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

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

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

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

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

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

113 Materials and methods

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

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

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

189 ## Results

190

191 **Generation of iPSCs from normal and CML CD34⁺ cells:**

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

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

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

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

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

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

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

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

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

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

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

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

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

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

388 **Reference**

389 1. Bhatia R, Holtz M, Niu N, Gray R, Snyder DS, Sawyers CL, et al. Persistence of
390 malignant hematopoietic progenitors in chronic myelogenous leukemia
391 patients in complete cytogenetic remission following imatinib mesylate
392 treatment. Blood. 2003 Jun;101(12):4701–7.

2. Imagawa J, Tanaka H, Okada M, Nakamae H, Hino M, Murai K, et al. Discontinuation of dasatinib in patients with chronic myeloid leukaemia who have maintained deep molecular response for longer than 1 year (DADI trial): a multicentre phase 2 trial. Lancet Haematol. 2015 Dec;2(12):e528–35.
3. Etienne G, Guilhot J, Rea D, Rigal-Huguet F, Nicolini F, Charbonnier A, et al. Long-term follow-up of the French Stop Imatinib (STIM1) study in patients with chronic myeloid leukemia. J Clin Oncol. 2017 Jan 20;35(3):298–305.
4. Corbin AS, Agarwal A, Loriaux M, Cortes J, Deininger MW, Druker BJ. Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity. J Clin Invest [Internet]. 2011 Jan 4 [cited 2023 Jan 7];121(1):396–409. Available from: http://www.jci.org
5. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. Cell [Internet]. 2007 Nov 30 [cited 2023 Apr 11];131(5):861–72. Available from: http://www.cell.com/article/S0092867407014717/fulltext
6. Papapetrou EP. Modeling Leukemia with Human Induced Pluripotent Stem Cells. Cold Spring Harb Perspect Med [Internet]. 2019 Dec 1 [cited 2023 Apr 19];9(12). Available from: /pmc/articles/PMC6886454/
7. Miyauchi M, Koya J, Arai S, Yamazaki S, Honda A, Kataoka K, et al. ADAM8 Is an Antigen of Tyrosine Kinase Inhibitor-Resistant Chronic Myeloid Leukemia Cells Identified by Patient-Derived Induced Pluripotent Stem Cells. Stem Cell Reports. 2018 Mar;10(3):1115–30.
8. Corbin AS, Agarwal A, Loriaux M, Cortes J, Deininger MW, Druker BJ. Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity. J Clin Invest. 2011 Jan;121(1):396–409.
9. Imeri J, Desterke C, Marcoux P, Telliam G, Sanekli S, Barreau S, et al. Modeling Blast Crisis Using Mutagenized Chronic Myeloid Leukemia-Derived Induced Pluripotent Stem Cells (iPSCs). Cells [Internet]. 2023 Feb 1 [cited 2023 Apr 11];12(4):598. Available from: https://www.mdpi.com/2073-4409/12/4/598/htm
10. Hu K, Yu J, Suknuntha K, Tian S, Montgomery K, Choi KD, et al. Efficient generation of transgene-free induced pluripotent stem cells from normal and

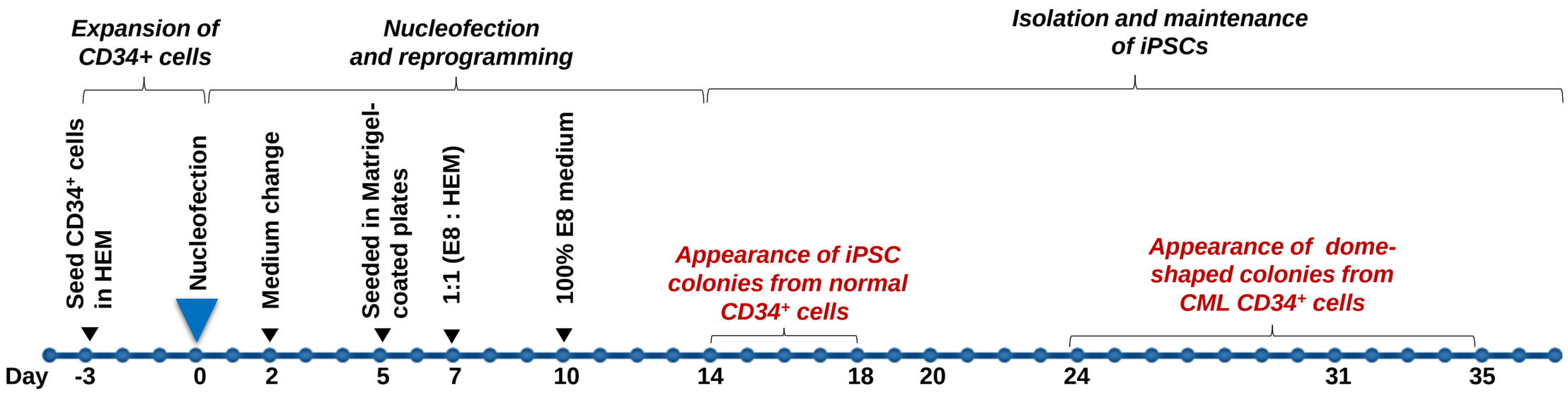
425 neoplastic bone marrow and cord blood mononuclear cells. Blood.
426 2011;117(14):109–19.
427 11. Telliam G, Féraud O, Griscelli F, Opolon P, Divers D, Bennaceur-Griscelli A, et
428 al. Generation of an induced pluripotent stem cell line from a patient with
429 chronic myeloid leukemia (CML) resistant to targeted therapies. Stem Cell Res.
430 2016;17(2):235–7.
431 12. Kumano K, Arai S, Hosoi M, Taoka K, Takayama N, Otsu M, et al. Generation of
432 induced pluripotent stem cells from primary chronic myelogenous leukemia
433 patient samples. Blood. 2012 Jun;119(26):6234–42.
434 13. Hosoi M, Kumano K, Taoka K, Arai S, Kataoka K, Ueda K, et al. Generation of
435 induced pluripotent stem cells derived from primary and secondary
436 myelofibrosis patient samples. Exp Hematol. 2014;42(9):816–25.
437 14. Bedel A, Pasquet JM, Lippert É, Taillepierre M, Lagarde V, Dabernat S, et al.
438 Variable Behavior of iPSCs Derived from CML Patients for Response to TKI
439 and Hematopoietic Differentiation. PLoS One. 2013;8(8).
440 15. Charaf L, Mahon FX, Lamrissi-Garcia I, Moranvillier I, Beliveau F, Cardinaud B,
441 et al. Effect of tyrosine kinase inhibitors on stemness in normal and chronic
442 myeloid leukemia cells. Leukemia. 2017;31(1):65–74.
443 16. Manian K V., Bharathan SP, Maddali M, Srivastava VM, Srivastava A,
444 Velayudhan SR. Generation of an integration-free iPSC line (CSCRi005-A) from
445 erythroid progenitor cells of a healthy Indian male individual. Stem Cell Res.
446 2018 May 1;29:148–51.
447 17. Hamilton A, Helgason GV, Schemionek M, Zhang B, Myssina S, Allan EK, et al.
448 Chronic myeloid leukemia stem cells are not dependent on Bcr-Abl kinase
449 activity for their survival. Blood [Internet]. 2012 Feb 9 [cited 2023 Jan
450 7];119(6):1501–10. Available from:
451 https://ashpublications.org/blood/article/119/6/1501/30195/Chronic-myeloid-
452 leukemia-stem-cells-are-not
453 18. Markose P, Chendamarai E, Balasubramanian P, Velayudhan SR, Srivastava
454 VM, Mathews V, et al. Spectrum of BCR-ABL kinase domain mutations in
455 patients with chronic myeloid leukemia from India with suspected resistance to
456 imatinib-mutations are rare and have different distributions.

