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Immunophenotyping and cell sorting of human MKs from human primary sources or differentiated in vitro from hematopoietic progenitors
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1 **TITLE:**

2 **Immunophenotyping and cell sorting of human MKs from human primary sources or**

3 **differentiated *in vitro* from hematopoietic progenitors**

4

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31 **SUMMARY**

32 Here, we present an immunophenotyping strategy for the characterization of megakaryocyte

33 differentiation, and show how that strategy allows the sorting of megakaryocytes at different

34 stages with a fluorescence-activated cell sorter. The methodology can be applied to human

35 primary tissues, but also to megakaryocytes generated in culture *in vitro*.

36

37 **ABSTRACT**

38 Megakaryocyte (MK) differentiation encompasses a number of endomitotic cycles that result

39 in a highly polyploid (reaching even >64N) and extremely large cell (40-60 μm). As opposed to

40 the fast-increasing knowledge in megakaryopoiesis at the cell biology and molecular level, the

41 characterization of megakaryopoiesis by flow cytometry is limited to the identification of

42 mature MKs using lineage-specific surface markers, while earlier MK differentiation stages

43 remain unexplored. Here, we present an immunophenotyping strategy that allows the

44 identification of successive MK differentiation stages, with increasing ploidy status, in human

45 primary sources or *in vitro* cultures with a panel integrating MK specific and non-specific

46 surface markers. Despite its size and fragility, MKs can be immunophenotyped using the

47 above-mentioned panel and enriched by fluorescence-activated cell sorting under specific

48 conditions of pressure and nozzle diameter. This approach facilitates multi-Omics studies,
49 with the aim to better understand the complexity of megakaryopoiesis and platelet
50 production in humans. A better characterization of megakaryopoiesis may pose fundamental
51 in the diagnosis or prognosis of lineage-related pathologies and malignancy.
52
53 **INTRODUCTION**
54 Megakaryocytes (MKs) develop from hematopoietic stem cells (HSCs) following a complex
55 process called megakaryopoiesis, which is orchestrated mainly by the hormone
56 thrombopoietin (TPO). The classical view of megakaryopoiesis describes the cellular journey
57 from HSCs through a succession of hierarchical stages of committed progenitors and
58 precursor cells, leading ultimately to a mature MK. During maturation, MKs experience
59 multiple rounds of endomitosis, develop an intricate intracellular demarcation membrane
60 system (DMS), which provides enough membrane surface for platelet production, and
61 efficiently produce and pack the plethora of factors that are contained in the different
62 granules inherited by mature platelets[1-3]. As a result, mature MKs are large cells (40-60 μm)
63 characterized by a highly polyploid nucleus (reaching even >64N). Recent studies suggest
64 alternative routes by which HSCs differentiate into MKs bypassing traditional lineage
65 commitment checkpoints in response to certain physio-pathological conditions[4-11]. These
66 findings highlight that hematopoietic differentiation towards the mature MK is a continuum
67 and adaptive process that responds to biological needs.
68
69 With the increasing knowledge on the cell biology and the molecular aspects characterizing
70 megakaryopoiesis[12], most of the research dedicated to the study of the process by flow
71 cytometry are limited to the identification of mature MKs using lineage-specific surface
72 markers (*i.e.,* CD42A/B, CD41/CD61), while earlier MK differentiation stages remain
73 unexplored. We previously documented a strategy to stage megakaryopoiesis in mouse bone
74 marrow and bone marrow-derived MK cultures[13,14], which we have adapted and applied to
75 humans[15]. In the present article we show an immunophenotyping strategy that allows the
76 characterization of megakaryopoiesis, from HSCs to mature MKs, in human primary sources
77 (bone marrow -BM- and peripheral blood -PB-) or *in vitro* cultures using a panel integrating
78 MK specific and non-specific surface markers (CD61, CD42B, CD49B, CD31, KIT and CD71,
79 amongst others). Despite its large size and fragility, MKs can be immunophenotyped using the
80 above-mentioned cell surface markers and enriched by fluorescence-activated cell sorting
81 under specific conditions of pressure and nozzle diameter to minimize cell rupture and/or
82 damage. This technique facilitates multi-Omics approaches, with the aim to better
83 understand the complexity of megakaryopoiesis and platelet production in human health and
84 disease. Noteworthy, it will pose as a useful tool to aid diagnosis and prognosis in a clinical
85 context of growing demand.
86
87 In this manuscript we document a strategy to stage human megakaryopoiesis with a panel
88 integrating MK-specific and non-specific surface markers from primary sources or generated
89 *in vitro*. Additionally, we provide a protocol to sort, with a fluorescence-activated cell sorter,
90 the preferred fractions and mature MKs (**Figure 1**). This step is not popular, as it is technically
91 difficult due to the large size and fragility of MKs, although it has been employed both in
92 mouse and human bone marrow samples previously, and due to technological advancement,
93 with a better result each time[16-18]. Human primary sources where MKs or MK precursors can
94 be studied include bone marrow, cord blood and peripheral blood, amongst other. The proper

95 sample processing to isolate the relevant cell fraction for analysis on each sample is of
96 importance. Standard procedures are incorporated, with some considerations to take into
97 account when aiming at the study of megakaryopoiesis.

99 **PROTOCOL**

101 Whole blood and bone marrow samples were obtained and processed in accordance with the
102 1964 Declaration of Helsinki. Whole blood samples were obtained from healthy donors after
103 giving informed consent (ISPA), within a study approved by our institutional medical ethical
104 committee (Hospital Universitario Central de Asturias -HUCA-). Bone marrow samples were
105 obtained from bone marrow aspirate discard material of patients managed at the Dept. of
106 Hematology of the Hospital Clínico San Carlos (HCSC).

108 **[FIGURE 1]**

110 **1.   Whole blood and bone marrow processing prior to immunophenotyping**

112 1.1.   When using whole blood (WB) from donations as a primary source, optionally isolate
113 the peripheral blood mononuclear cell (PBMC) component. This can be achieved by using
114 standard differential centrifugation combined with density gradient cell separation, as
115 previously described.[15]

117 1.1.1. In brief, centrifuge blood at 193 x g for 15 min (brake 3) at room temperature. Discard
118 the upper plasma fraction and collect the buffy ring. Dilute 1:1 with phosphate buffer saline
119 (PBS)/Trisodium Citrate Dihydrate (38 g/L, pH 7) buffer and pipette carefully a volume of 25
120 mL on top of 15 mL of a density gradient solution (1.076 g/mL) in 50 mL tubes.

122 1.1.2. Centrifuge for 20 min at 1114 x g (accelerator 3, brake 3, room temperature). Discard
123 the plasma fraction and collect the buffy ring containing PBMCs. Wash by adding the same
124 volume of PBS, centrifuge at 435 x g for 5 min, and resuspend in PBS for further use.

126 1.2.   Alternatively, use a WB sample (around 100 μL) for immunophenotyping after lysing
127 the red blood cells (RBCs) and thorough washing.

129 1.2.1. In brief, dilute 1:1 in ice cold RBC lysing buffer (4.15 g of NH_4Cl, 0.5 g of $KHCO_3$ and
130 18.5 mg of EDTA (triplex III) to 500 mL of H_2O, pH 7.1-7.4). Wait until the cell suspension
131 becomes translucid red (3-5 min).

133 1.2.2. Centrifuge at 435 x g for 5 min, at 4 °C, and resuspend the cells in PBS. Repeat the
134 procedure as many times as necessary to obtain a white cell pellet.

136 1.3.   Similarly, directly process the samples obtained from bone marrow (aspiration) with
137 RBC lysing buffer (see point 1.2) and thorough washing, as to begin with a clear single-cell
138 suspension (**Figure 1**).

140 1.3.1. Avoid the use of vortexing to mix samples during processing, as it may damage the
141 fragile MKs. Mix by flicking or inverting the tube.

142

143 NOTE: Density gradient to obtain PBMCs may result in a richer and cleaner cell fraction as
144 compared to RBC-lysed WB. However, we should bear in mind that high-density, mature MKs
145 might be lost in the "neutrophil" fraction. This will be discussed in the representative results.
146

147 **2.   In vitro MK differentiation from PBMCs**
148

149 NOTE: MKs can be differentiated *in vitro* from earlier precursors, such as CD34^{+} cells, present
150 in different primary sources (*i.e.,* WB/PBMCs, cord blood, bone marrow) and from iPSCs.
151 There are different protocols that have been applied to this end. Here we use a culture
152 method developed by us that allows MK differentiation from PBMCs, without the need of
153 enriching for CD34^{+} precursors.[15,19-22]
154

155 2.1.   Use three culture phases, where the concentration of thrombopoietin (TPO) gradually
156 increases at the expense of growth factors favoring the proliferation of earlier precursors (i.e.,
157 SCF, EPO), which gradually decrease (**Figure 2**).[15]
158

159 2.1.1. For the basis medium, use StemSpan SFEM supplemented with 0.4% of cholesterol
160 rich lipid mix and 1% Penicillin/Streptomycin.
161

162 2.1.2. For the Phase I medium, use the basis medium supplemented with SCF (100 ng/mL),
163 Erythropoietin (EPO, 0.5 U/mL) and Thrombopoietin (TPO, 30 ng/mL). The Phase II medium is
164 the basis medium supplemented with SCF (50 ng/mL), EPO (0.25 U/mL) and TPO (50 ng/mL).
165

166 2.1.3. For the Phase III medium, use the basis medium supplemented with TPO (100 ng/mL).
167

168 2.1.4. Culture PBMCs in Phase I medium. At day 6-8, place the PBMCs in Phase II medium,
169 and at day 9-12 place the PBMCs in Phase III medium.
170

171 2.1.5. Replace medium by centrifuging cells at 435 x g for 5 min at room temperature from
172 Phase I to Phase II, and at 95 x g for 5 min at room temperature from Phase II to Phase III, and
173 resuspending them in fresh medium.
174

175 2.1.6. Culture cells in an incubator at 37 °C, 5% CO_2. In these primary cultures, MK
176 differentiation lasts 10-14 days, and samples can be drawn at different time points
177 throughout the culture period, as to follow MK differentiation.
178

179 **[FIGURE 2]**
180

181 2.2.   Sample collection: Wash cells by applying low-speed centrifugation 5 min (app. 95 x g)
182 and resuspend them in PBS or PBS containing 1% of bovine serum albumin (BSA). For
183 immunophenotyping, the ideal density is 10^5-10^6 cells/100 μL (see point 3.1). Depending on
184 the status of the cultures (*i.e.,* presence of dead cells, debris, etc.), 1 or 2 washes may be
185 needed. Collect your cells at the time-points of interest during culture.
186

187 **3.   Immunophenotyping of MK differentiation – incubation with a panel of tagged-**
188 **antibodies**

3.1.   Incubate the cell samples with a panel of tagged-antibodies following standard procedures, paying attention to centrifuge at low speed (95 x g) when processing megakaryocytes. We normally incubate the samples with 1% BSA in PBS in volumes of 100 μL at 4 °C, 20 min, with a range of 10^5-10^6 cells.

