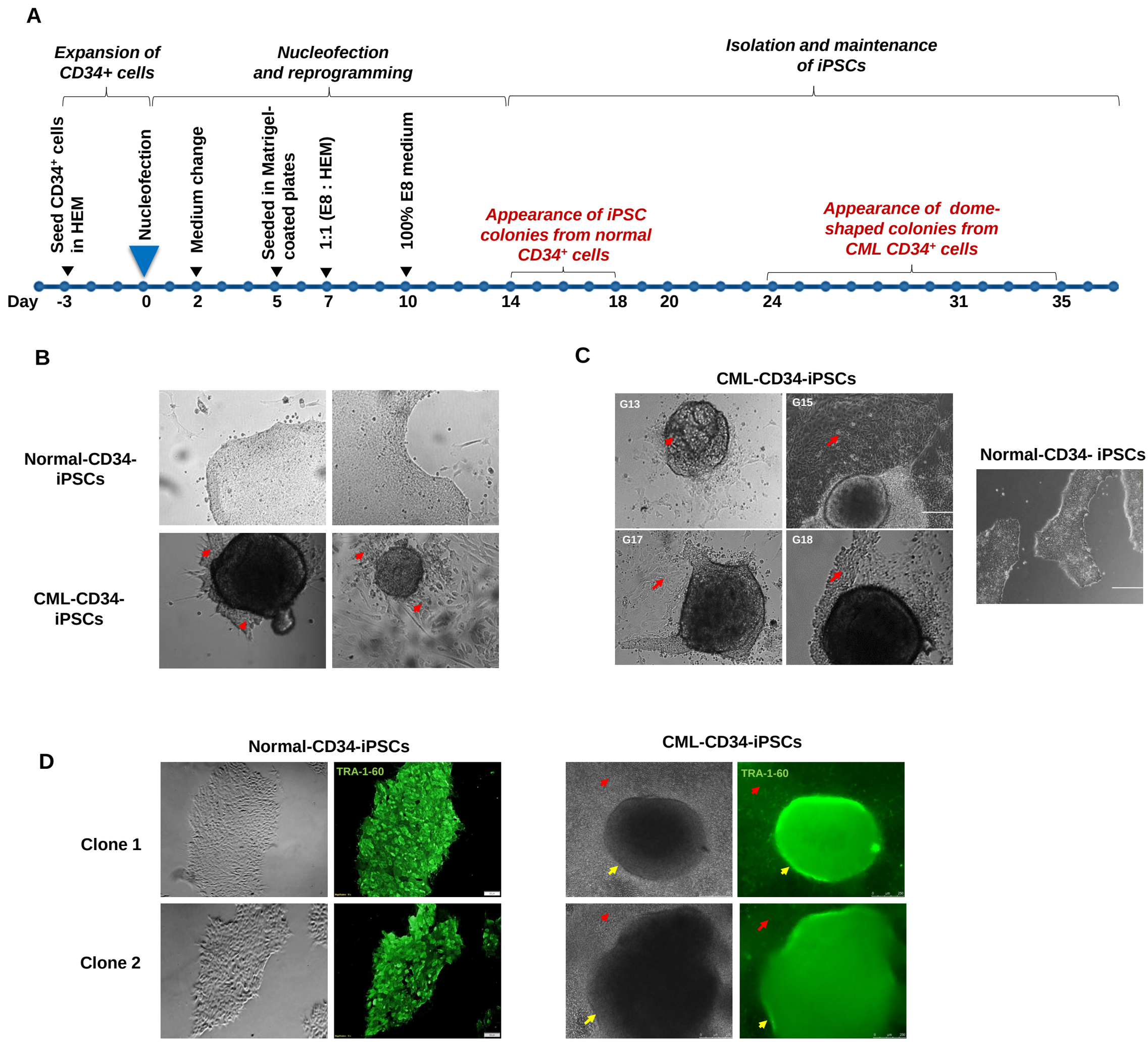
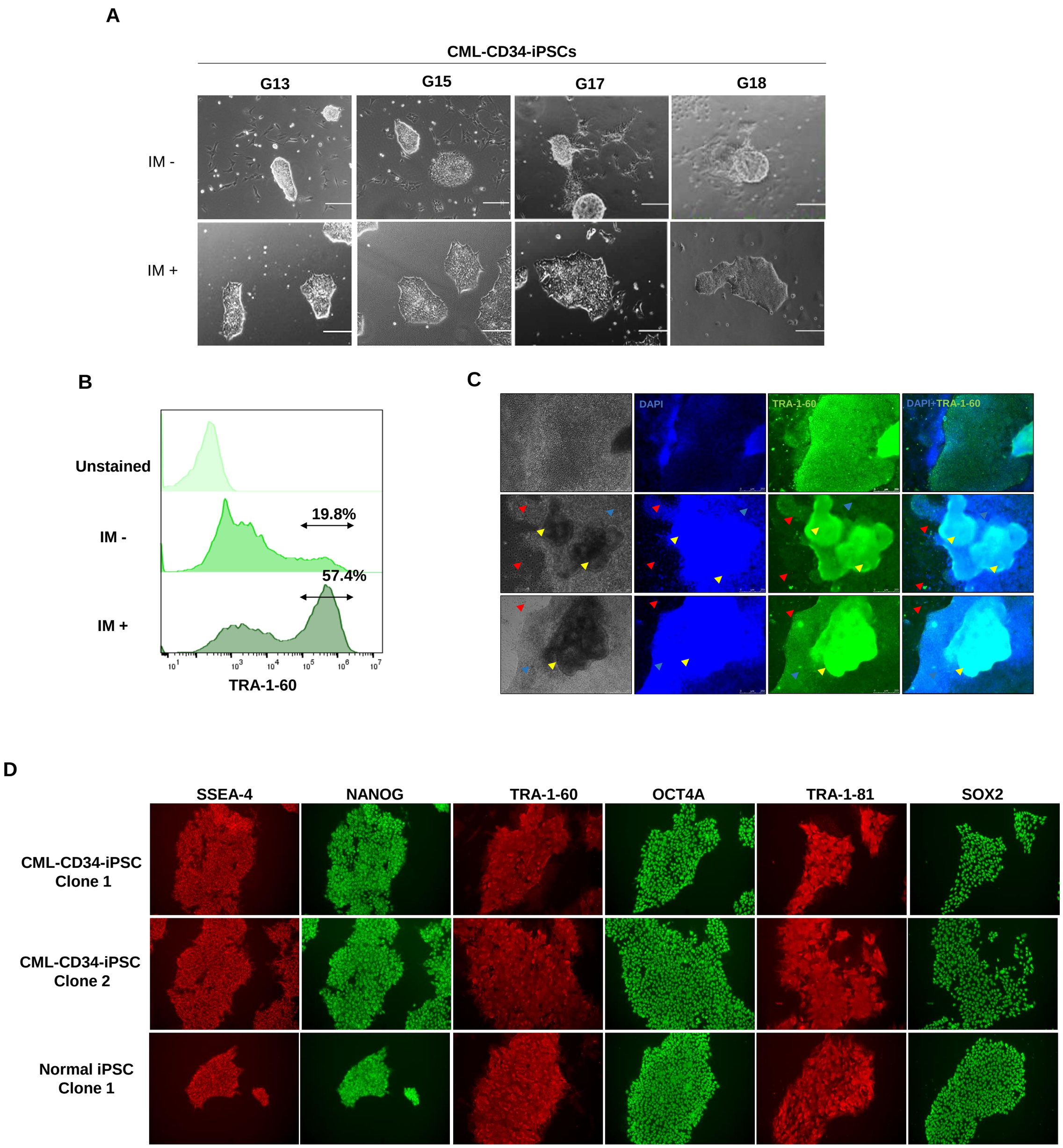
Figure 1: Reprogramming of Normal and CML CD34⁺ cells



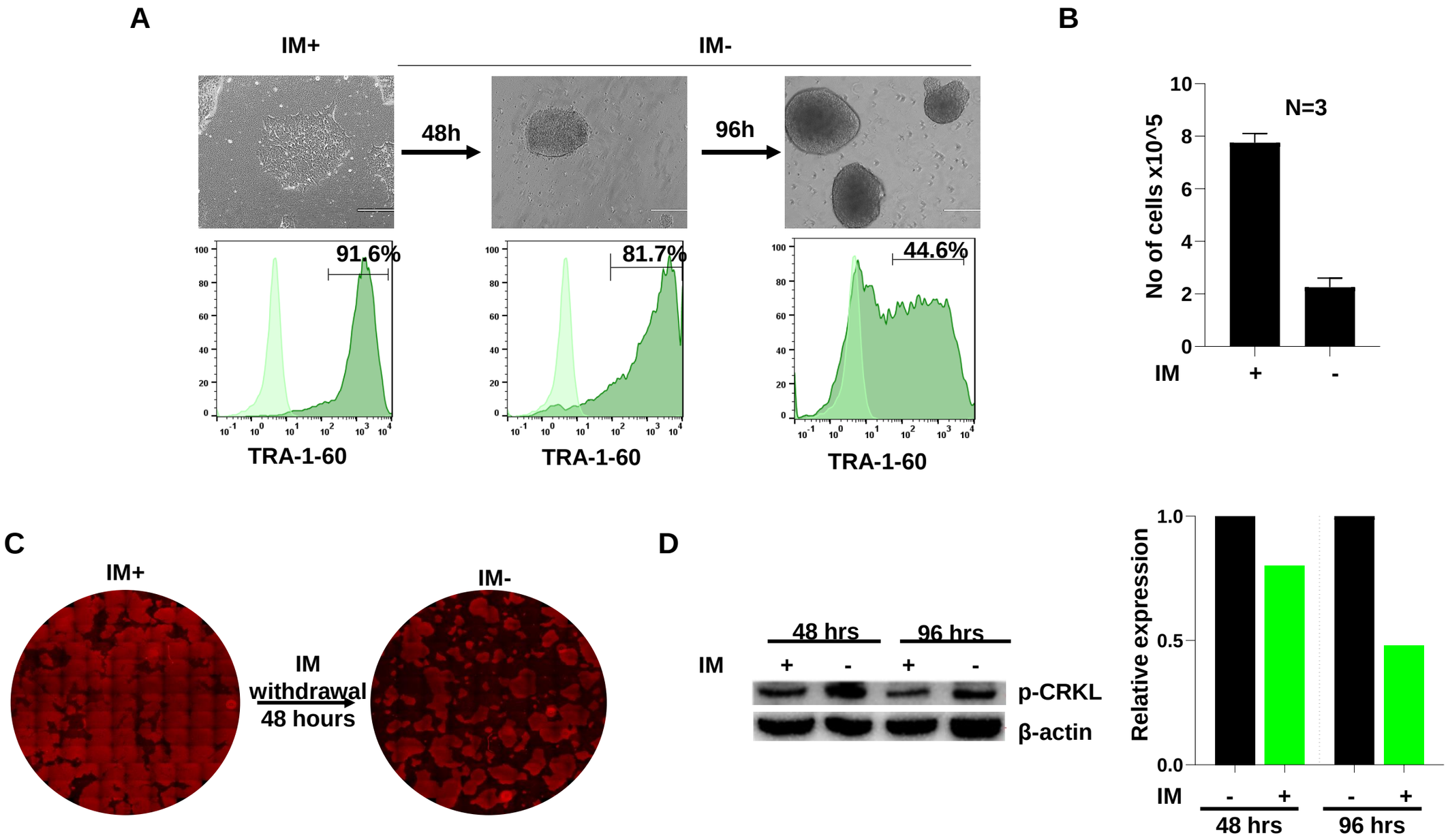
(**A**) Experimental outline for reprogramming of CML-CD34⁺ and normal-CD34⁺ cells. The purified CD34⁺ cells were expanded in culture, nucleofected with episomal plasmids, transferred to Matrigel-coated plates containing the reprogramming medium, and isolated colonies and passaged. HEM: Hematopoietic expansion media comprising SFEM II containing CD34+ expansion supplement and UM729. (**B**) Representative images of the emerging iPSC colonies generated from CML-CD34⁺ and normal-CD34⁺ cells**.** CML-CD34-iPSCs had "dome-shaped" colonies and normal-CD34-iPSCs had typical flat morphology. (**C**) CML-CD34-iPSC and normal-CD34-iPSC colonies. Red arrows indicate the non-iPSC cells spontaneously differentiated from CML-CD34-iPSCs. Scale bar: 50μM. (**D**) TRA-1-60 immunofluorescence of normal-CD34-iPSC and "dome-shaped" CML-CD34-iPSC colonies. Scale bar: 250μM. The green color indicates TRA-1-60 Yellow arrows indicate TRA-1-60+ "dome-shaped" CML-CD34-iPSC colonies and red arrows indicate differentiated cells.