457 https://doi.org/103109/10428190903332486 [Internet]. 2009 Dec [cited 2023
458 Apr 10];50(12):2092–5. Available from:
459 https://www.tandfonline.com/doi/abs/10.3109/10428190903332486
460 19. Bharathan SP, Nandy K, Palani D, Janet A NB, Natarajan K, George B, et al.
461 Generation of an induced pluripotent stem cell line that mimics the disease
462 phenotypes from a patient with Fanconi anemia by conditional
463 complementation. Stem Cell Res. 2017 Apr;20:54–7.
464 20. Hamilton A, Elrick L, Myssina S, Copland M, Jørgensen H, Melo J V., et al.
465 BCR-ABL activity and its response to drugs can be determined in CD34+ CML
466 stem cells by CrkL phosphorylation status using flow cytometry. Leuk 2006 206
467 [Internet]. 2006 Mar 30 [cited 2022 Dec 20];20(6):1035–9. Available from:
468 https://www.nature.com/articles/2404189
469 21. Nichols G, Raines M, Vera J, Lacomis L, Tempst P, Golde D. Identification of
470 CRKL as the Constitutively Phosphorylated 39-kD Tyrosine Phosphoprotein in
471 Chronic Myelogenous Leukemia Cells. Blood. 1994 Nov 1;84(9):2912–8.
472 22. Kumano K, Arai S, Hosoi M, Taoka K, Takayama N, Otsu M, et al. Generation of
473 induced pluripotent stem cells from primary chronic myelogenous leukemia
474 patient samples. Blood [Internet]. 2012 Jun 28 [cited 2022 Dec
475 28];119(26):6234–42. Available from:
476 https://ashpublications.org/blood/article/119/26/6234/105596/Generation-of-
477 induced-pluripotent-stem-cells-from
478 23. Mahon FX, Etienne G. Deep molecular response in chronic myeloid leukemia:
479 The new goal of therapy? Clin Cancer Res [Internet]. 2014 Jan 15 [cited 2023
480 Mar 27];20(2):310–22. Available from:
481 https://aacrjournals.org/clincancerres/article/20/2/310/78489/Deep-Molecular-
482 Response-in-Chronic-Myeloid
483 24. Ito K, Nagata K, Ohta S, Matsuda Y, Ukai T, Yasuda I, et al. The oncogene-
484 dependent resistance to reprogramming unveils cancer therapeutic targets.
485 Cell Rep. 2022 Apr;39(4):110721.
486 25. Suknuntha K, Ishii Y, Tao L, Hu K, McIntosh BE, Yang D, et al. Discovery of
487 survival factor for primitive chronic myeloid leukemia cells using induced
488 pluripotent stem cells. Stem Cell Res. 2015 Nov 1;15(3):678–93.

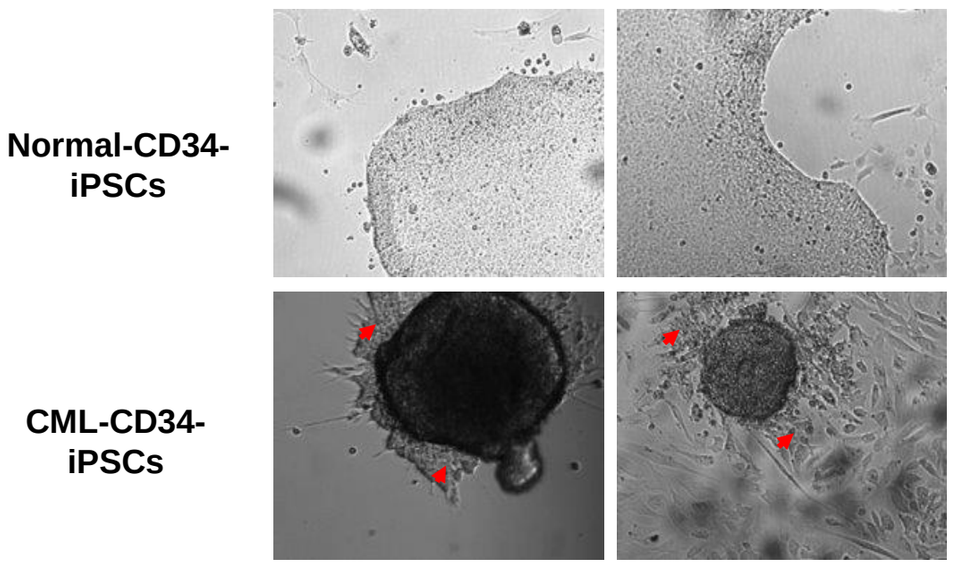
https://cmcv-my.sharepoint.com/personal/esther_sathya_cmcvellore_ac_in/_layouts/15/onedrive.aspx?id=%2Fpersonal%2Frvshaji%5Fcmcvellore%5Fac%5Fin%2FDocuments%2FStudents%2FEsther%2FManuscript%2FFinal%20CML%2DiPSCs%20manuscript%2Fbiorvix%2FSupplementary%20data1%2Epdf&listurl=%2Fpersonal%2Frvshaji%5Fcmcvellore%5Fac%5Fin%2FDocuments&parentview=1