3.2.   Scale up when necessary.

3.3.   After incubation, add 5 mL of PBS/BSA 1%, centrifuge at low speed (95 x g), aspirate the supernatant and resuspend the sample in PBS/BSA 2% to preserve MK viability (2 mL). Add 1-5 mM EDTA to disrupt cell-cell aggregates (which are naturally seen in MK cultures).

3.4.   Transfer samples into a 12 x 75 mm round bottom tube (FACS tube) or plate, keeping them in the dark until flow cytometry analysis or cell sorting.

3.5.   Preparation of the single antibodies and panel mix; setting up the flow cytometer:

3.5.1.   Titrate the antibodies prior to their use to determine the optimal concentration in the antibody panels. The optimal concentration of antibodies is the lowest concentration that separates clearly positive from negative cells (and allows distinction of intermediate levels of expression). As an example, most of the antibodies are used in a 1:200 dilution (stock 100 μg/mL) unless otherwise titrated or indicated by the manufacturer.

3.5.2.   Once the antibody titration is determined, prepare a 10x dilution of each antibody. These dilutions are used for the single-color controls and for preparing the panel mixes. The dilutions and panel mixes are fine for use even a month after preparation (stored at 4 °C, unless manufacturer´s indications preclude this storage conditions), which allows the staining of samples with the same panel through a time period.

3.5.3.   Use 10 mL per 100 μL of the 10x dilution both for the single-color controls and for the panel mix.

3.5.3.1.   For the single-color controls, use antibody affinity beads, which can be directly measured after adding the antibody. The single-color controls should be measured with every experiment, to allow proper compensation adjustment (and fine-tuning post-measurement with the analysis software).

3.5.3.2.   Alternatively, perform the single-color controls with cell-samples. However, the beads allow the fast measurement of a given number of events, which, depending on the antibody/surface marker, might not be possible to obtain on complex primary or cultured cell sources. We also advise to run cell samples stained with "Fluorescence Minus One" (FMO) panel mixes to set up the appropriate compensation settings (before running experiments). This is relevant as to carefully identify compensation problems, and especially in cultured MKs, to identify autofluorescence interference (which will be present if using culture medium containing phenol red).

236 3.5.4. Prepare enough volume of the panel mix, depending on the number of samples,
237 containing the antibodies of the designed panel. Most of our panels include six antibodies (6-
238 color panels, see **Tables 1-2**).

240 3.5.5. For these panels, use 488-nm and 633-nm lasers of the flow cytometer, however,
241 panels can be adapted to other technical scenarios. Furthermore, the compensation
242 considerations can be obviated when using mass-spectrometry-based flow cytometry or
243 cytometers with acoustic focusing technology.

245 3.5.5.1. Dyes for measuring viability may give false information regarding MKs, especially
246 when they mature. MKs are very active uptaking cells, and positivity with Hoechst, 7-AAD or
247 PE, might not always reflect actual cell death. An alternative (if cell death measurement is
248 required) might be the use of mitochondria stains (CMX Ros) or amine reactive dyes (Zombie
249 or Ghost dyes).

251 **[TABLES 1 and 2]**

253 **4.   Ploidy analysis combined with 6-color panels**

255 4.1.   For ploidy analysis in combination with a 6-color antibody panel, proceed with fixation
256 and permeabilization of cells after incubation with the antibody-panel. This strategy will allow
257 the preservation of the surface marker staining, while allowing the staining of the DNA of
258 cells. We use Hoechst 33342 to stain DNA, as it can be visualized with the available violet 405-
259 nm laser.

261 4.2.   For 10^5-10^6 cells, after incubation with the antibody panel, centrifuge cells (95 x g for
262 5 min), resuspend in 200 mL of fixation buffer and incubate 10 min at room temperature (RT).

264 4.3.   Centrifuge cells as indicated above, resuspended for a second time in 200 mL fixation
265 buffer, and incubate another 10 min at RT.

267 4.4.   Prepare permeabilization buffer, containing 0.1% Triton X-100, 200 mg/mL RNase and
268 20 mg/mL Hoechst 33342 (permeabilization Hoechst MIX).

270 4.5.   Centrifuge cells as above, resuspend them in 300 mL of the permeabilization Hoechst
271 MIX and incubate 30 min at 37 °C. This step is very important, since, in order to obtain a clean
272 ploidy measurement, RNA has to be degraded.

274 4.6.   After the incubation time, measure samples directly with a flow cytometer. Otherwise,
275 keep the samples at 4 °C, in the dark. Measure them promptly. However, since these samples
276 are fixed, measurement can be delayed even 24-48 hours. Be sure the sample is flicked
277 thoroughly prior to measuring, or passed through a cell strainer, to assure a single cell
278 suspension.

280 4.6.1. Morphometric parameters such as FSC and SSC are not maintained after cell fixation.
281 The FSC/SSC plot will show a shrinkage of the cell distribution after fixation. However, the
282 surface marker staining is mostly preserved, and the gating strategy is barely altered, allowing

the analysis of the ploidy status at the different stages of differentiation defined by surface marker combinations.

5.   MK differentiation analysis

NOTE: We have seen that the combination of CD31/CD71 allows to set a number of gates which correspond to different stages of MK differentiation. Further back-gating with MK-specific markers allows the separation of mature and immature MKs. Furthermore, in fresh samples, back-gating to verify the presence of other markers used, or to place the populations in the FCS/SSC axes, refines the assessment of MK differentiation stages and allows to discard other cell types that could be present on the same populations.

5.1.   Use a panel of antibodies that includes early precursor markers (KIT, CD34), common precursor markers (CD31, CD71), and lineage markers, some of them specific (CD42A/CD42B, CD49B, CD41/CD61, CLEC2, GPVI, etc) (see **Tables 1-2**). The use of Lineage (Lin) cocktail (CD3, CD14, CD16, CD19, CD20, and CD56), also allows to "filter out" mature hematopoietic cells that might add noise to the analysis (when selecting the Lin⁻ population). As an example, we will go through the analysis of MKs in PBMCs, bone marrow and through PBMC-derived cell cultures (see representative results).

6.   MK and MK precursor cell sorting

NOTE: The stained cells were analyzed and sorted on a fluorescence-activated cell sorter FACS Aria IIu equipped with 488-nm and 633-nm standard solid-state lasers using FACSDiva software; data were additionally analyzed and presented using FlowJo software and Cytobank (viSNE analysis). Purity of sorted fractions was confirmed by flow cytometry analysis of each of the sorted fractions (purity above 85%).

6.1.   Perform cell sorting as soon as possible or within 1 hour after antibody incubation in order to avoid cell deterioration.

6.2.   Filter the sample with a 100 μm cell strainer to assure single cell suspension and the integrity of large MK.

6.3.   Use a 130-μm ceramic nozzle, a sheath pressure set to 11 pounds per square inch (PSI) and the drop-drive frequency set to 12 kHz to break the stream into drops.

6.4.   Prior to sorting, sterilize the nozzle, sheath, and sample lines by performing a 30 min acquisition of Penicillin/Streptomycin diluted 1:5 in sterile water, followed by a 10 min acquisition with sterile water to remove remaining decontaminant.

6.5.   Once the stream has stabilized, adjust the drop-delay with recommended beads in order to sort in fine tune mode more than 97.5% of the reflected drops at a flow rate of 400-1200 events per second.

6.6.   Prepare collection FACS tubes with 500 μL of PBS/BSA 2%. The percentage of BSA can be increased up to 5-10%.

330
331 6.7.   Generate the experiment template with the proper compensation matrix parameters.
332
333 6.8.   Load FACS tube into cytometer.
334
335 6.9.   Perform a measurement of the sample to set the desired gates and purity of the target
336 cell populations. Maintain the record activated to show up to 200,000 events in the selected
337 population gates during the cell-sorting.
338
339 6.10.   FACS Aria IIu allows the separation of up to 4 different cell populations at the same
340 time. Create a new sort layout and select the collection device (4 tubes) and appropriate
341 precision mode (intermediate mask of purity and recovery is recommended). Finally, add the
342 population(s) of interest to each sort location field (**Figure 3**).
343
344 **[FIGURE 3]**
345
346 6.11.   Load the collection tubes and start to sort the target populations.
347
348 6.12.   Centrifuge the collection tubes for 5 minutes at 95 x g and resuspend the cell pellet in
349 the appropriate volume of 2% BSA in PBS.
350
351 6.13.   Measure again a fraction of each sorted sample, to calculate the purity.
352
353 6.14.   Store cells appropriately for further use. Sorted cells can be used for cytological and
354 molecular analyses, or may be re-cultured with the aim of studying the differentiation process
355 of a selected cell population.
356
357 **7.   Post-sort sample preparation**
358
359 7.1.   Preparing cytospins for cytological analysis with a cytocentrifuge
360
361 7.1.1. Bring sorted cells to an easy-to-handle working volume of 100-200 µL. Take into
362 consideration that cell density will depend on the sorted population yield in each case.
363
364 7.1.2. Place a clean slide on the metal holder and place a filter top. Remember to label the
365 slide and the filter to avoid mixing samples.
366
367 7.1.3. Add 100 µL PBS on the filter hole against the slide, so the filter gets humidified on the
368 hole-rim.
369
370 7.1.4. Place the funnel, close the metal holder and place it on its spot in the centrifuge.
371
372 7.1.5. Add the sample (100-200 µL) inside the funnel.
373
374 7.1.6. Centrifuge at 36 x g for 5 minutes. The cytospin slides can be allowed to air dry at RT
375 (properly covered to prevent dust) and can be kept at RT for 1 week prior to the
376 immunostaining or histochemistry.