Figure 2: IM aids in the maintenance of CML-CD34-iPSCs.



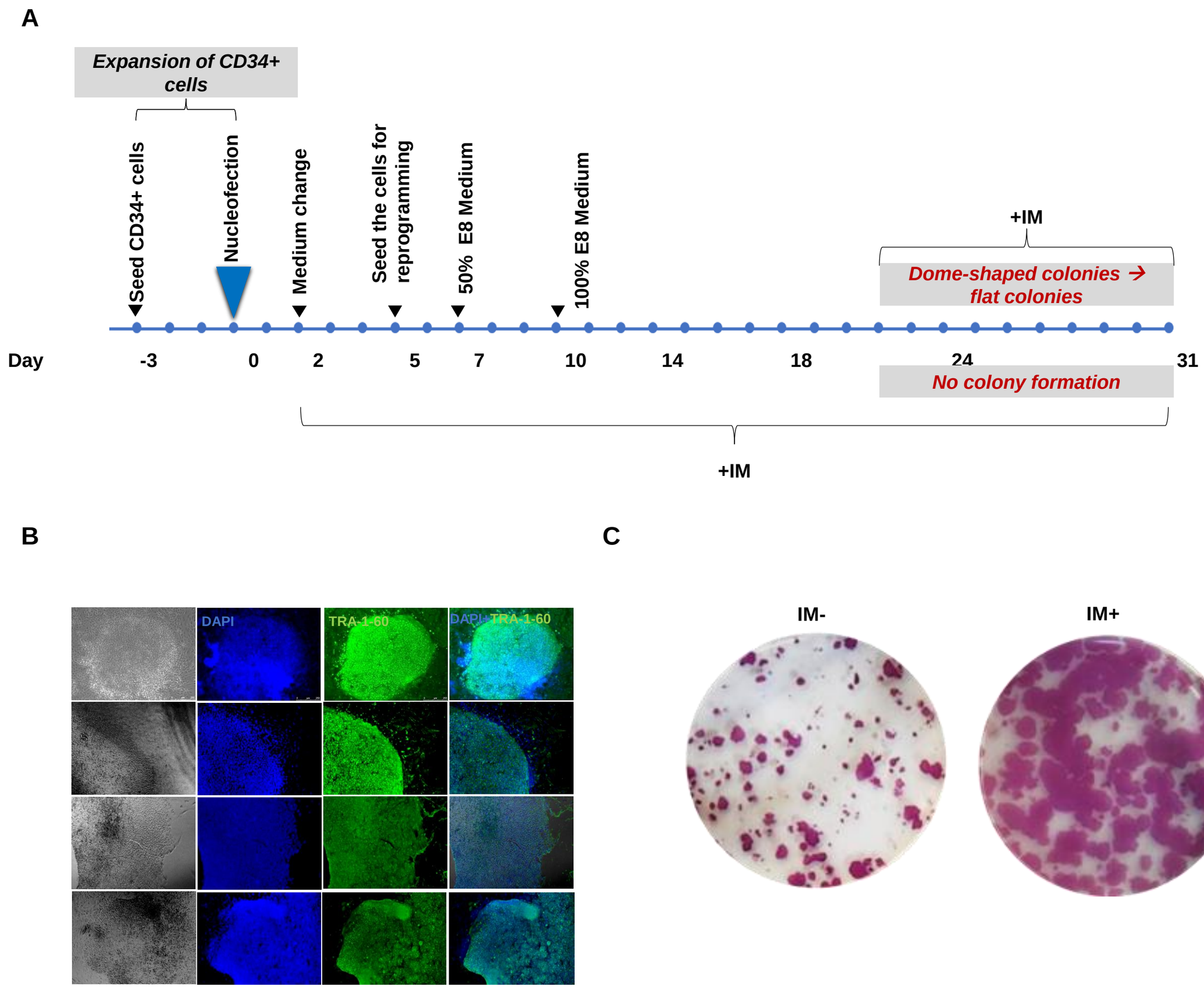
(**A**) Representative images of the "dome-shaped" CML-CD34-iPSC colonies, which exhibit significant differentiation generated in the absence of IM and with typical iPSC morphology after IM supplementation for 24 hours. Scale bar: 20μM. (**B)** Flow cytometry analysis of TRA-1-60 expression in CML-CD34-iPSCs cultured in the presence and absence of IM. CML-CD34-iPSCs cultured in the presence of IM showed a significantly reduced level of differentiation compared to the colonies maintained in the absence of IM. (**C**)Transformation of "dome-shaped" colonies to typical iPSCs colonies after IM treatment. "Dome-shaped" colonies transform to TRA-1-60+ iPSC colonies with flat morphology. Red arrows: differentiated cells, yellow arrows: "dome-shaped" TRA-1-60+ colonies, and blue arrows: typical iPSC colonies formed beneath the "dome-shaped" colonies. Scale bar: 250μM. (**D**) Representative immunofluorescence of pluripotency markers in CML-CD34-iPSC and normal-CD34-iPSC clones stained with anti-SSEA-4, anti-NANOG, anti-TRA-1-60, anti-OCT4A, anti-TRA-1-81 and anti-SOX2 antibodies. Scale bar:100μM

Figure 3: Withdrawal of IM affects cell proliferation, morphology, and pluripotency of CML-CD34-iPSCs.



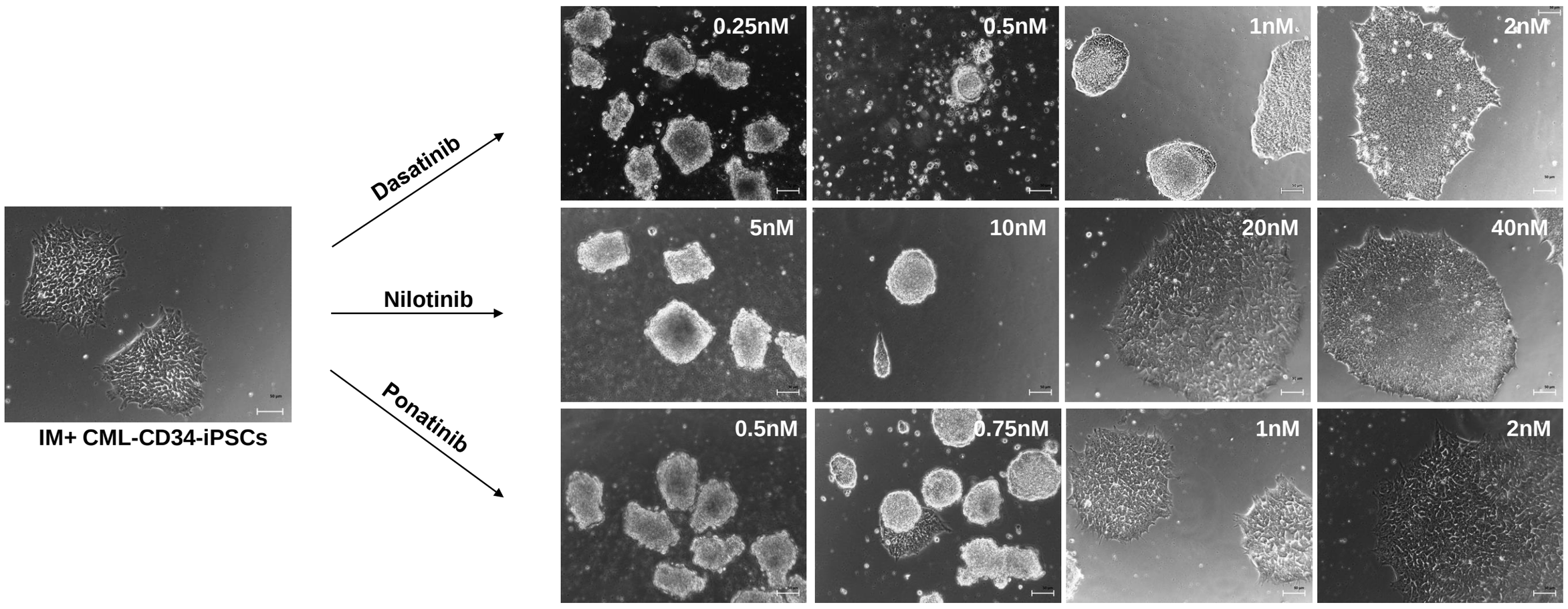
(**A**) The morphology and flow cytometry analysis of CML-CD34-iPSCs after IM withdrawal for 48 and 96 hours. The IM-supplemented CML-CD34-iPSCs formed "dome-shaped" colonies after IM withdrawal resulting in reduced TRA-1-60 expression. (**B**) Number of CML-CD34-iPSCs after 48h of IM withdrawal. CML-CD34-iPSCs showed reduced cell viability after IM withdrawal (**C**) Immunofluorescence analysis showing TRA-1-60 expression in the CML-CD34-iPSC clones cultured with (IM+) and without IM (IM-). (**D**) Immunoblot