Figure 1

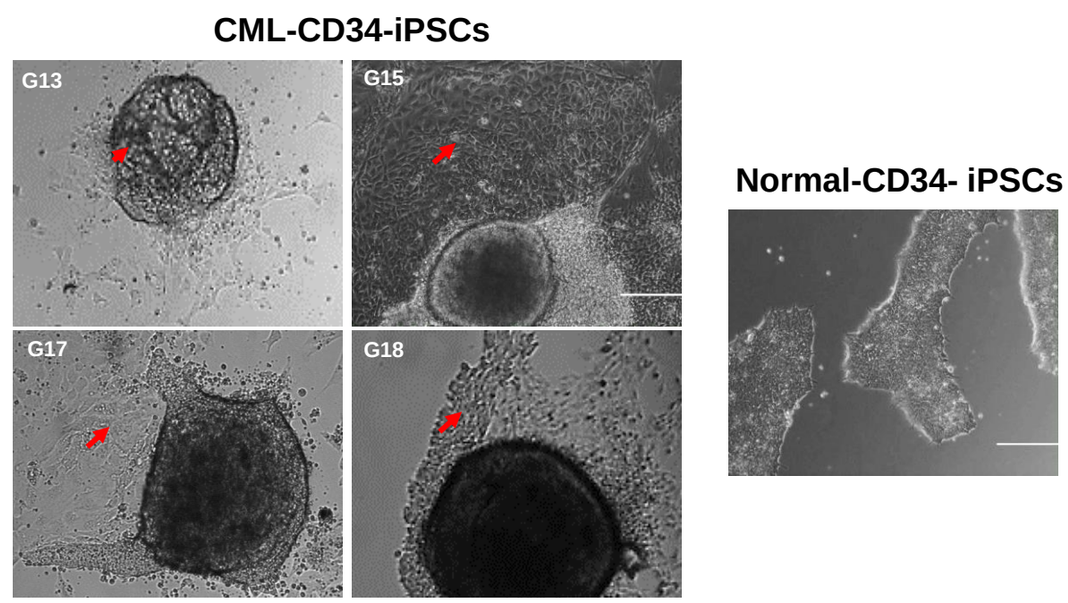
A



B



C



D

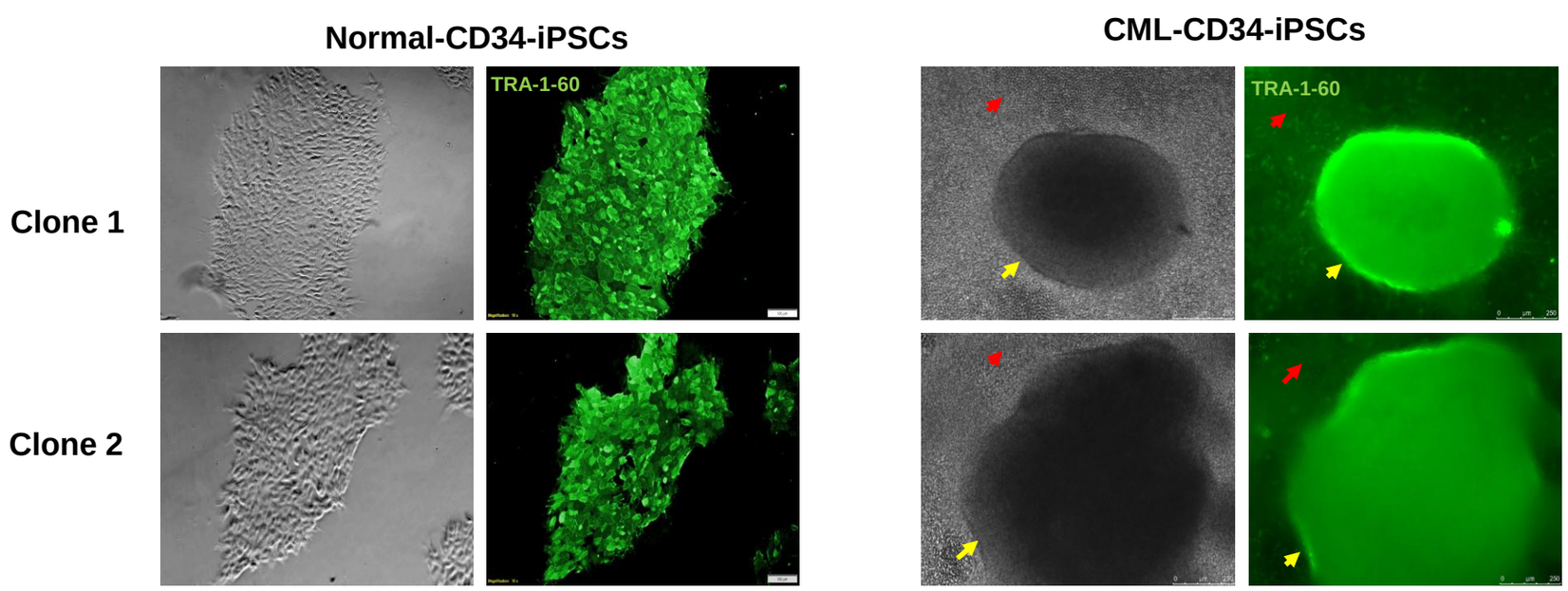


Figure 2

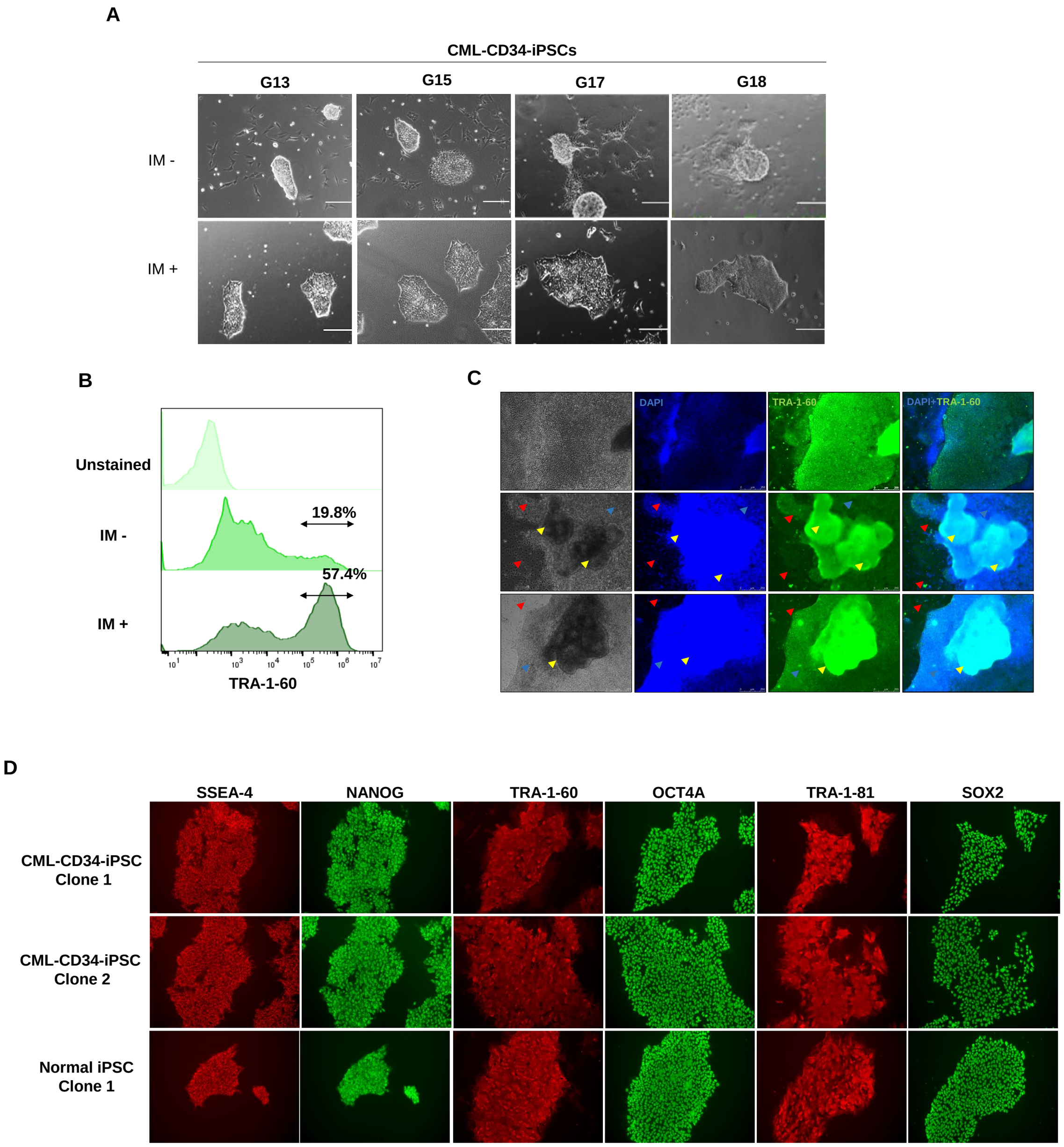

A
CML-CD34-iPSCs
G13
G15
G17
G18
IM -
IM +
B
Unstained
IM -
IM +
19.8%
57.4%
TRA-1-60
C
DAPI
TRA-1-60
DAPI+TRA-1-60
D
SSEA-4
NANOG
TRA-1-60
OCT4A
TRA-1-81
SOX2
CML-CD34-iPSC Clone 1
CML-CD34-iPSC Clone 2
Normal iPSC Clone 1

Figure 3

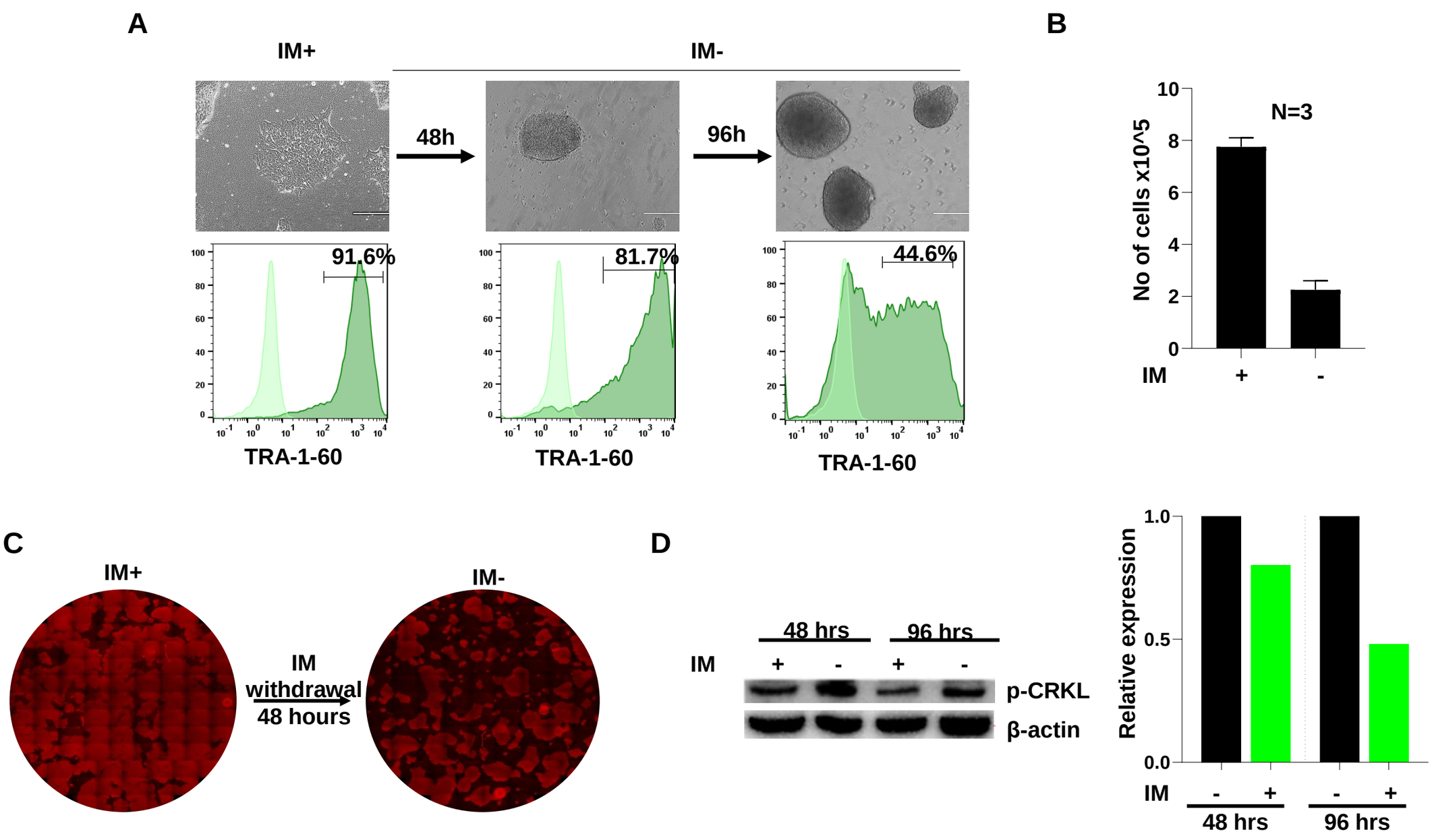
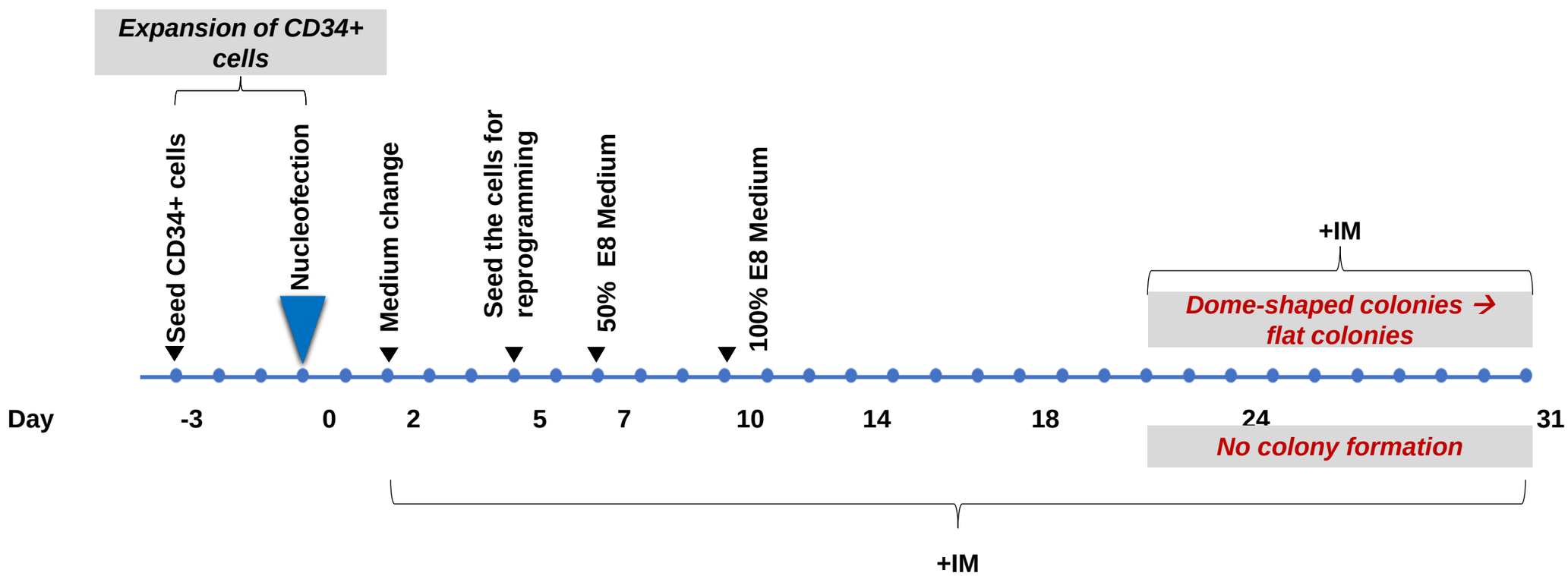
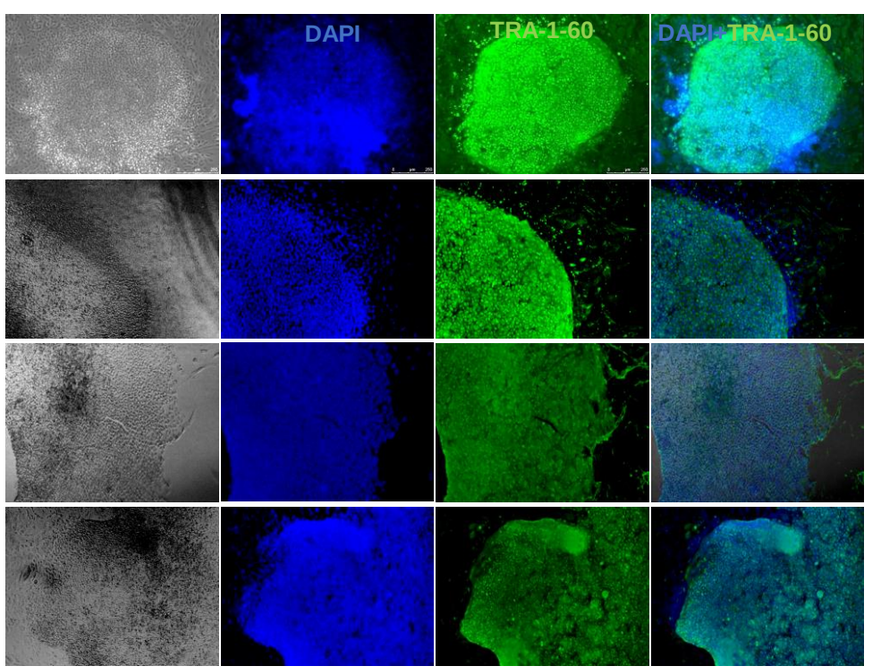

