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Differentiation of an embryonic stem cell to hemogenic endothelium by defined factors: essential role of bone morphogenetic protein 4

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SUMMARY

Current approaches to differentiate embryonic stem (ES) cells to hematopoietic precursors in vitro use either feeder cell, serum, conditioned culture medium or embryoid body, methods that cannot avoid undefined culture conditions, precluding analysis of the fate of individual cells. Here, we have developed a defined, serum-free and low cell-density differentiation program to generate endothelial and hematopoietic cells within 6 days from murine ES cells. Our novel approach identifies a set of factors that are necessary and sufficient to differentiate ES cells into definitive hematopoietic precursors, as documented by the time-lapse video microscopy of the stepwise differentiation processes from single progenitors. Moreover, this defined milieu revealed the essential role of bone morphogenetic protein 4 (BMP4) in determining the hematopoietic/endothelial fate and demonstrated that the hemogenic fate in mesoderm is determined as early as day 4 of our differentiation protocol. Our ability to directly convert ES cells to endothelial and hematopoietic precursors should have important utilities for studies of hematopoietic development and personalized medicine in the future.

KEY WORDS: Hemangioblast, Hemogenic endothelium, Serum-free differentiation, Single cell colonial differentiation, Time-lapse microscopy

INTRODUCTION

The differentiation of embryonic stem (ES) cells to endothelial and blood cells has important clinical implications. The emergence of induced pluripotent stem cell technology (Takahashi et al., 2007) makes it possible to obtain isogenic endothelial and blood cells, provided it is possible to drive the differentiation process in a well-defined manner. An appropriate differentiation program requires a system that is stable and has minimal unknown factors. It is also preferable that the differentiation process could be monitored directly and continuously. In order to reduce uncontrolled cell-to-cell interaction, either directly or indirectly, the formation of three-dimensional structures and high density culture should also be avoided (Nishikawa et al., 2007). Finally, to minimize potential contamination and cost, a system that is simple and without tedious sorting for both intermediate and end-stage cell populations is highly desirable.

Murine ES cells are a popular tool employed to understand the mechanisms of differentiation. Current approaches to differentiate ES cells to hematopoietic precursors use either feeder cells (Eilken et al., 2009; Nakano et al., 1994), serum (Lancrin et al., 2009), conditioned culture medium (Kennedy et al., 1997) or embryoid body (Lancrin et al., 2009). The use of defined conditions to differentiate embryonic stem cells to hematopoietic precursors has important clinical applications. However, differentiation in serum or with a feeder layer, currently two of

the most commonly used methods used to obtain hematopoietic precursors, raises significant concerns regarding pathogen contamination and potential allergens in these xenogenic substances. Furthermore, the batch-to-batch differences in serum may lead to variation in the efficiency of differentiation. The use of the feeder layer also suffers from issues of passage limitation and senescence of the cell line. Moreover, the complex composition of the factors in serum or secreted by the feeder cells may result in uncontrollable multi-lineage differentiation that requires tedious selection of a pure population of desired cells. Although the formation of embryoid bodies is one way to acquire hematopoietic precursors, the floating and tightly packed nature of differentiating cells prevents the real-time follow-up of cells with high resolution. Moreover, the culture of differentiating cells in a tightly confined three-dimensional space also cannot avoid complex intercellular signaling via intercellular contacts or secreted molecules. Thus, a defined, adherent and low-density system with high differentiation efficiency and minimal manipulation remains an unmet need.

By using a combination of recombinant cytokines and small molecules, we demonstrate a defined differentiation system showing stepwise transition from ES cells to endothelial cells through the Vegfr2⁺ (Kdr⁺ – Mouse Genome Informatics) mesoderm intermediate (Yamashita et al., 2000). Importantly, we have discovered the crucial role of bone morphogenetic protein 4 (BMP4) in our differentiation program to yield CD41⁺ (Itga2b⁺ – Mouse Genome Informatics) hematopoietic precursors with high levels of efficiency and purity. Finally, we demonstrate that our system could be used for high resolution follow-up of the differentiation process through time-lapse video recording of the emergence of hematopoietic precursors from hemogenic endothelium (Bertrand et al., 2010; Boisset et al., 2010; Kissa and Herbomel, 2010).