377
378 7.2.   For immunostaining

379
380 7.2.1. Fix slides in 2% paraformaldehyde (PFA) diluted in PBS and incubate during 5min.

381
382 7.2.1.1. A range of 0.5-4% PFA/PBS may be used. In our hands, we use 4% to obtain proper
383 fixation of tissues or some cell types, and 0.5% PFA/PBS is sufficient for platelets. When
384 setting this technique up, the right percentage of PFA requires optimization per cell
385 type/source.

386
387 7.2.2. Incubate 5 min in PBS.

388
389 7.2.3. Incubate 5 min in 50% EtOH.

390
391 7.2.4. Incubate 5 min in 70% EtOH.

392
393 7.2.5. Store in 70% EtOH at -20ºC.

394
395 7.2.6. When performing the immunostaining, rehydrate, and follow standard procedures
396 (permeabilization, washing, blocking, primary and secondary antibody incubations,
397 preserving, etc).

398
399 7.3.   For cytochemistry:

400
401 NOTE: The slides can be stained with May-Grünwald Giemsa staining or the convenient
402 staining for each purpose.

403
404 7.4.   For immediate morphology examination

405
406 7.4.1. Add one drop of mounting medium on the cell-containing spot of the cytospin and
407 place a coverslip.

408
409 7.4.2. Keep slides at 4 °C not longer than a week, unless sealed, which allows long-term
410 storage even at RT. Mounting medium fixation allows long-term storage at RT.

411
412 **REPRESENTATIVE RESULTS**
413 **Bone Marrow and Ploidy**
414 In **Figure 4** we show a representative immunophenotyping analysis of megakaryopoiesis in
415 BM samples (aspiration) from patients. When plotting the cellular fraction against CD71 and
416 CD31, we have gated six main populations: CD31$^-$ CD71$^-$ (red), CD31$^-$ CD71$^+$ (blue), CD31$^+$
417 CD71$^-$ (orange), CD31$^+$ CD71^{mid} (light green), CD31$^+$ CD71$^+$ (dark green) and CD31^{++} CD71^{mid}
418 (cream). These populations are not always present in the same positions (considering
419 constant cytometer settings), as it can be seen, and that should be taken into account. This
420 might be inherent to the pathological condition and bone marrow status. Back-gating these
421 six populations overlaid in histograms against a maturation marker contained in the panel
422 (CD42A/CD42B), and to the Forward Scatter, is shown on the right. The figure also shows a
423 plot against CD31/CD71 depicting an overlay of the CD42B$^+$ cells with the total cellular fraction

as to visualize the distribution of the more mature MKs. In general terms, the population CD31$^-$ CD71$^-$ does not contain MKs or lineage precursors, does not present MK maturation markers, and is smaller in size. The CD31$^-$ CD71$^+$ population contains mainly cells of the erythroid lineage, although it can also contain common lineage precursors, as we hypothesize from our observations. It remains negative for MK maturation markers, and depending on the proportion of earlier or later erythroid cells, the Forward Scatter might fluctuate as well.

As an example, the MDS patient has a larger proportion of immature (i.e., larger) erythroid cells, compared to other patients. MK progenitors and mature cells will distribute in the CD31$^+$ cells, with some variation. However, as it can be seen comparing the BM analysis on each pathology, there are some constant features. More mature MKs are present in the CD31^{++} CD71^{mid} population (cream), and pathologies where this maturation is "blocked" include Immune Thrombocytopenia (ITP) and a reactive BM sample (with underlying inflammation). While in the lymphoma patient there seems to be an equilibrium between early precursors and late MKs (orange and cream gates), this proportion is altered in the MDS patient (with more "blocked" precursors) and in the AML patient (with more mature MKs). Of interest, the "blockade" seems to be different comparing pathologies amongst themselves: in pathologies concurring with underlying inflammation the blockade might be due to a combination of maturation defects, destruction (i.e., autoimmunity) and platelet production by MK rupture.

[FIGURE 4]

We next set out to analyze the ploidy status of the above-mentioned populations on human BM samples. **Figure 5** shows an increasing ploidy status within the CD31$^+$ populations, that we foresee will be different and characteristic for each pathological context. Note that due to the cell fixation and permeabilization used in these experiments, the dot plots are not completely comparable to those of unfixed, unpermeabilized samples (**Figure 4**).

[FIGURE 5]

PBMCs and Cell Culture
In **Figure 6** we show a representative immunophenotyping analysis of PBMCs and the MK culture set with those PBMCs at day 7 and day 11. We use a panel containing the Lin cocktail, and we show the cellular fraction of live cells before and after Lin$^-$ selection. This negative selection might result in the loss of some fraction of MKs co-expressing, for example, CD14, but it also allows a more refined analysis. When plotting the Lin$^-$ fraction against CD71 and CD31, we gated five main populations: CD31$^-$ CD71$^-$, CD31$^-$ CD71$^+$, CD31$^+$ CD71$^-$, CD31$^+$ CD71^{mid} and CD31^{++} CD71^{mid}. These populations are not always present in the same positions (considering constant cytometer settings), as it can be seen, and that should be taken into account. In this case, the differences are not only inherent to the individual health or pathological status, but also to the MK differentiation capacity of the precursors in the PBMC fraction. Back-gating these five populations overlaid in histograms against other markers contained in the panel (KIT and CD42B), and to the Forward Scatter, is shown on the right. MK precursors and MKs at different stages of maturation are within the CD31$^+$ populations.

[FIGURE 6]

471   **[TABLE 3]**

472

473   In PBMCs, MKs are within the CD31^{++} CD71^{mid} gate, although their size is not as large as we
474   have seen with cultured MKs. This might be due to either one of the two following options:
475   MKs in PBMCs represent a fraction of immature MKs that enter the circulation, or a fraction
476   of mature circulating MKs that have lost cytoplasmic complexity. Our sort experiments
477   suggest that the latter might be the reason explaining that size difference. Supporting this
478   notion, the expression of CD42B, is not present in 100% of the cells on those populations (as
479   it can also be seen in BM samples in **Figure 4**), probably due to the loss of surface markers
480   upon proplatelet formation and/or platelet shedding. However, in cultured samples, the MKs
481   in the "mature" gates, are almost 100% CD42B positive, and larger in size. These hypothesized
482   cellular dynamics should be further studied and validated.

483

484   Due to the pleiotropic effects of TPO, these cultures are heterogeneous and asynchronous,
485   which *per se* makes it difficult to further analyze discrete MK differentiation stages.[19,20]
486   Following the expansion of earlier progenitors, MKs at different maturation stages will appear
487   in the culture from day 6-10 (or earlier, due to donor variability) and will gradually increase in
488   numbers as the MK lineage-committed cells mature towards MK. Some MKs will undergo
489   terminal differentiation and start forming proplatelets. Towards the end of the culture, there
490   will be a gradual increment of cell loss due to "extenuation/exhaustion" after proplatelet
491   formation and platelet shedding or cell death (**Figure 2**).

492

493   If using TPO analogues different than recombinant TPO, the concentrations should be tested.

494

495   The source of progenitors (adult, cord blood) will condition the cultures, as MKs from sources
496   of different developmental stages have their own characteristics. Furthermore, cultures from
497   enriched CD34$^+$ progenitors, while appearing more homogeneous at the beginning of the
498   culture, will reach a stage of heterogeneity and asynchronicity once MK commitment and
499   differentiation commences.[19,20]

500

501   **MK Cell Sorting**
502   In **Figure 7** we show a representative gating strategy of a sample of MK *in vitro* culture at day
503   10 of differentiation. When plotting the nucleated cell fraction against CD31 and CD71 or the
504   Forward Scatter, we can distinguish two main populations characterized by the presence or
505   absence of CD31.

506

507   When plotting the CD31$^+$ fraction against CD41 and CD71, we can gate four main populations:
508   CD41$^-$ CD71$^-$ (1), CD41^{low} CD71$^+$ (2), CD41^{low} CD71^{+++} (3) and CD41^{+++} CD71$^+$ (4), in which other
509   surface markers (KIT and CD49B) were expressed differentially, allowing the identification of
510   the MK compartment as CD41 highly positive cells (**Figure 7A**). The purity of the sorted
511   fractions is shown, as well as cytological stainings of cytospins of the sorted fractions.

512

513   In addition, to characterize the cell population spectrum in PBMC-MK cultures, viSNE analysis
514   was employed (**Figure 7B**). The viSNE map separates cells into spatially distinct subsets based
515   on the combination of markers that they express. Each point in the viSNE analysis represents
516   an individual cell colored according to the expression levels of CD31, CD42A, CD71 and KIT.

517

518   Double negative cells (CD31$^-$ and CD71$^-$) are mainly residual lymphocytes (population 5) that
519   persist throughout the culture in these conditions. This population has been sorted in a
520   second round of cell sorting using the remaining fraction of the sample.
521
522   Gates 6 and 7 are CD71 mid and high expressing cells, which include erythroid progenitors
523   and, potentially, other precursors.
524
525   We must take into account the technical difficulties due to the cell heterogeneous nature of
526   this culture method and the sticky nature of mature MKs that results in the presence of
527   mature MKs in subpopulations where they do not belong (see remarks section).
528
529   **[FIGURE 7]**
530
531   **Post-sort processing**
532   The sorted populations can be analyzed by different methods, including cytological and
533   molecular analysis by immunofluorescence (see section 7) in order to evaluate the MK
534   features in a specific physio-pathological context, or to better study the process of
535   megakaryopoiesis. Some examples are presented (**Figure 8**).
536
537   **[FIGURE 8]**
538
539   Remarks:
540   MKs can be sorted from unexpected regions due to the capacity to attach cells to their
541   membrane (**Figure 9**). This issue can be a problem to get high purity cell subpopulations. To
542   minimize cell-cell aggregates, 1-5 mM EDTA can be added to the panel mix buffers.
543
544   Furthermore, when using WB as starting material, we proposed either obtaining PBMCs or
545   lysing RBCs to use the cellular fraction directly. In **Figure 10** we show flow cytometry analysis
546   of the two methodologies. On one hand, when lysing RBCs and analyzing the whole cellular
547   compartment, we still have a large amount of neutrophils. These can be filtered out with a
548   specific marker (or using the Lin cocktail strategy). However, we may lose some MKs due to
549   their promiscuous nature related to surface marker expression. On the other hand, PBMC
550   isolation, will allow us to get rid of neutrophils as well as RBCs, although we may lose the MKs
551   with higher density (i.e., the ones with more complex cytoplasmic features or more
552   immature). As shown in **Figure 10**, MKs from lysed WB show a higher expression of
553   maturation markers, which appears lower when isolating PBMCs. The density gradient might
554   result in the loss of the MKs that still contain a complex cytoplasm, while the ones contained
555   in the PBMC fraction, might be already "exhausted" in the circulation after releasing platelets,
556   and thus maintaining the polyploid nucleus within a less complex cytoplasm. They should not
557   be confused with immature MKs. Each procedure has its advantages and its drawbacks.
558
559   **[FIGURE 9]**
560
561   **[FIGURE 10]**
562
563   **FIGURE LEGENDS**

Figure 1: Schematic representation of the protocol documented in this manuscript. The primary human sources or primary cultures where MK differentiation can be staged by using immunophenotyping are indicated. This immunophenotyping strategy can be applied to the study of the process in different lineage-related pathologies or malignancy in primary sources. In addition, it makes possible the cell sorting of MKs and precursors with a fluorescence-activated cell sorter, which allows further analysis of enriched fractions. Images used are part of Servier Medical Art (SMART) by Servier and are licensed under CC BY 3.0.