A
IM+
IM-
48h
96h
91.6%
81.7%
44.6%
TRA-1-60
TRA-1-60
TRA-1-60
B
N=3
No of cells x10^5
IM
+
-
C
IM+
IM-
IM
withdrawal
48 hours
D
48 hrs
96 hrs
IM
+
-
+
-
p-CRKL
β-actin
Relative expression
IM
-
+
-
+
48 hrs
96 hrs

Figure 4

A


Expansion of CD34+ cells
Seed CD34+ cells
Nucleofection
Medium change
Seed the cells for reprogramming
50% E8 Medium
100% E8 Medium
+IM
Dome-shaped colonies → flat colonies
Day
-3
0
2
5
7
10
14
18
24
31
No colony formation
+IM

B


DAPI
TRA-1-60
DAPI+TRA-1-60

C

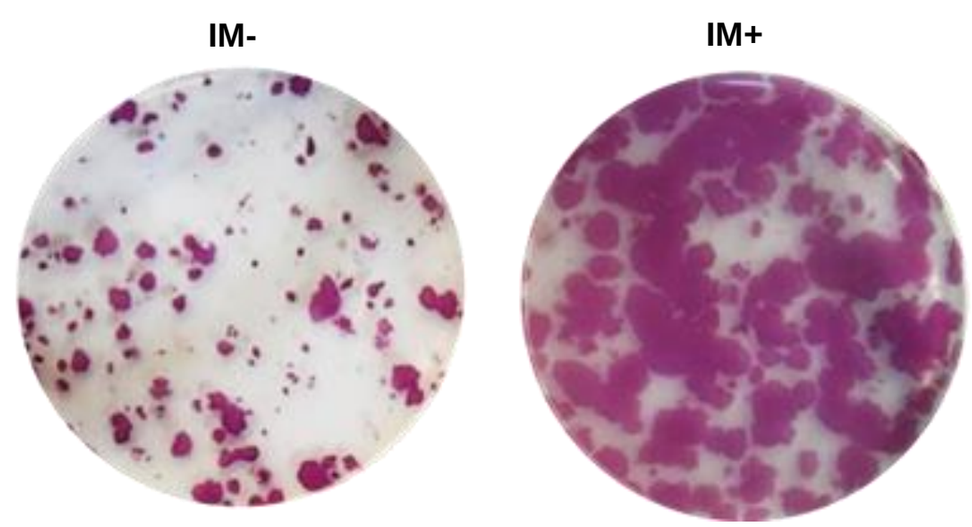

IM-
IM+

Figure 5

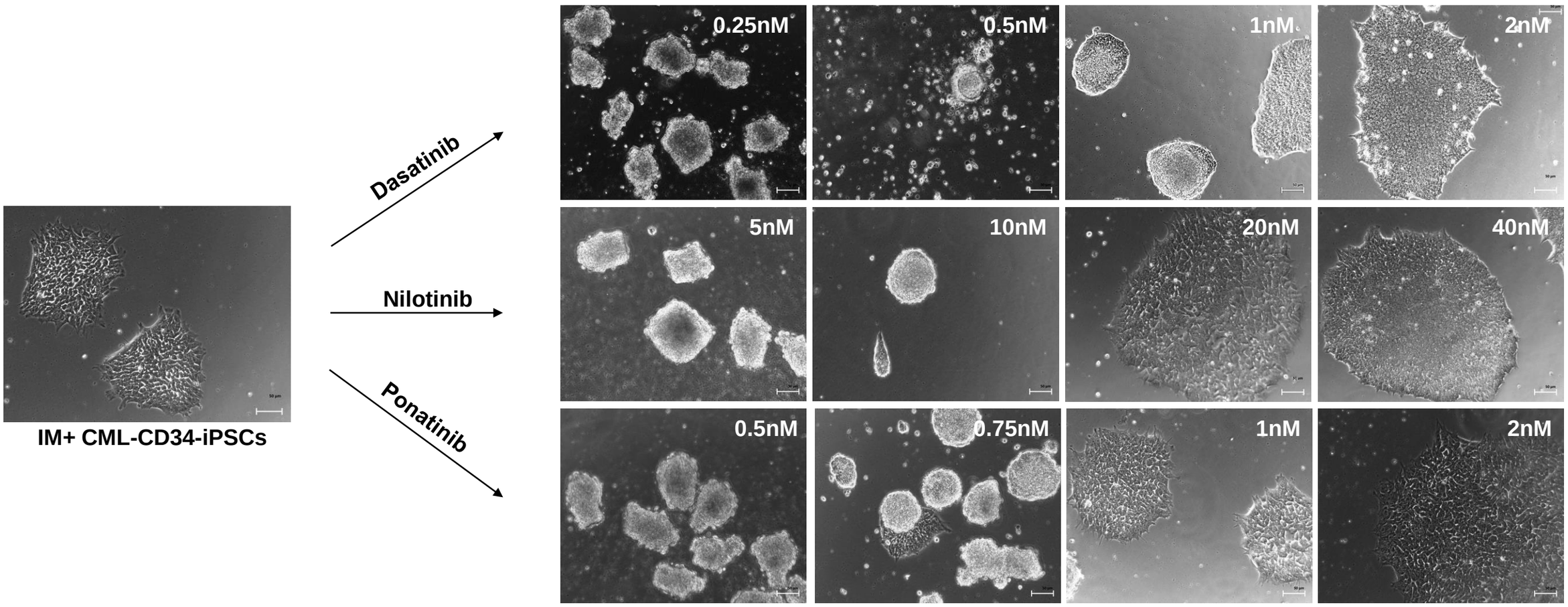

IM+ CML-CD34-iPSCs
Dasatinib
Nilotinib
Ponatinib
0.25nM
0.5nM
1nM
2nM
5nM
10nM
20nM
40nM
0.5nM
0.75nM
1nM
2nM