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MATERIALS AND METHODS

ES culture and differentiation

ES cells were cultured in serum- and feeder-free conditions using established protocols (Ying et al., 2008). Briefly, cells were split in modified (vitamin A free) N2B27 (basal medium, BM) with combinations of factors (see text) on either laminin-, Matrigel- or Cell-Tak-coated plates during differentiation. This in vitro differentiation protocol had been replicated for more than 50 consecutive times on germline competent v26.2 B6 line (Humpherys et al., 2001) and tested on two other independent germline competent ES cell lines, v17.2, and an in-house germline competent strain 129S6/SvEvTac ES cell line, in order to ensure reproducibility and generality of the method. Only ES cells with less than 50 passages were used.

Cytokines and chemicals used for ES cell culture and differentiation

Recombinant human BMP4, PD173074 (FGFRi), PD0325901 (ERKi), LDN-193189 (BMPRI) and human FGF2 (Stemgent); CHIR99021 (GSKi, Stemgent or Axon Medchem); dexamethasone, 8-bromoadenosine 3',5'-cyclic monophosphate (BrcAMP, a cell permeable cAMP analog), mIL3 and SB431542 (ALKi, Sigma); recombinant human activin-A, mouse TPO and mouse CSF-GM (Invitrogen); human IL6, human EPOa, mouse VEGF165, human SCF, mouse CSF-G and mouse CSF-M (Prospec); and mouse IGFII (R&D Systems) were used.

Limiting dilution assay of endothelial or hemogenic colony formation

Day 4 mesodermal cells cultured in the presence of mesodermal inducer (Mi, BM plus 3 μ M GSKi, 4 ng/ml activin, and 12.5 ng/ml FGF2) or Mi plus BMP4 were dissociated (enzyme-free cell dissociation buffer) and replated on 96-well plates at a concentration of four or two cells per well, respectively, in Vm medium (BM plus FGF2 12.5 ng/ml, BMP4 20 ng/ml, VEGF 20 ng/ml, BrcAMP 0.25 mM and ALKi 4 μ M). The number of colonies with typical endothelial or hemogenic budding was determined 48 hours later; only colonies with cell number equal to or greater than four were used.

Semi-quantitative RT-PCR

Total RNAs extracted from cells were subjected to reverse transcription followed by PCR analysis using the primer sets shown in Table S1 in the supplementary material.

Immunocytochemistry and live cell immunofluorescence

Adherent cells were fixed in 4% paraformaldehyde for 10 minutes for immunohistochemical analysis using the following antibodies: α E-cadherin (DECMA-1, Sigma), α -Vegfr2 (Avas12 α 1, BD), α CD31 (390, eBio; MEC13.3, BD), α VE-cadherin (eBioBV13, eBio), α Runx1 (EPR3099, Epitomics), α CD41 (MWRReg30, BD), α PU.1 (Cell Signaling), α CD117 (ACK2, eBio), α Tie-2 (TEK4, eBio), α CD11b (M1/70, eBio), α Ter-119 (TER119, eBio) and α CD45 (Ly-5, eBio). Except for FITC-CD41 (Biolegend), antibodies were conjugated with Alexa 594 for live immunofluorescence at an incubation concentration of 100 ng/ml. Human AcLDL Alexa 594 (Invitrogen) was used at 5 μ g/ml. Nuclear counterstain was performed with H33342. All time-lapse live-cell imaging was initiated 1 hour after plating and continued at 10-minute intervals, except for Movie 6 in the supplementary material, which was performed 24 hours after plating.

Flow cytometry

Cells were harvested by dissociation medium (adherent cells) or trituration (floating cells), incubated with fluorescent labeled antibodies for 30 minutes at 4°C, washed twice and analyzed with FACSCalibur. The following antibodies were used: FITC- α CD41 (MWRReg30, BD), PE- α E-cadherin (DECMA-1, eBio), PE- α CD31 (390, eBio; MEC13.3, BD) and PE- α -Vegfr2 (Avas12 α 1, BD).