Figure 2: Schematic representation of the PBMC-derived MK culture method. PBMCs from healthy donors were cultured according to the three-phase protocol developed by us to generate MK *in vitro* (scheme adapted from Salunkhe et al).[15] Pictures taken at day 10 and day 13 of culture are shown. Pictures are taken with a 20X objective.

Figure 3: Schematic representation of the principle of fluorescence-activated cell sorting (FACS). The particles go through the 130 µm-nozzle and are forced to break up into a stream of regular droplets due to the application of vibration to the nozzle. Next, the droplets are interrogated by the laser (point of analysis) and the signals are processed to give the "sort decision" by applying a charge to those droplets. When a charge droplet passes through a high voltage electrostatic field (detection plate), it is deflected and collected into the corresponding collection tube.

Figure 4: Immunophenotyìng of megakaryopoiesis in human bone marrow samples from patients with different pathologies. Representative bone marrow (BM) samples from patients diagnosed with lymphoma (i.e., normal BM), myelodysplastic syndrome (MDS), acute myeloid leukemia (AML), immune thrombocytopenia (ITP) and a patient with a reactive BM due to an infection were analysed. When plotting the cellular fraction (i.e., nucleated cells) against CD71 and CD31, we have gated six main populations: CD31- CD71- (red), CD31- CD71+ (blue), CD31+ CD71- (orange), CD31+ CD71mid (light green), CD31+ CD71+ (dark green) and CD31++ CD71mid (cream). These populations are not always present in the same positions (considering constant cytometer settings), as it can be seen, and that should be taken into account. Back-gating these six populations overlaid in histograms against a maturation marker contained in the panel (CD42A/CD42B), and to the Forward Scatter, is shown on the right. The figure also shows a plot against CD31/CD71 depicting an overlay of the CD42B+ cells with the total cellular fraction as to visualize the distribution of the more mature MKs. Numbers matching the gates in the dot plot and the histogram overlays are depicted.

Figure 5: Ploidy analysis of the populations selected based on CD31/CD71 expression, in human BM samples. Representative bone marrow (BM) samples from patients diagnosed with acute myeloid leukemia (AML) and immune thrombocytopenia (ITP). When plotting the cellular fraction (i.e., nucleated cells) against CD71 and CD31, we have gated six main populations: CD31- CD71- (red), CD31- CD71+ (blue), CD31+ CD71- (orange), CD31+ CD71mid (light green), CD31+ CD71+ (dark green) and CD31++ CD71mid (cream). These populations are not always present in the same positions (considering constant cytometer settings), as it can be seen, and that should be taken into account. Back-gating these six populations overlaid in histograms against Hoechst 33342 shows the different ploidy status of the populations, and the general tendency to increase ploidy with maturation (although MKs can reach maturity

610 without polyploidization). Numbers matching the gates in the dot plot and the histogram
611 overlays are depicted.
612
613 **Figure 6: Immunophenotyping of megakaryopoiesis during MK cell culture, including the**
614 **starting PBMC material.** Representative immunophenotyping analysis of PBMCs and the MK
615 culture set with those PBMCs at day 7 and day 11. We use a panel containing the Lin cocktail,
616 and we show the cellular fraction (i.e., nucleated cells) before and after Lin- selection. This
617 negative selection might result in the loss of some fraction of MKs co-expressing, for example,
618 CD14, but it also allows a more refined analysis. When plotting the Lin- fraction against CD71
619 and CD31, we gated five main populations: CD31- CD71-, CD31- CD71+, CD31+ CD71-, CD31+
620 CD71mid and CD31++ CD71mid. These populations are not always present in the same
621 positions (considering constant cytometer settings), as it can be seen, and that should be
622 taken into account. Back-gating these five populations overlaid in histograms against other
623 markers contained in the panel (KIT and CD42B), and to the Forward Scatter, is shown on the
624 right. MK precursors and MKs at different stages of maturation are within the CD31+
625 populations. The figure also shows a plot against CD31/CD71 depicting an overlay of the
626 CD42B+ cells with the total cellular fraction as to visualize the distribution of the more mature
627 MKs. Numbers matching the gates in the dot plot and the histogram overlays are depicted.
628
629 **Figure 7: Pre-sort immunophenotypic analysis of 10-day in vitro culture MKs. A)**
630 Representative gating strategy using CD31, CD41 and CD71, with two sorting rounds, resulting
631 in CD31$^+$ populations 1-4 and CD31$^-$ populations 5-7. The purity of each sorted fraction was
632 analysed after sorting, and is depicted, together with a representative microphotograph of
633 cytological analysis of cytospins from each sorted fraction. **B)** viSNE map of day 10 PBMC-
634 derived MK culture cells measured by flow cytometry. The map is constructed using the
635 expression levels of CD31, CD42B, CD71 and KIT, as measured by flow cytometry.
636
637 **Figure 8. Post-sort MK analysis. A)** Cytological analysis of sorted MK compartment from a 10-
638 day in vitro culture. May-Grünwald Giemsa staining allows the observation of key features of
639 mature MKs, i.e., pseudopod formation (red arrow), highly polyploid MK (blue arrow). **B)**
640 Immunofluorescence analysis. Cells were stained with anti-human von Willebrand factor
641 (shown in red, secondary antibody Alexa Fluor 555 Goat anti-mouse IgG), anti-human CD31
642 or tandem CD41/CD61, both conjugated with FITC (shown in green) and nuclear DNA was
643 labeled with DAPI (shown in blue).
644
645 **Figure 9. The sticky nature of MKs.** MKs can be found in unexpected gates due to their
646 capacity to attach cells to their membrane. **A-C)** MKs sorted in populations 1 **(B)** and 5 **(A and**
647 **C),** according the gating strategy for MK sorting. This issue can be a problem to get high purity
648 cell subpopulations.
649
650 **Figure 10. Flow cytometry immunophenotyping of MKs from PBMCs or WB after lysing the**
651 **RBCs. A)** Gating strategy. **B)** Size (FSC), cellular complexity (SSC) and expression of CD42A,
652 CD41, CD71 and KIT in MKs as identified in PBMCs or WB.
653
654 **DISCUSSION**
655 Most of the research focusing on the study of megakaryopoiesis by flow cytometry is to date
656 limited to the identification of MK subsets using only lineage-specific surface markers (i.e.,

CD42A/CD42B, CD41/CD61), while earlier MK differentiation stages have been poorly examined. In the present article we show an immunophenotyping strategy to address a comprehensive flow cytometry characterization of human megakaryopoiesis. Overall, we would like to highlight the utility of combining MK specific and non-specific surface markers (Tables 1 and 2) in the same flow cytometry antibody panel for the study of megakaryopoiesis in a more detailed way. Our novel approach could serve as a powerful tool to aid diagnosis and/or prognosis in hematological disorders. We consider that the presented panels and combinations are not definitive. We have observed a very dynamic behavior of surface markers in human primary sources (mainly in BM samples), which is also dependent on the subjacent pathology. More studies are required, and hopefully, the scientific community will start applying this approach as to feedback with observations from studies focusing on a single pathology and how megakaryopoiesis staging may aid at diagnosis or prognosis. The implementation of combined HSC and MK characterization in primary tissues, considering the diverse routes for MK commitment as commented in the introduction section, is of relevance and worth of further developments. The fact that we are also able to detect MKs in PBMCs is quite an observation. We also hypothesize, that the analysis of MKs in this primary source will aid in disease diagnosis and prognosis, as a very relevant alternative to BM aspirates, as extraction of a blood sample is not as invasive. That aspect also requires development.

Despite the technical limitations associated to the biological characteristics of MKs (large size, osmotic fragility, stickiness) we were able to sort different maturation stages in several human MK sources, specifically in bone marrow, PBMCs and PBMCs-derived cell cultures. Future studies will be conducted in order to analyze the peculiarities of megakaryopoiesis in other human sources such as cord blood and in different physio-pathological situations. In addition, the differentiation potential of each gated and sorted cell fraction should be evaluated in colony-forming unit (CFU) assays of MK progenitors (CFU-MK) as to objectively assess the differentiation potential of each population in the megakaryopoiesis journey. While surpassing the technical limitations that were once thought as to impede the sort of mature megakaryocytes, such a necessity still demands more perfection. On one hand, single-cell Omics would allow comprehensive studies when applying the immunophenotyping panels, but if physical sort is required, despite the purity of the sorted populations is above 85% (based on surface marker expression), other variables need to be taken into account, which are still difficult to solve. As mentioned, MK cultures are not synchronous and are very heterogeneous. MKs themselves are promiscuous in surface marker expression, sharing markers with other myeloid and non-myeloid hematopoietic and non-hematopoietic cells. Furthermore, they are very difficult to cluster with the morphometric variables (Forward and Side Scatter) as they distribute widely. Further studies are required to refine this application, but with collaborative efforts, we are closer to narrow down megakaryopoiesis.