Figure 6

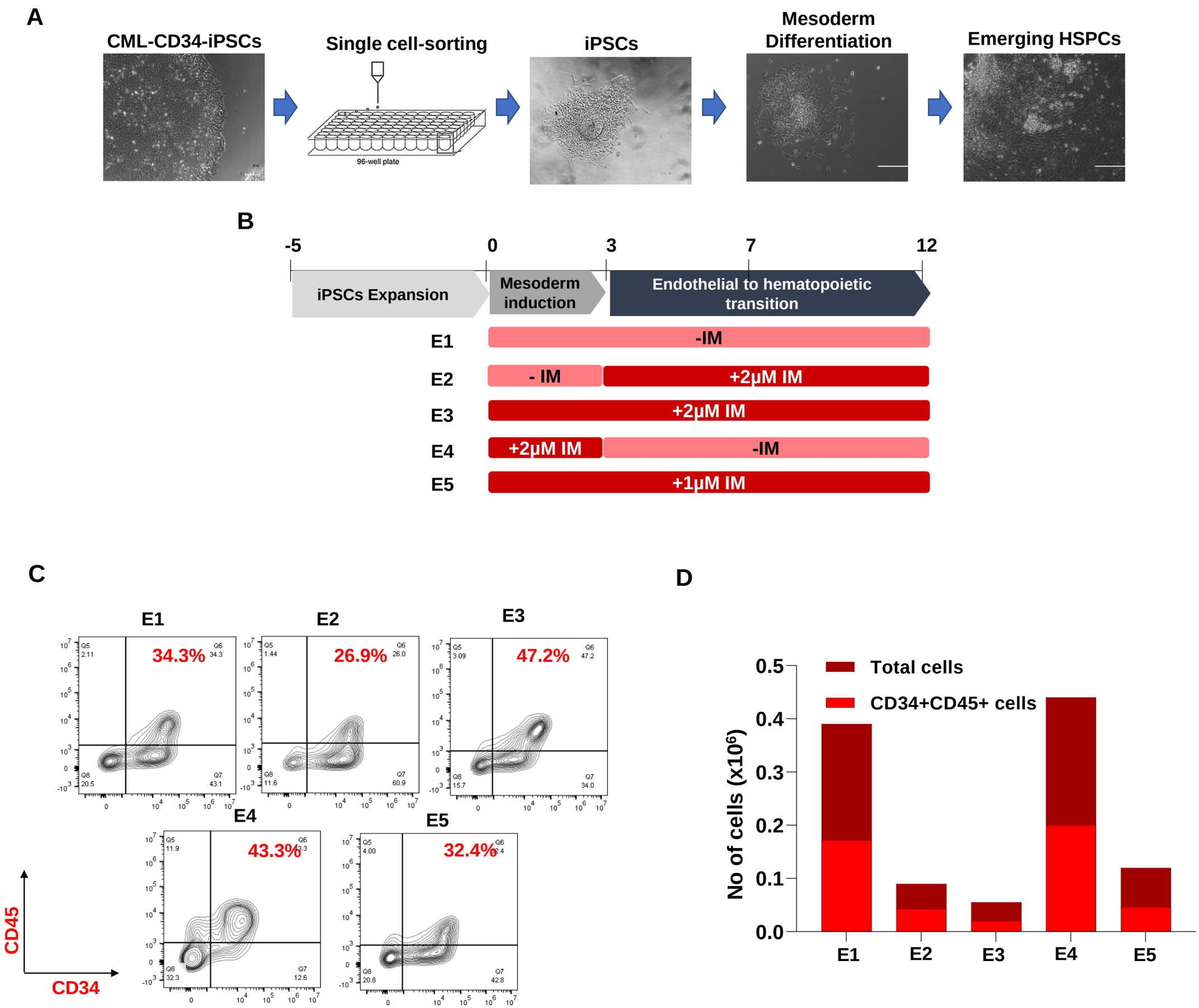

A
CML-CD34-iPSCs
Single cell-sorting
96-well plate
iPSCs
Mesoderm Differentiation
Emerging HSPCs
B
-5
0
3
7
12
iPSCs Expansion
Mesoderm induction
Endothelial to hematopoietic transition
E1
-IM
E2
- IM
+2μM IM
E3
+2μM IM
E4
+2μM IM
-IM
E5
+1μM IM
C
E1
34.3%
E2
26.9%
E3
47.2%
E4
43.3%
E5
32.4%
CD45
CD34
D
Total cells
CD34+CD45+ cells
No of cells (x10⁶)
0.0
0.1
0.2
0.3
0.4
0.5
E1
E2
E3
E4
E5