analysis of phospho-CRKL expression in CML-iPSC clones cultured with and without IM. Relative Phospho-CRKL protein expression was estimated using Image J software.

Figure 4: Supplementation of IM did not improve the reprogramming of CML-CD34-iPSCs.



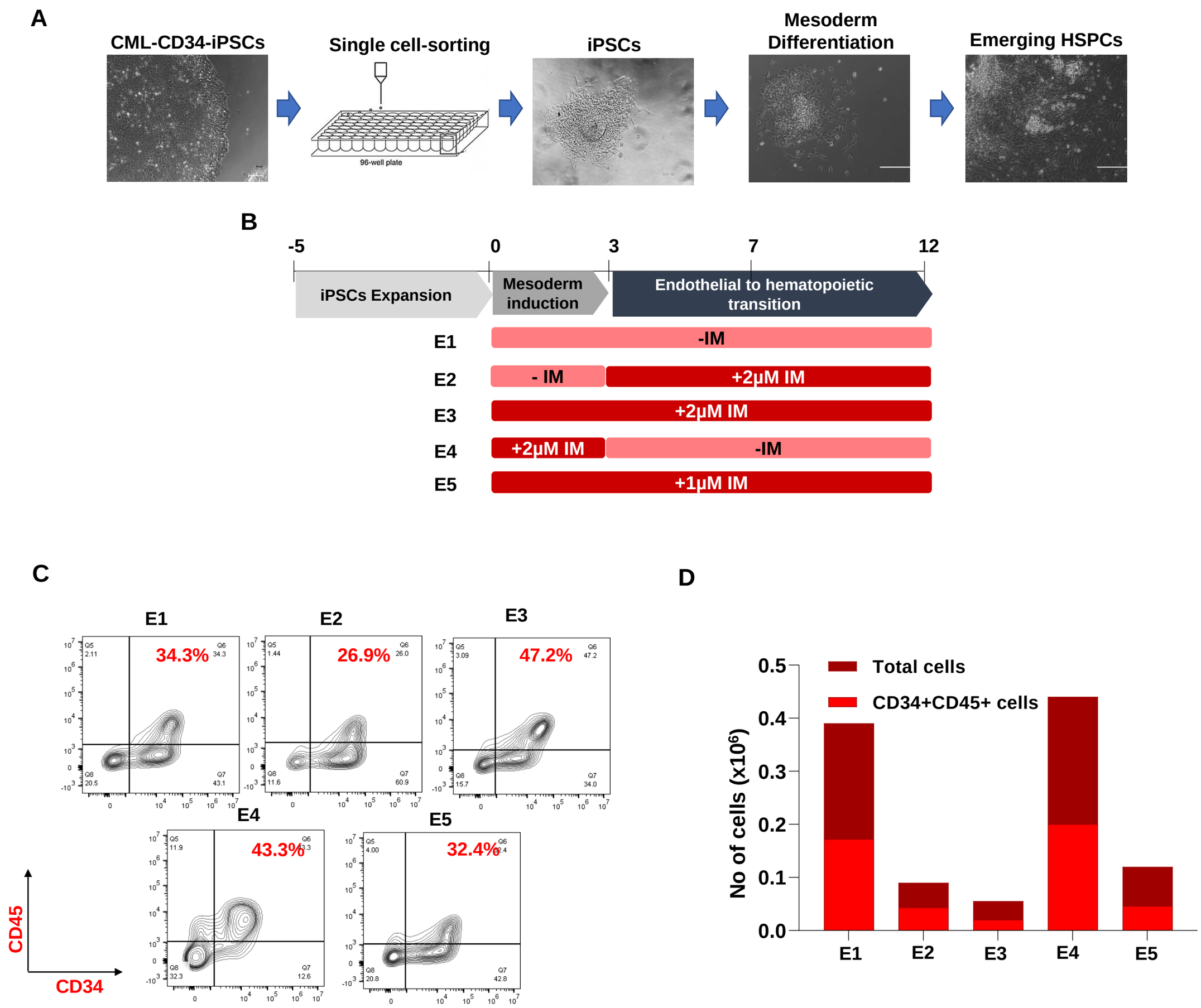
(**A**) Experimental outline to assess the effect of IM in reprogramming CML-CD34-iPSCs. (**B**) Immunofluorescence showing TRA-1-60 expression in CML-CD34-iPSCs after IM supplementation during reprogramming. (**C**) Alkaline Phosphatase staining of CML iPSC generated from an IM-refractory CML patient's $CD34^+$ in the absence (a) and presence of IM (b). Scale bar: 50μM

Figure 5: Effect of Second and third generation TKI in maintaining CML-CD34-iPSCs.



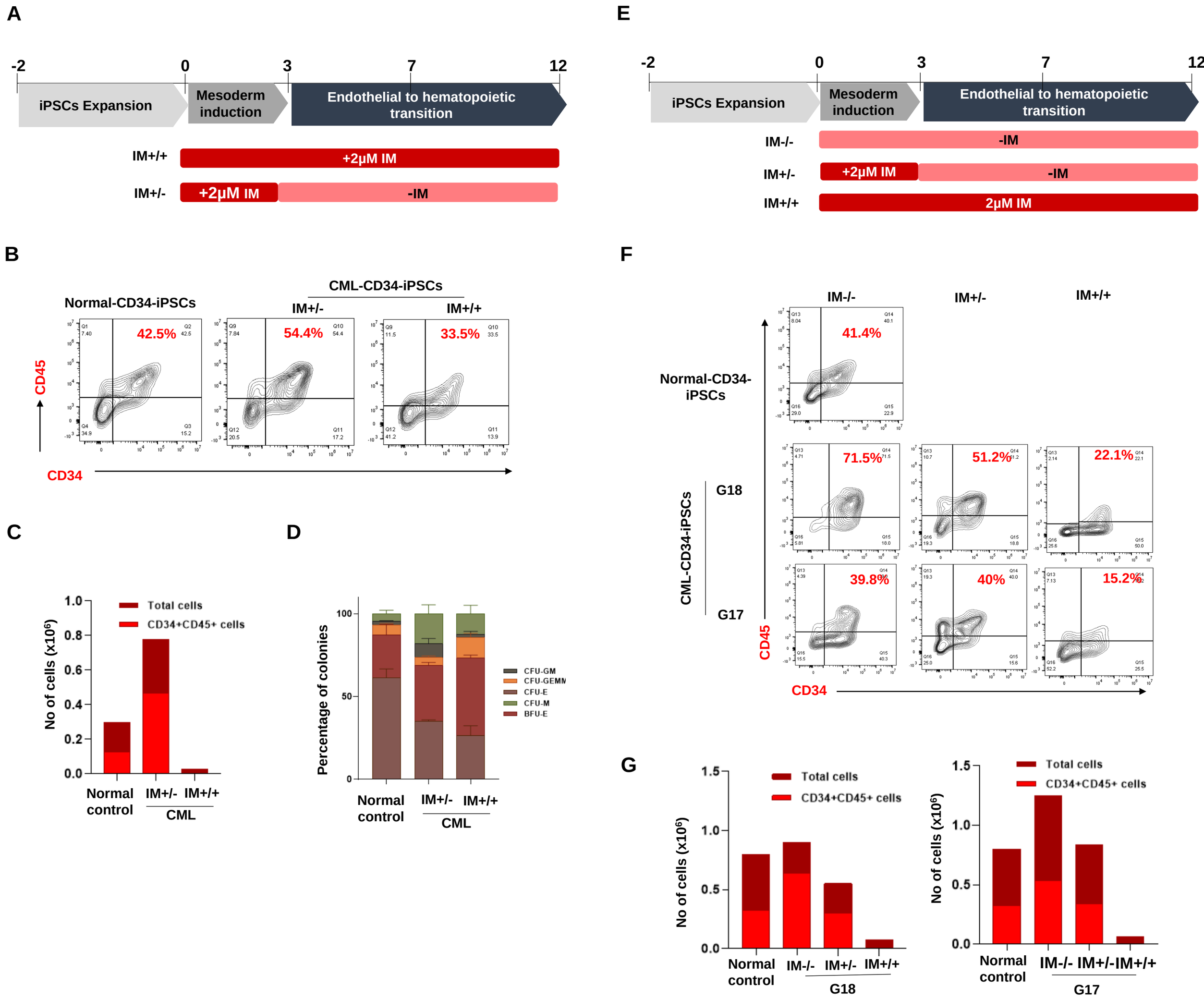
Images of iPSCs after treating the IM-supplemented CML-CD34-iPSCs with different concentrations of dasatinib (0.25,0.5,1and 2nM), nilotinib (5,10, 20 and 40nM) and ponatinib (0.5,0.75, 1 and 2 nM). Scale bar: 50μM