Acknowledgements

15

711

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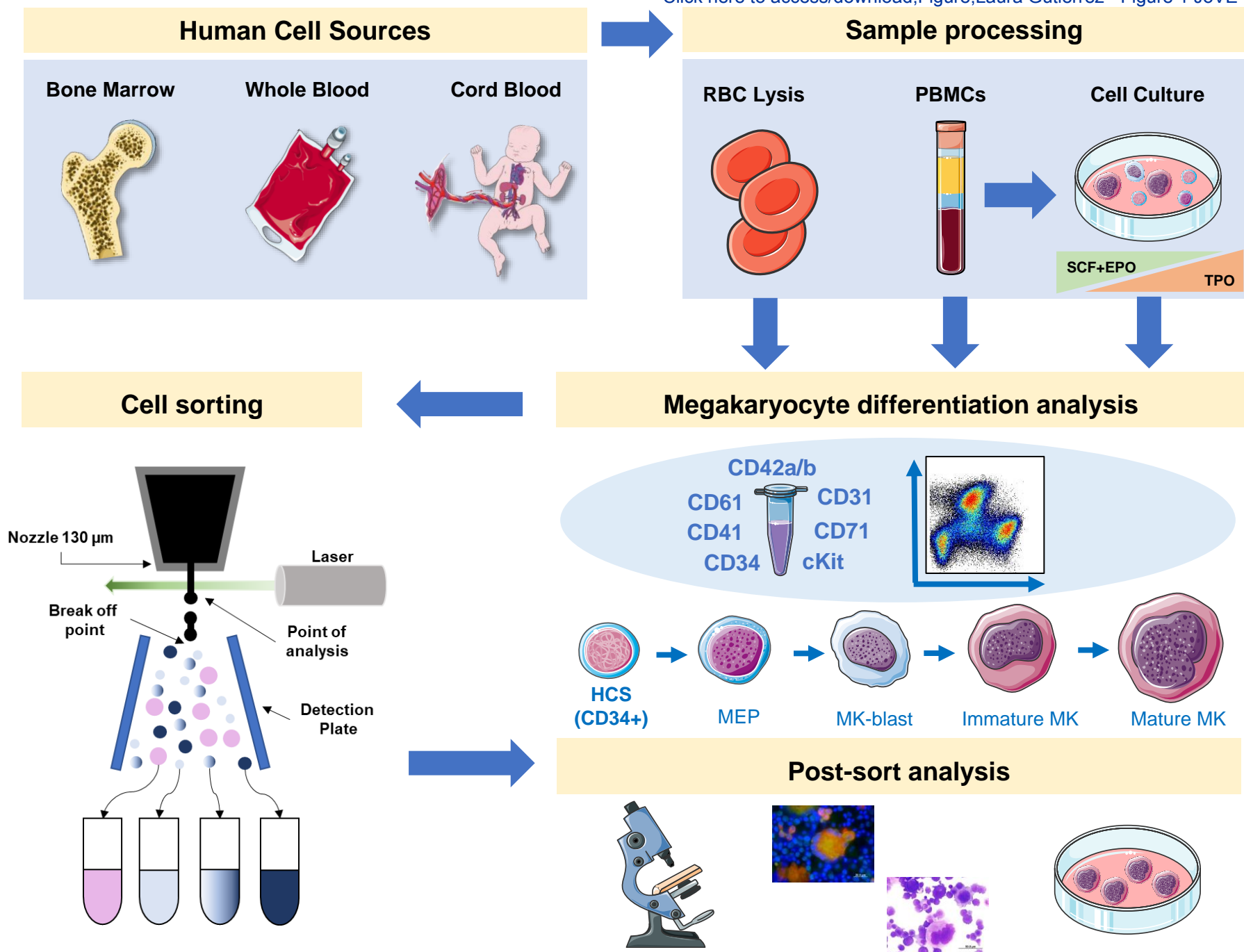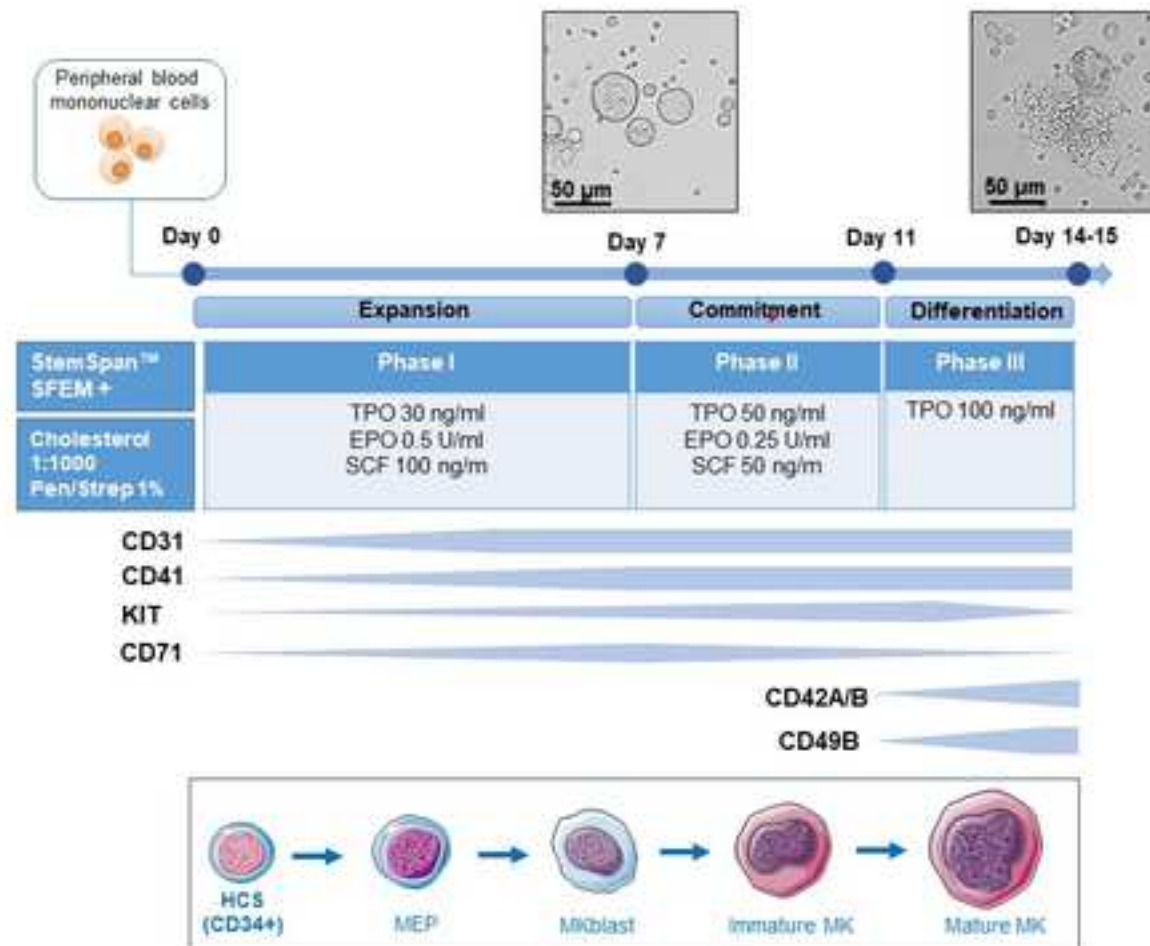762   10.1055/s-0040-1719028 (2020).
763