Figure 7

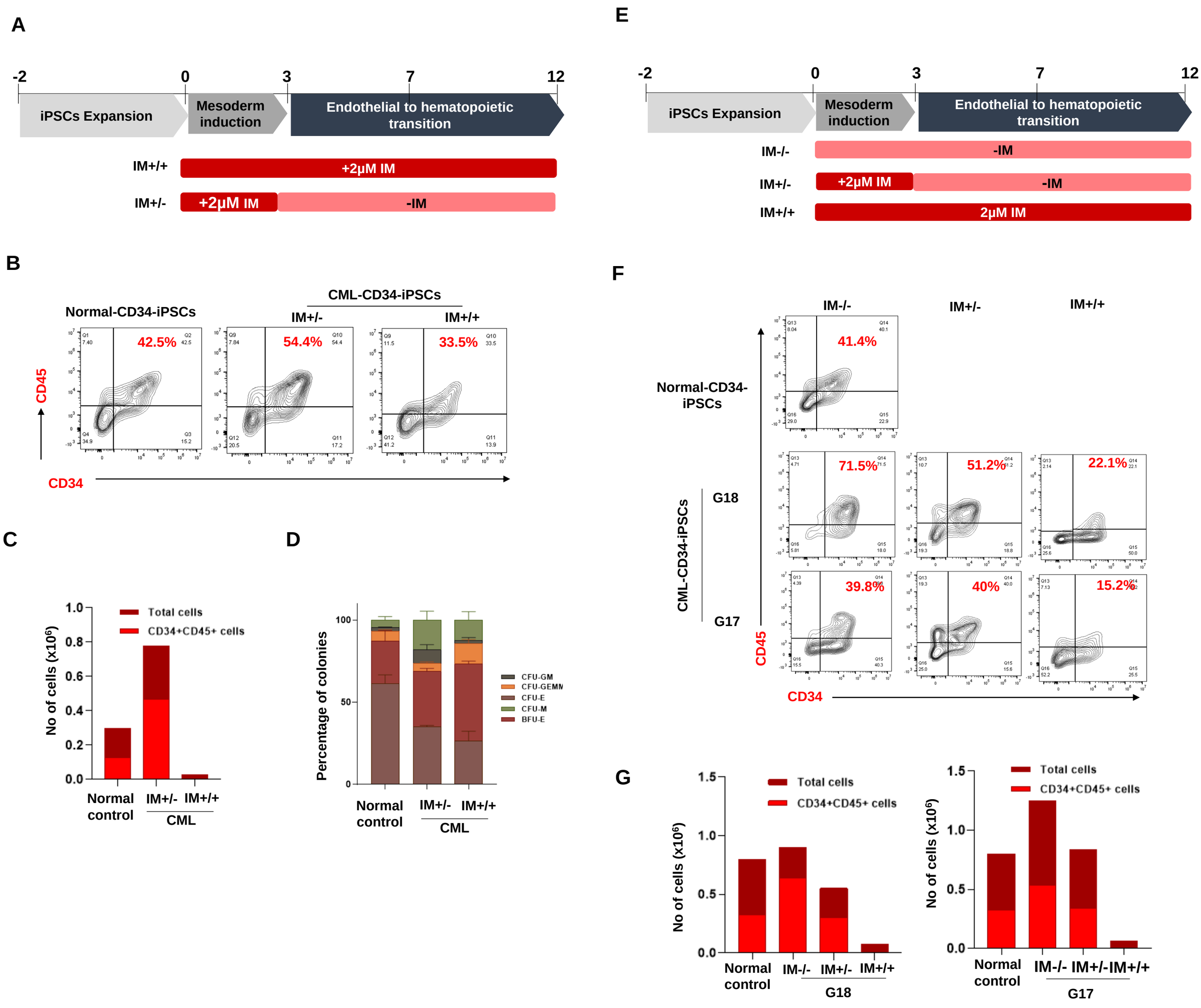

A
-2
0
3
7
12
iPSCs Expansion
Mesoderm induction
Endothelial to hematopoietic transition
IM+/+
+2μM IM
IM+/-
+2μM IM
-IM
B
Normal-CD34-iPSCs
CML-CD34-iPSCs
IM+/-
IM+/+
42.5%
54.4%
33.5%
CD45
CD34
C
Total cells
CD34+CD45+ cells
No of cells (x10⁶)
Normal control
IM+/-
IM+/+
CML
D
Percentage of colonies
CFU-GM
CFU-GEMM
CFU-E
CFU-M
BFU-E
Normal control
IM+/-
IM+/+
CML
E
-2
0
3
7
12
iPSCs Expansion
Mesoderm induction
Endothelial to hematopoietic transition
IM-/-
-IM
IM+/-
+2μM IM
-IM
IM+/+
2μM IM
F
IM-/-
IM+/-
IM+/+
Normal-CD34-iPSCs
CML-CD34-iPSCs
G18
G17
41.4%
71.5%
51.2%
22.1%
39.8%
40%
15.2%
CD45
CD34
G
Total cells
CD34+CD45+ cells
No of cells (x10⁶)
Normal control
IM-/-
IM+/-
IM+/+
G18
G17

Figure S1

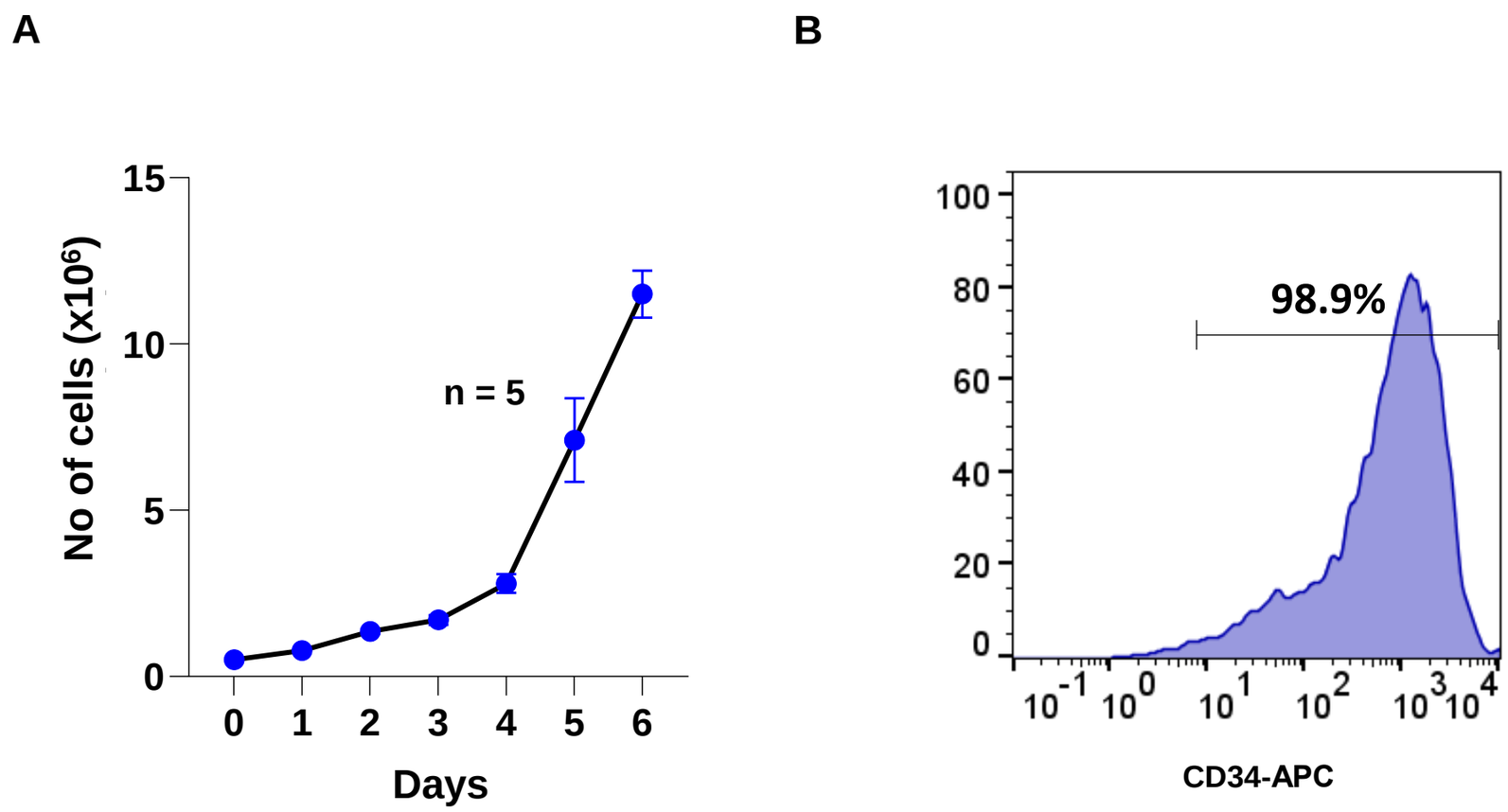
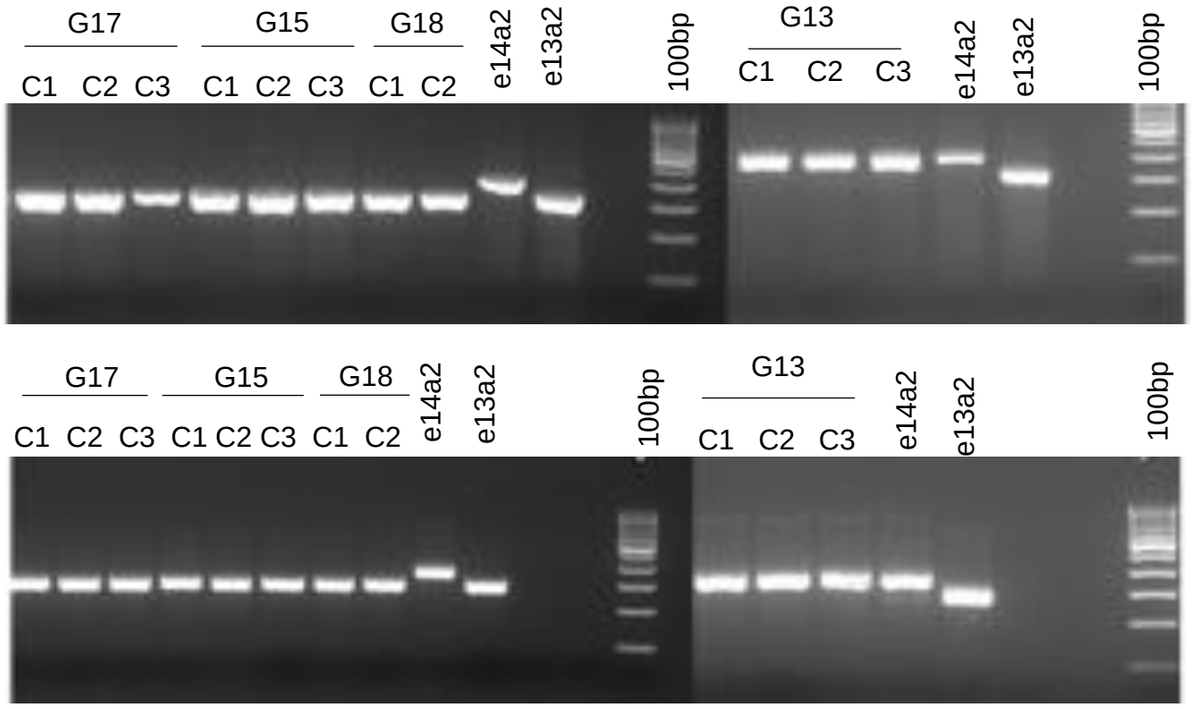

A
B
No of cells (x10^6)
15
10
5
0
n = 5
0
1
2
3
4
5
6
Days
100
80
60
40
20
0
98.9%
10^-1
10^0
10^1
10^2
10^3
10^4
CD34-APC