Figure 6: Effect of IM on hematopoietic differentiation of single cell expanded CML-CD34-iPSCs.



(**A**) Experimental outline of hematopoietic differentiation from single-cell sorted CML-CD34-iPSCs. (**B**) Representative work outline of different experiment groups (E1 to E5) with IM supplementation during different stages of hematopoietic differentiation. (**C**) Representative flow cytometry results showing the percentage of $CD34^+$ and $CD45^+$ generated under different conditions of hematopoietic differentiation. (**D**) Total cells and $CD34^+CD45^+$ cells generated during hematopoietic differentiation.

Figure 7: IM affects the HSPCs differentiation of CML-CD34-iPSCs.



(**A**) Schematic representation of hematopoietic differentiation of CML-iPSCs to analyze the effect of IM after mesoderm differentiation (**B**) Representative flow cytometry results of the percentages of $CD34^{+}CD45^{+}$ cells generated from healthy donor and CML-CD34-iPSCs. (**D**) Hematopoietic colony forming capacity of HSPCs derived from CML-CD34-iPSCs and normal CD34-iPSCs. (**E**) Experimental outline HSPC differentiation with and without IM at different stages of differentiation. (**F**)Representative flow cytometry results of the percentages of $CD34^{+}CD45^{+}$ cells generated from healthy donor and CML-CD34-iPSCs generated from two different patients. (**G**) Bar graphs showing the relative cell numbers and $CD34^{+}CD45^{+}$ cells generated by CML-iPSC colonies (G17 and G18) generated from two different patients during differentiation under different conditions. IM-/- represents CML-CD34-iPSCs was subjected to HSPC differentiation without IM; IM+/- represents IM was withdrawn after mesoderm induction. IM+/+ represents IM was supplemented throughout HSPCs differentiation.