Figure 1

Human Cell Sources

Bone Marrow   Whole Blood   Cord Blood

Sample processing

RBC Lysis   PBMCs   Cell Culture

SCF+EPO   TPO

Cell sorting

Nozzle 130 µm

Laser

Break off point

Point of analysis

Detection Plate

Megakaryocyte differentiation analysis

CD42a/b
CD61   CD31
CD41   CD71
CD34   cKit

HCS (CD34+)   MEP   MK-blast   Immature MK   Mature MK

Post-sort analysis

Figure 2

Adapted from *Culture of Megakaryocytes from Human Peripheral Blood Mononuclear Cells. Salunkhe V., et al. Bio-Protocol 2015*
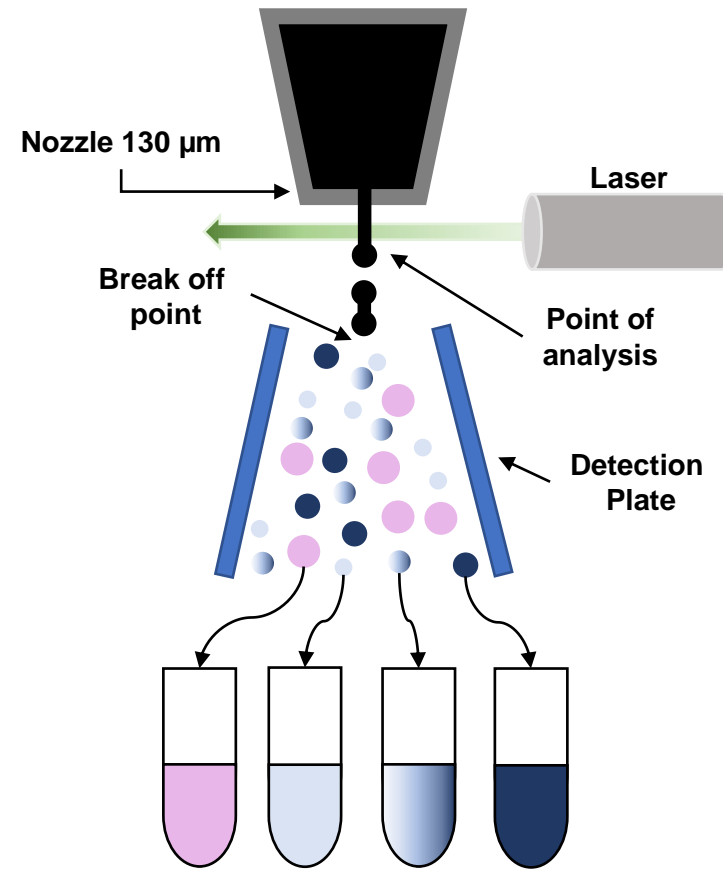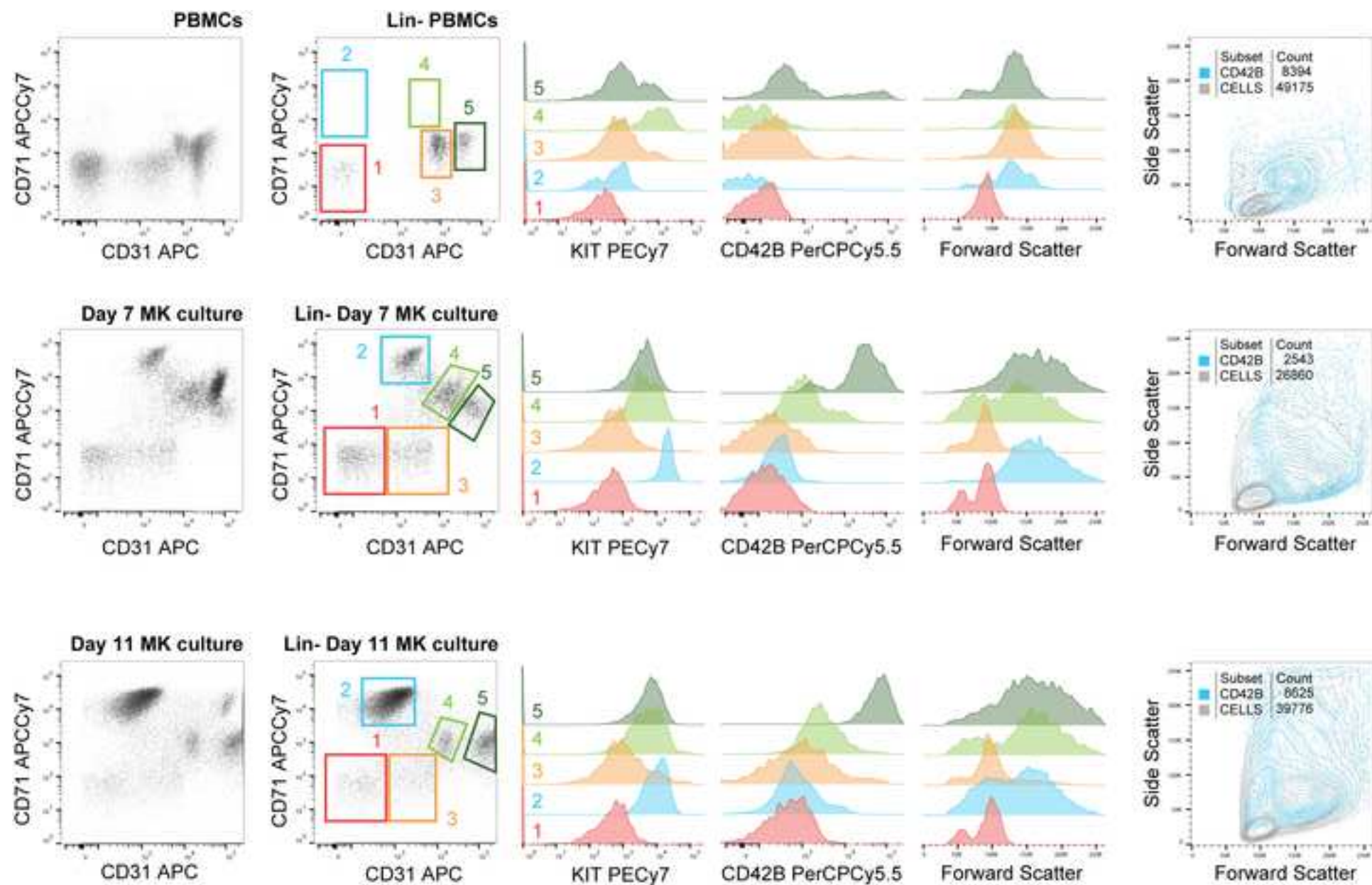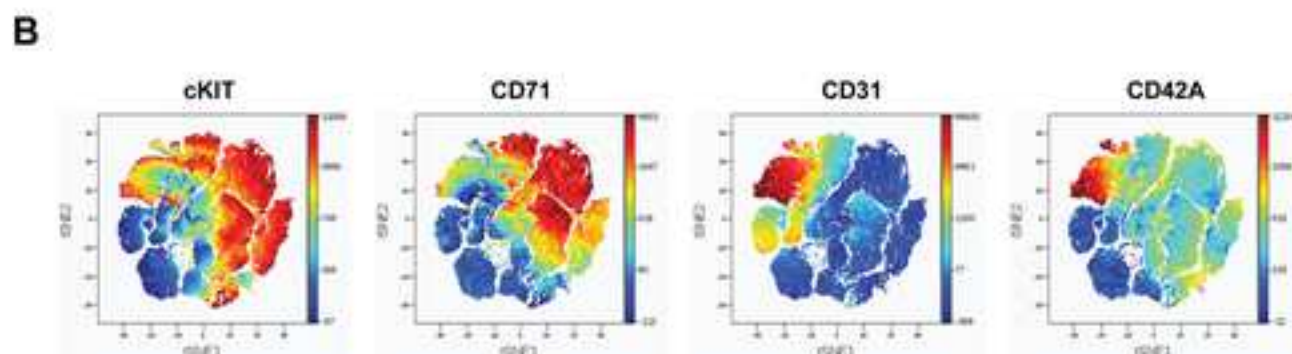
Figure 3
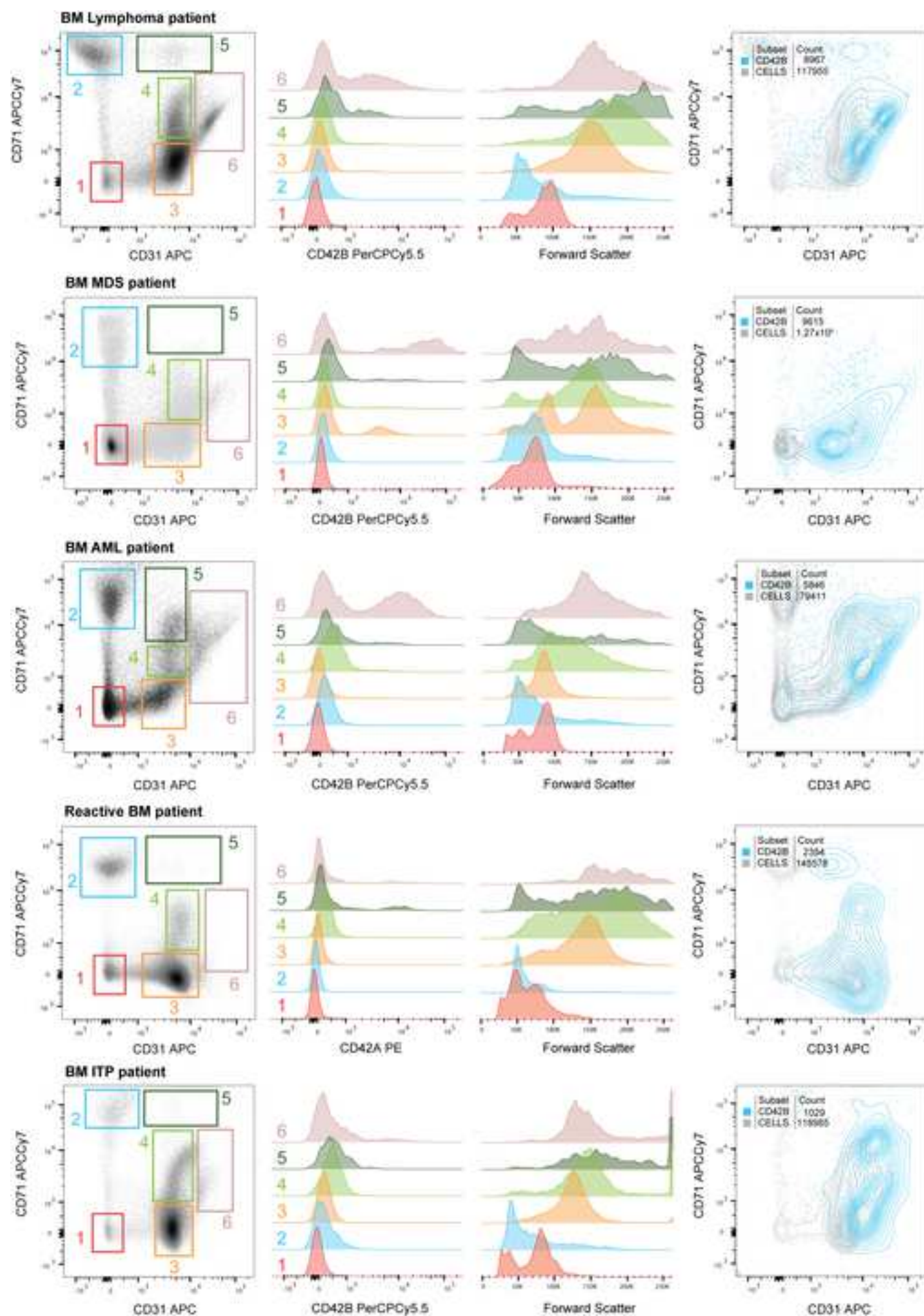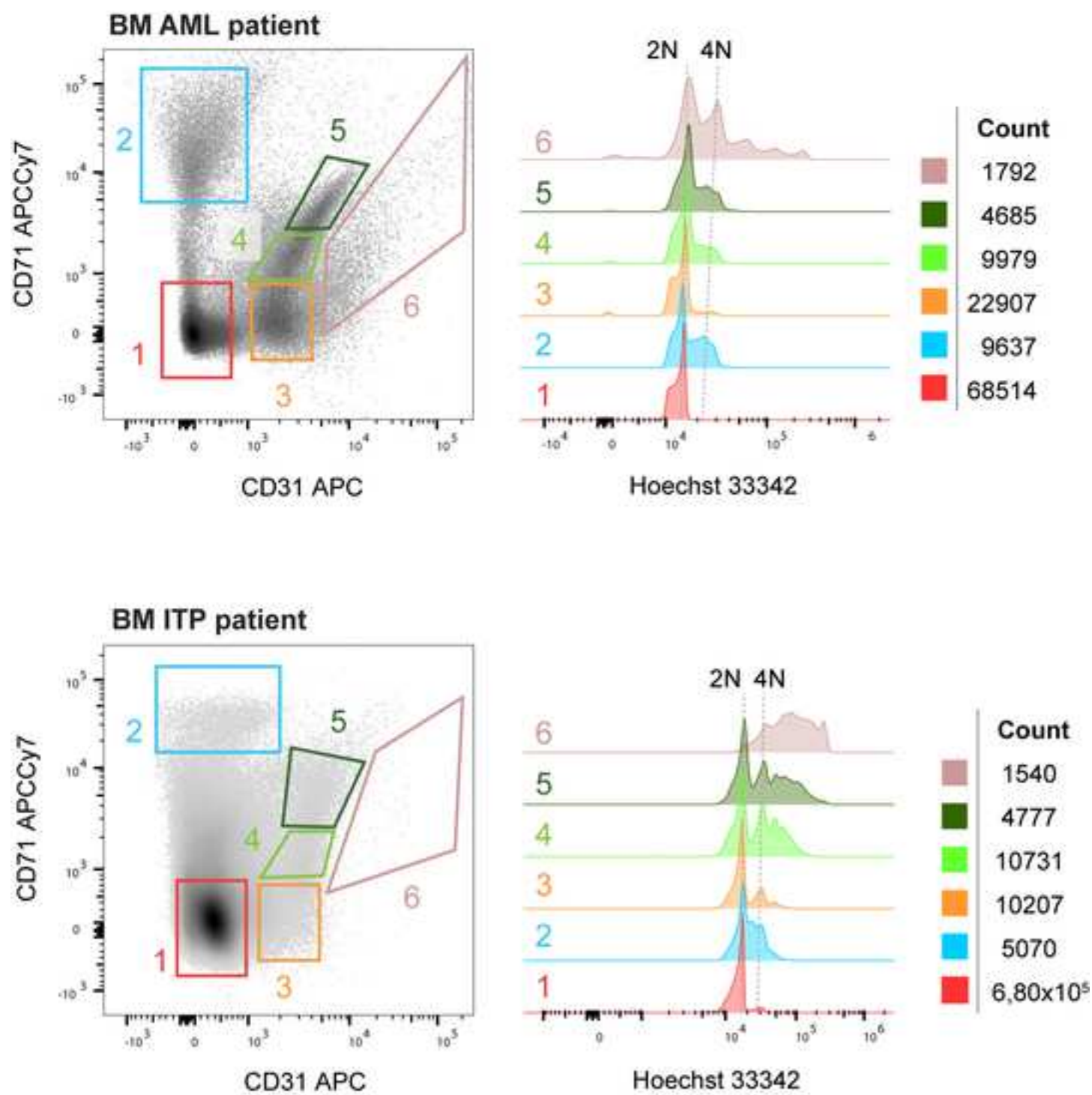
Figure 5