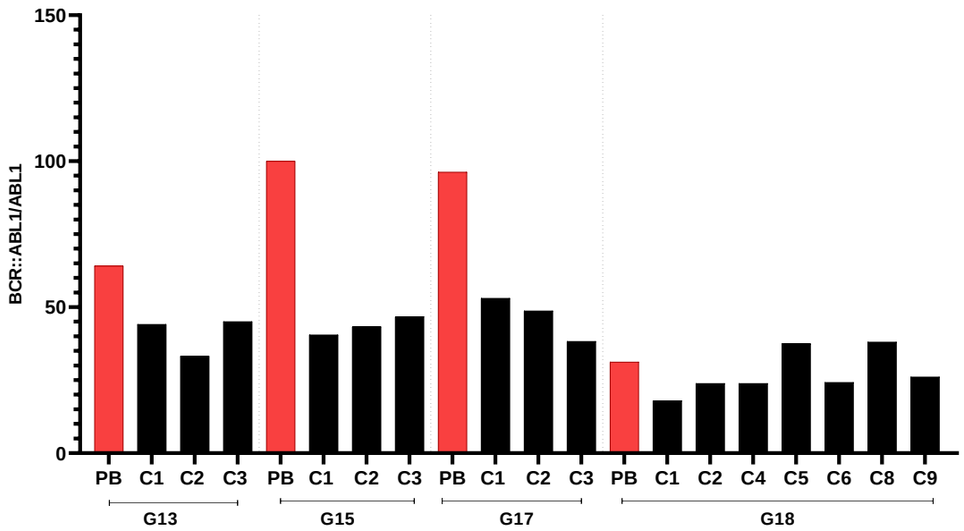
Figure S2

A



Patient ID	Transcript Type
G17	e13a2
G15	e13a2
G18	e13a2
G13	e14a2

B



Patient ID	Clones	Transcript Type
G17	C1,C2 and C3	e13a2
G15	C1,C2 and C3	e13a2
G13	C1,C2 and C3	e14a2
G18	C1,C2,C4,C5,C6,C8 and C9	e13a2
	C7	Nil

C

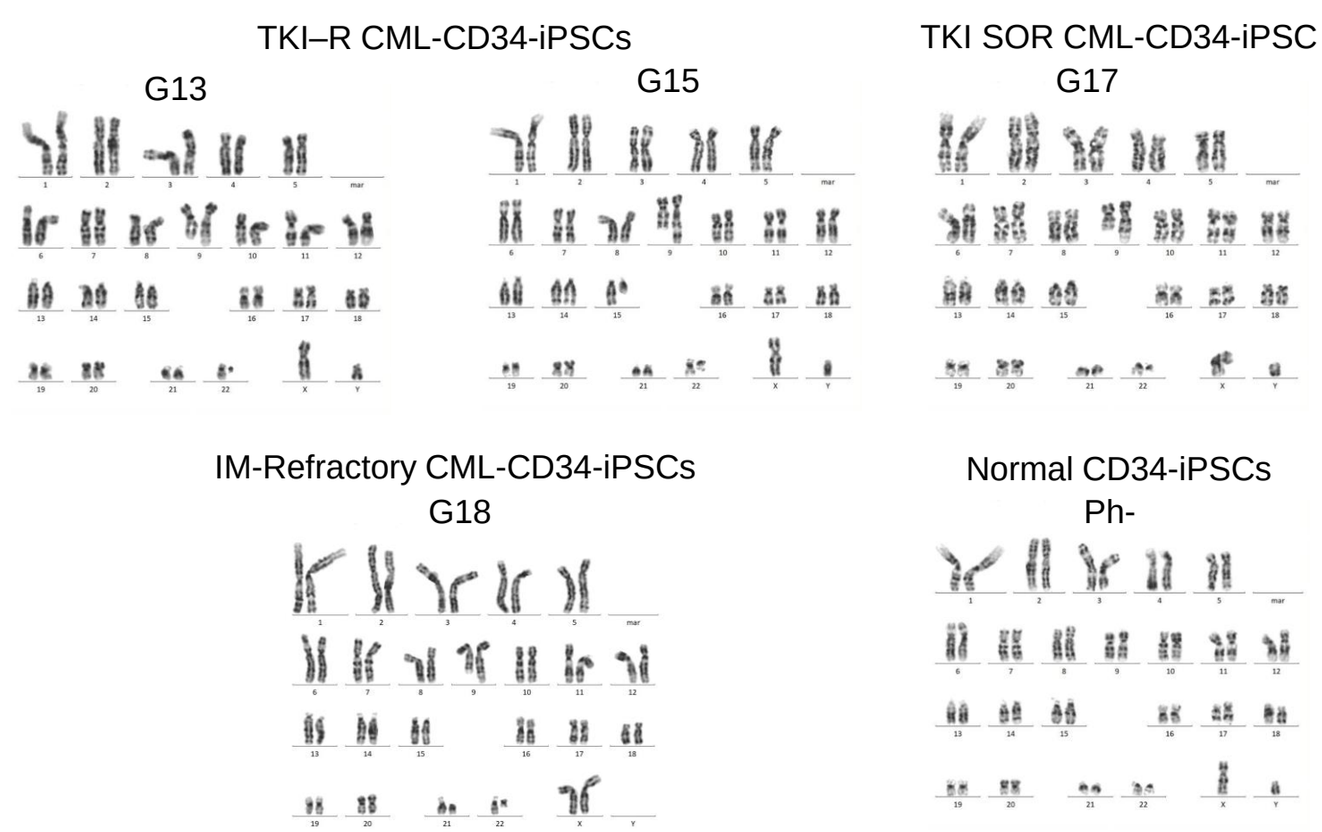


Figure S3:

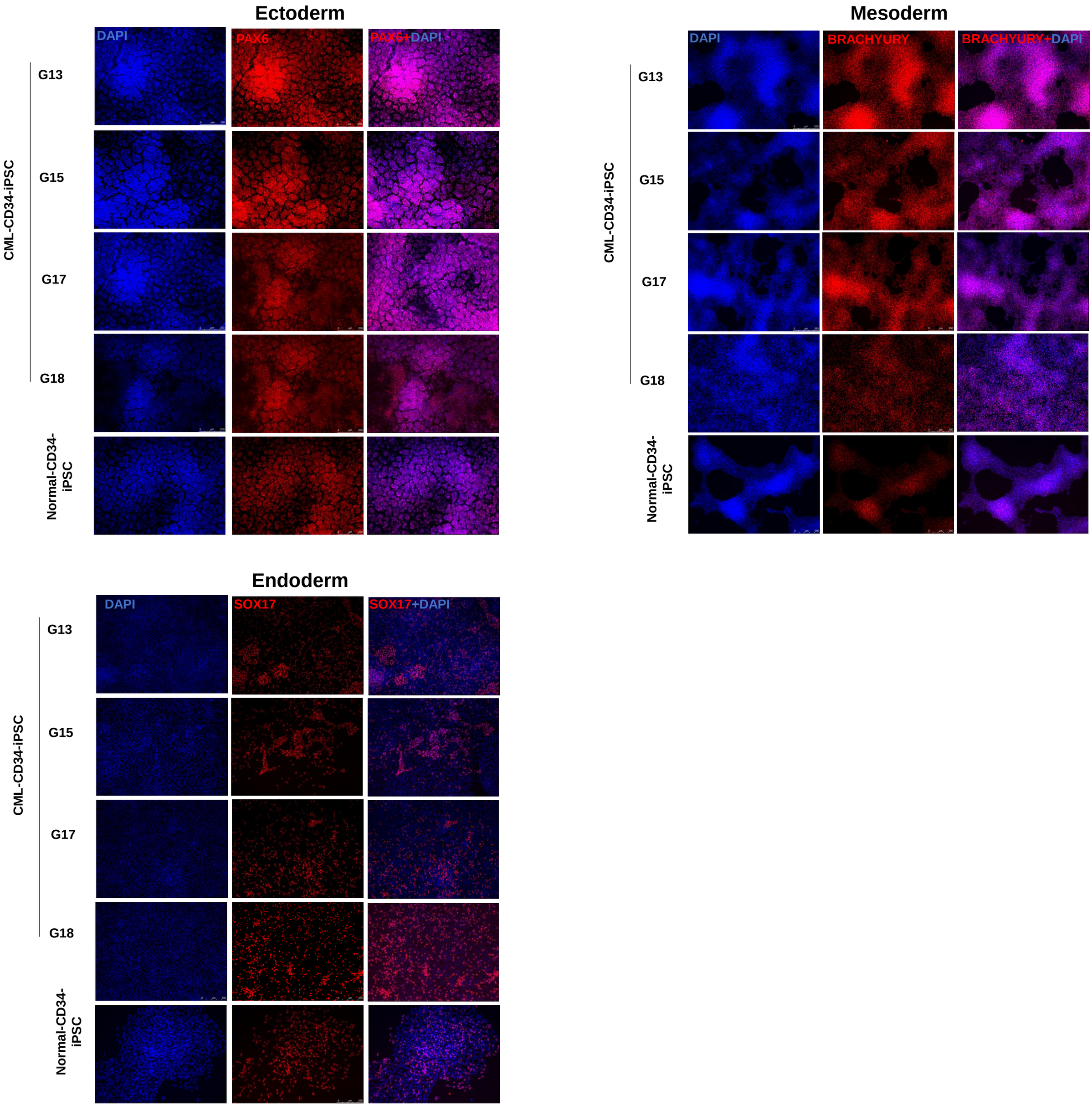
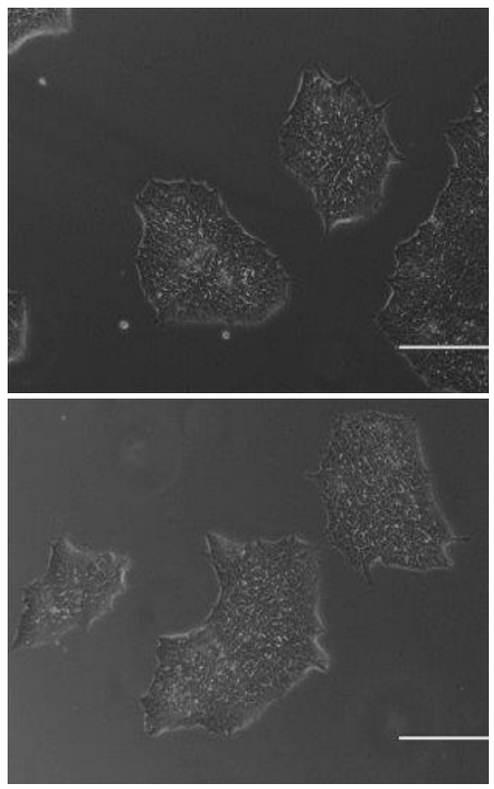

Ectoderm
DAPI
PAX6
PAX6+DAPI
CML-CD34-iPSC
G13
G15
G17
G18
Normal-CD34-iPSC
Mesoderm
DAPI
BRACHYURY
BRACHYURY+DAPI
CML-CD34-iPSC
G13
G15
G17
G18
Normal-CD34-iPSC
Endoderm
DAPI
SOX17
SOX17+DAPI
CML-CD34-iPSC
G13
G15
G17
G18
Normal-CD34-iPSC

Figure S4

Figure S5

A

IM-Refractory iPSC Ph- clone G18
Morphology



B

IM-Refractory iPSC Ph- clone G18
Karyotype

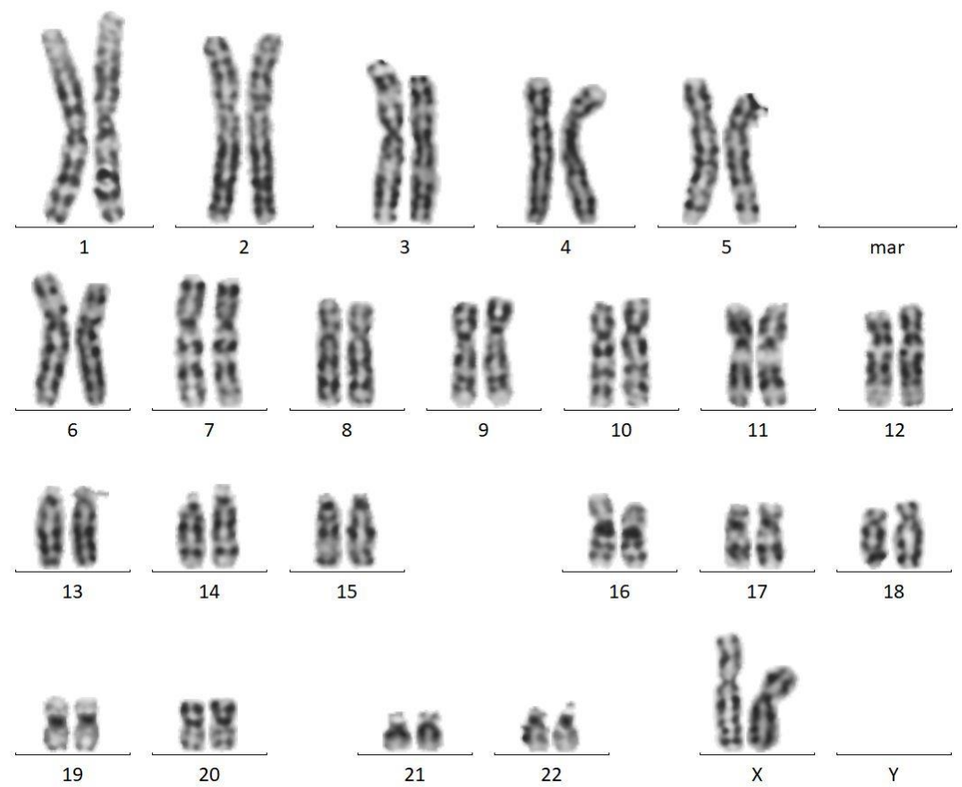


Figure S6

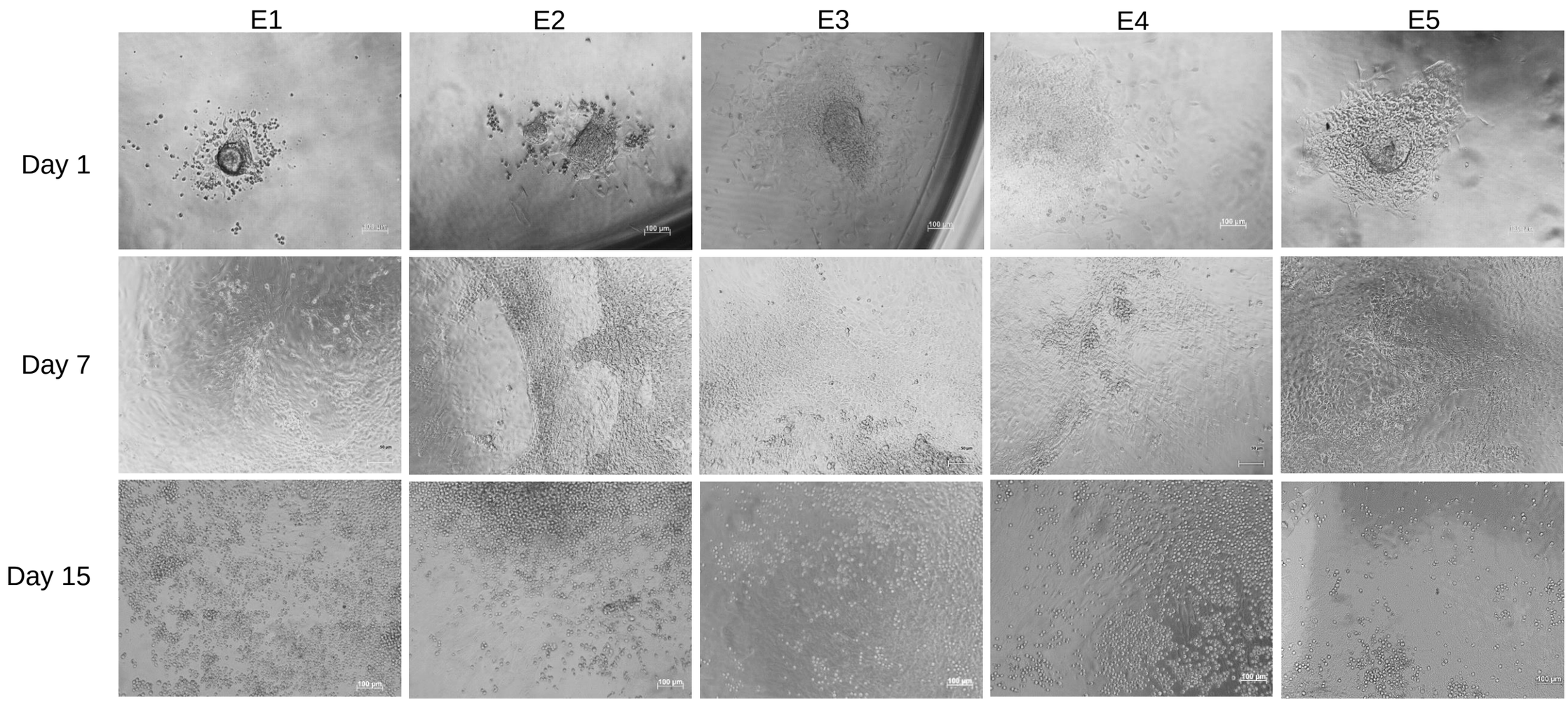


Table S1

Sample ID	Age/Gender	TKI/Dose	Phase	Response status
G13	58/M	DA/50mg	CP	CCYR*
G15	53/M	DA/50mg	CP	SOR**
G17	28/M	DA/50mg	CP	CCYR*
G18	51/F	IM/400mg	CP	REFRACTORY***

****Complete cytogenetic response - <1% BCR::ABL1 cells***
*****Sub-optimal response ->1% BCR::ABL1 cells***
******No hematological response at 3 month evaluation***