Figure 6
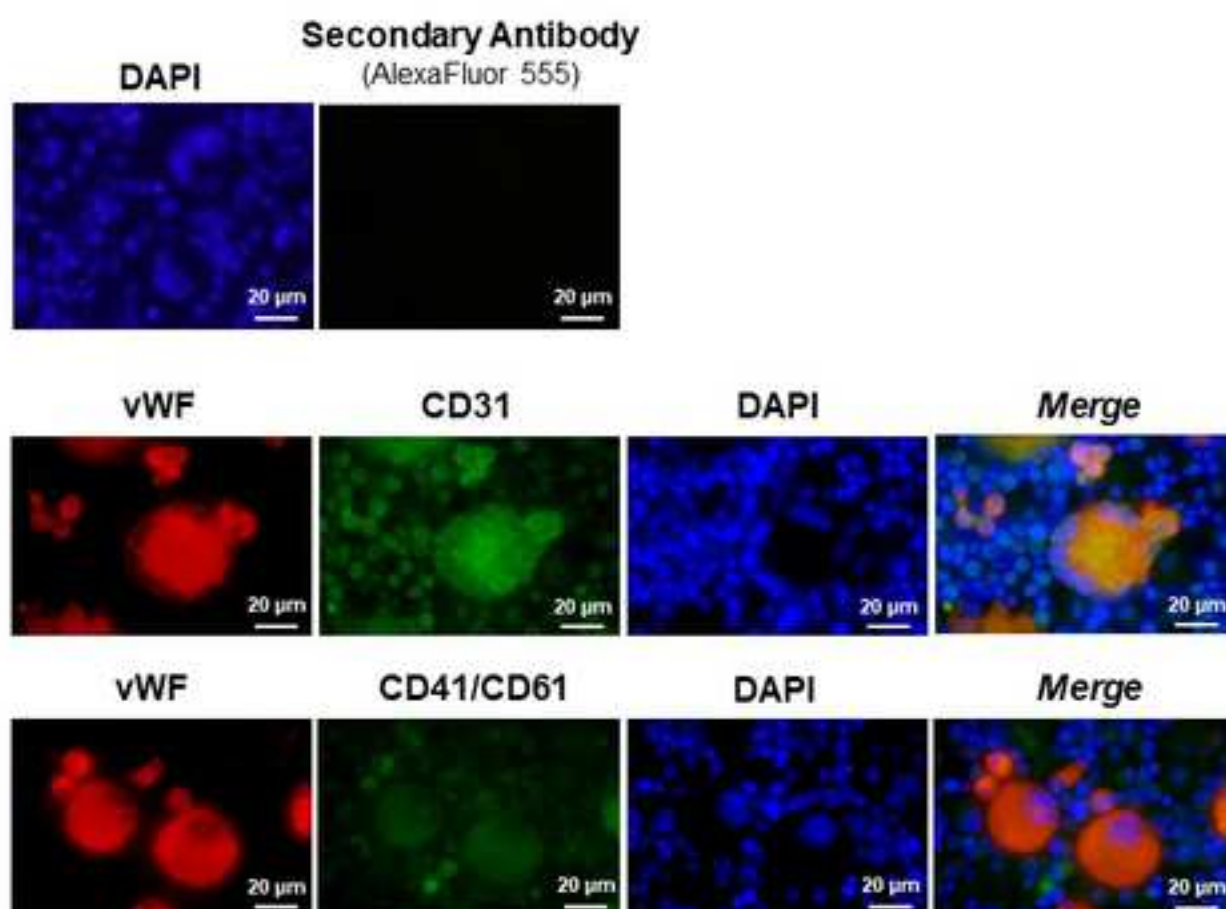
Click here to access/download;Figure;Figure 6 JoVE 62569.jpg

A
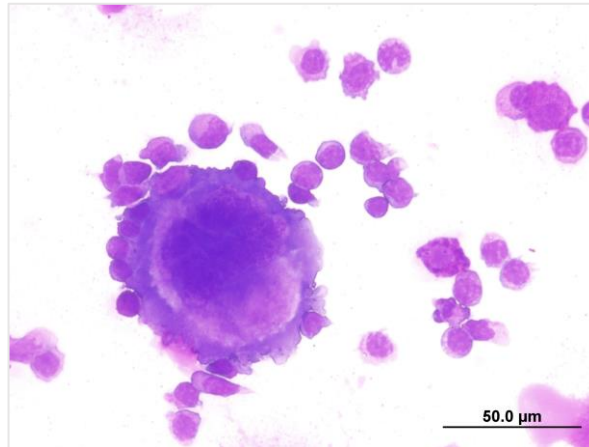
B

A   *Cytologic analysis*
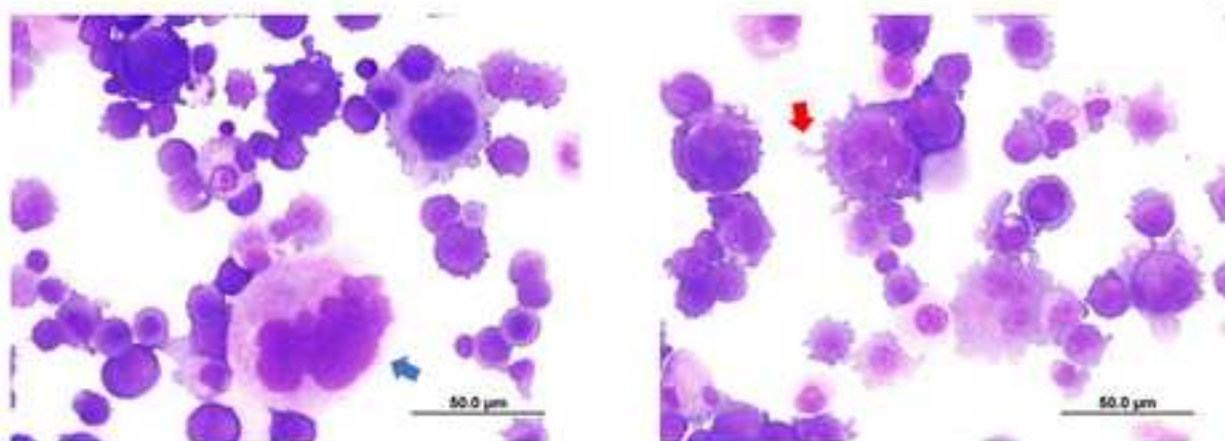
B   *Immunofluorescence*

Figure 9

The sticky nature of megakaryocytes

A

B

C

50.0 µm

Figure 10

Click here to access/download;Figure;Figura 10 R2.JPG ±
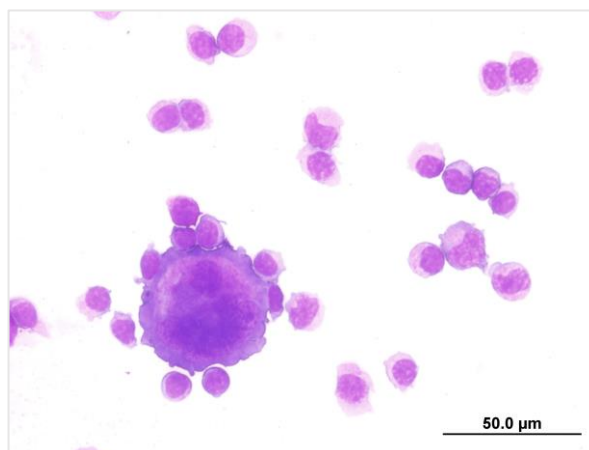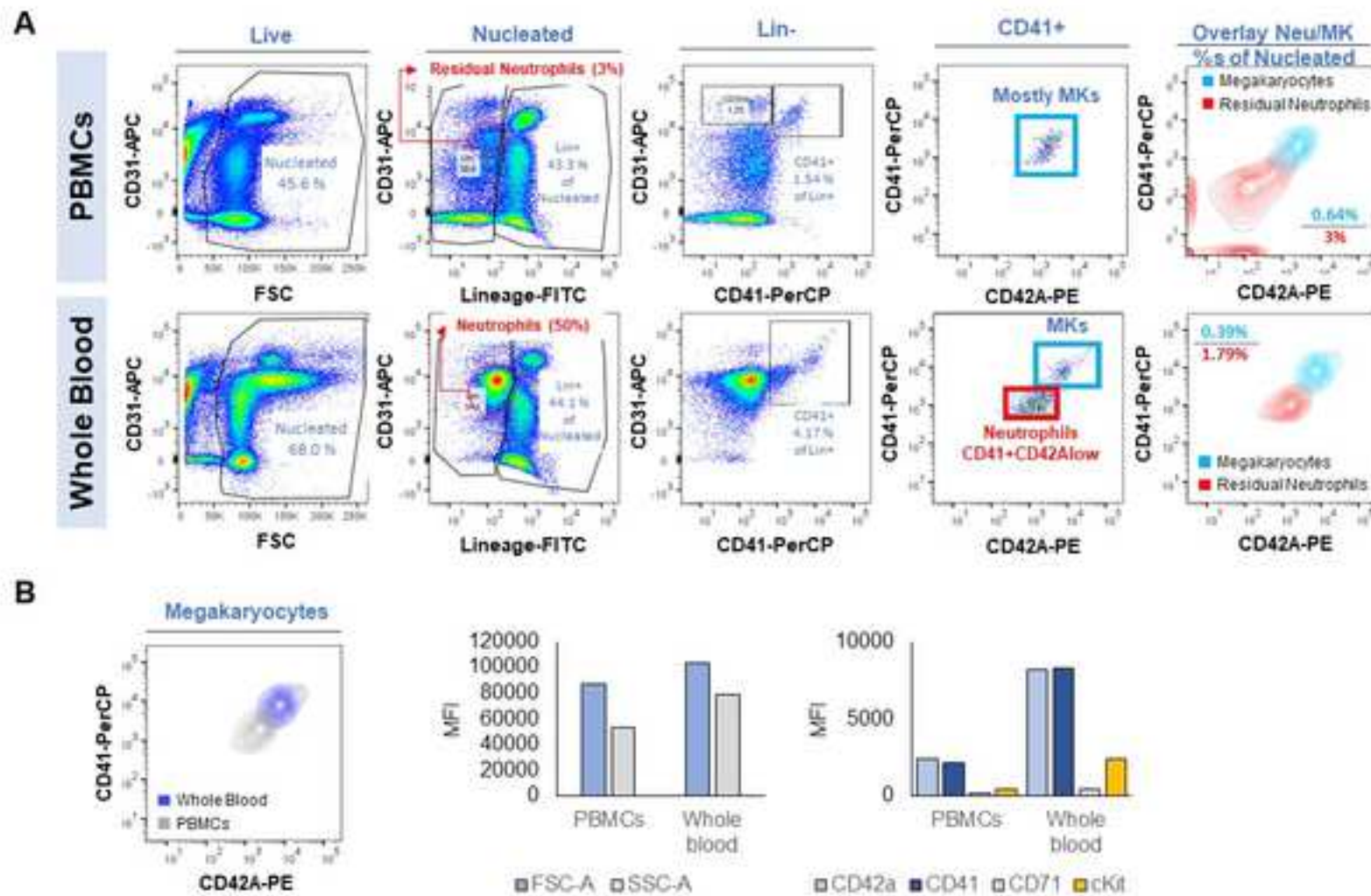
Table 1

Table 1: notes on cell surface markers of the megakar

Surface markers

Earlier hematopoietic precursors:
CD34
MPL
KIT - *also present in erythroblasts, mast cells, precursors*
Earlier megakaryocytic precursors:
CD34
MPL
KIT – *also present in erythroblasts, mast cells, precursors*
CD31 – *also present in endothelial cells*
CD71 – *also present in erythroblasts*
MKs:
CD9 – *also present in some leukocytes*
CD41 / CD61
CLEC2 - *also present in some leukocytes*
GPVI
Mature MKs:
CD42A / CD42B
CD49B - *also present in some leukocytes*
Optional:
Lineage cocktail – *to avoid 'noise'*
(i.e. combination of CD3, CD14, CD19, CD20, and CD56 human antibodies)

Table 1. Rationale of the surface markers that are combin results section for flow cytometry phenotypical analysis of

yocytic lineage

Comments

They are present as well in MKs, although KIT seems to be downregulated through maturation. Detection of MPL might fluctuate or be difficult depending on the antibody used.

CD31 and CD71 are essential in our immunophenotyping strategy. Combined with one or two MK/mature MK markers, and depending on the needs of the study, with earlier markers such as KIT, MPL or CD34.

These markers Will partially overlap with CD42A / CD42B or CD49B, and some of them are present in other cells tan MKs, which might not be useful for staging megakaryopoiesis. They could be included when required for the specific study.

Culture conditions partially induce CD42B shedding. This may also occur in circulating MKs, whereas CD42A remains more stable.

It helps to filter out cells from other lineages. However, we should be cautious, as the surface marker promiscuity of MKs is acknowledged. As an example, some MKs might be CD14+.

ed in the panels used and presented in the representative MK subpopulations.

Table 2

Table 2: Antibody panels

Fluorochromes and flow cytometer laser and channels			PANEL SORT	PANEL SORT, PBMCs & CULTURES	BM	BM II
488	**Green**	*FITC, AF 488*	CD31	LIN -	CD61	CD34
	Yellow	*PE*	CD49B	CD42A	CD41	CD42A
	Orange	*PE-Texas Red*				
	Red	*PerCP, PECy5, PerCPCy5.5*	CD41	CD41	CD42B	CD42B
	Infrared	*PE-Cy7*	KIT	KIT	KIT	KIT
561	**Yellow**	*PE*				
	Orange	*PE-Texas Red*				
	Red	*PE-Cy5*				
	Infrared	*PE-Cy7*				
640	**Red**	*APC, AF 647*	CD42A	CD31	CD31	CD31
	Far red	*Alexa Fluor 700*				
	Infrared	*APC-Cy7, BD APC-H7*	CD71	CD71	CD71	CD71
405	**Green**	*AmCyan, V500*				
	Blue	*Pacific Blue, V450*				

Table 2. Some of the combinations/panels used in the representative results section.

Ploidy
Use the panel of preference
Hoechst 33342

Table 3

Table 3: Population percentages flow cytometry analyses

% of nucleated (% of CD42B$^+$ on each gate)	Figure 4				
GATE	Lymphoma BM	MDS BM	AML BM	Reactive BM	ITP BM
1	2.99 (1.81)	96.06 (0.037)	29.86 (0.63)	7.79 (0.26)	2.41 (0.17)
2	10.38 (2.8)	0.47 (0.36)	15.27 -1	8.87 (0.070)	3.33 (0.30)
3	57.66 (5.69)	2.13 (23.5)	19.31 (3.86)	69.00 (1.43)	72.2 (0.23)
4	12.98 (8.98)	0.71 (7.72)	9.97 (6.84)	8.11 (1.60)	10.4 (0.93)
5	0.73 (32.9)	0.14 (14.3)	0.38 (10.8)	0.39 -17	0.31 (4.07)
6	5.68 (46.3)	0.07 (51.8)	7.36 (51.6)	0.26 (12.4)	3.47 (12.6)

Table 3A. Representative flow cytometry analyses of bone marrow samples. The percentage of nucleated cells o
in Figures 4 and 5, and the percentage of CD42B$^+$ cells within each gate, is indicated.

% of Lin- (% of CD42B$^+$ on each gate)	Figure 6		
GATE	PBMCs	Day 7	Day 11
1	11.21 0	11.60 0	5.68 (0.11)
2	1.22 (1.96)	11.45 (0.29)	41.41 (1.93)
3	29.80 (4.11)	9.40 (0.92)	5.93 (6.72)
4	1.45 (0.83)	13.91 (14.9)	8.34 (9.34)
5	17.20 (22.2)	9.35 (90.8)	9.89 (98.2)

Table 3B. Representative flow cytometry analyses of PBMCs and corresponding PBMC-derived MK cultures at d
percentage of nucleated cells of each gate (1-5), as shown in Figure 6, and the percentage of CD42B$^+$ cells with

Figure 5 (Fix&Perm)

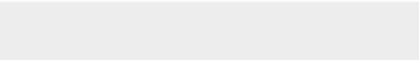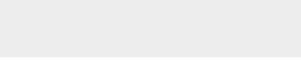| AML BM	ITP BM
50.29	74.72
(0.29)	(0.015)
7.07	0.55
(0.45)	(0.18)
16.84	1.12
(15.6)	(21.4)
7.32	1.18
(10.1)	(66.5)
3.43	0.52
(26.2)	(80.1)
1.31	0.19
(65.3)	(94.6)

f each gate (1-6), as shown

ay 7 and day 11. The
in each gate, is indicated.

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Table of Materials

JoVE_Materials Acebes-Huerta et al 62569 R3 280621.xlsx

ISPA
Instituto de Investigación Sanitaria
del Principado de Asturias

Universidad de
Oviedo

To:	**Nam Nguyen, Ph.D.,**	**June 28th 2021**
	Manager of Review	
	JoVE	**Oviedo, Spain**

Subject: Revision **JoVE62569R3** "Immunophenotyping and cell sorting of human MKs from human primary sources or differentiated in vitro from hematopoietic progenitors".

Dear Editor,

We are very thankful for the thorough revision of our manuscript. We provide a revised version of it, with changes highlighted in yellow and red font. We have paid especial attention to the text remarks given by the Editor, and have provided an answer to every point raised. We have updated the Materials Table and provide the Tables as individual Excel files. We have corrected and updated all centrifugation speeds (in x g). We have removed all commercial names, with the exception of the culture medium (StemSpan, because it is irreplaceable as far as we know) and the flow cytometry analysis software packages used. However, if the Editor decides this should not be mentioned in the text, we can correct it to:

- The culture medium used as indicated in the Materials Table…
- Flow Cytometry analysis software packages, as indicated in the figure legends of the representative results, or as indicated in the Materials Table…

We have also removed all the NOTES as they were within the protocol part of the manuscript, and have either moved them to representative results, or have incorporated them in sub-points. We have also corrected some volume units that need to be in Symbol font, to indicate microliters instead of mililiters.

We think that our manuscript has improved considerably, and we hope that we have not missed anything on this revision, otherwise, please let us know whether anything should be improved or updated. We hope you find it of interest to the readership of JoVE.

With Kind Regards,

Laura Gutiérrez