Capillary formation assay in Matrigel

In one well of a 24-well plate, 140,000 cells from day 6 culture were seeded and coated with 200 μ l of Matrigel after cells were attached. After the formation of gel matrix, 800 μ l of BM, 4 μ M ALKi and 20 ng/ml

VEGF were added, and the cells were incubated for 2 days. The culture medium was replaced with BM containing 12.5 ng/ml FGF2, 4 μ M ALKi and 20 ng/ml VEGF, and the cells were incubated for another 48 hours before AcLDL uptake assay and immunohistochemical analysis were performed.

Determination of multi-lineage hematopoiesis

The ES cells were differentiated in the presence of: BM during days 0-2, Mi plus 5 ng/ml of BMP4 during days 2-4, and Vm during days 4-6. Cells (40,000) derived from the floating fractions were harvested and incubated in each well of a 12-well plate coated with Cell-Tak (BD) in BM containing SCF (100 ng/ml), mIL3 (50 ng/ml), mIGFII (100 ng/ml), BMP4 (20 ng/ml) with either hEPOa (10 IU/ml) and dexamethasone (1 μ M) for 48 hours for erythroid differentiation; mTPO (100 ng/ml) and hIL6 (100 ng/ml) for 96 hours for megakaryocytic differentiation; or mCSF-G, mCSF-GM, mCSF-M and hIL6 (100 ng/ml each) for 96 hours for myeloid differentiation. Cells (40,000) were cultured in each well of a 24-well plate in BM supplemented with SCF (100 ng/ml), mIL3 (25 ng/ml), mIGFII (100 ng/ml), BMP4 (20 ng/ml), hEPOa (10 IU/ml), mIL3 (25 ng/ml), mTPO (25 ng/ml), hIL6 (25 ng/ml), mCSF-G (25 ng/ml), mCSF-GM (25 ng/ml) and mCSF-M (25 ng/ml) for 96 hours before RNA harvest for RT-PCR or fixation for staining.

Colony-forming assay

The ES cells were differentiated in the presence of: BM during days 0-2, Mi plus 5 ng/ml of BMP4 during days 2-4, and Vm during days 4-6. Floating live cells (150,000) were harvested and seeded into Methylocult M3434 on cell culture plate coated with 0.1% agarose. Hematopoietic colonies were counted 8 days after seeding.

Statistical analysis

Statistical analyses were performed using one-way ANOVA with Dunnett's post-ANOVA test, with $n \geq 3$: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

RESULTS

Epiblast forms in the absence of canonical Wnt agonist

To identify factors required for in vitro differentiation of ES cells to hematopoietic precursors, we initially freed murine ES cells from feeder mouse embryonic fibroblasts and fetal bovine serum by more than five passages in ES medium (ESM) comprising N2B27 basal medium (BM) supplemented with extracellular-signal-regulated kinase inhibitor (ERKi, 1 μ M PD0325901), glycogen synthase kinase inhibitor (GSKi, 3 μ M CHIR99021) and leukemia inhibitory factor (LIF, 10 ng/ml), conditions that would maintain ES cells in an undifferentiated state (Ying et al., 2008). To trigger differentiation (Fig. 1A, ESC to epiblast), the dissociated ES cells were cultured in BM only, or in BM supplemented with either ERKi (1 μ M), LIF (10 ng/ml) or GSKi (3 μ M GSKi) for 48 hours. As expected, a greater number of ES colonies was observed in cultures with the ES medium or BM with LIF alone (Fig. 1C, ESM and LIF), confirming that LIF enhances survival of ES cells. Morphologically, cells cultured in BM containing only GSKi (Fig. 1B, GSKi) tended to develop domed, ball-like colonies – a feature that is similar to the pre-differentiation stage (undifferentiated ES cells in ES medium, Fig. 1B, ESM). By contrast, most of the ES cells cultured in the absence of GSKi became flattened and failed to form compact round colonies, a phenotype that was also observed with or without LIF or ERKi (Fig. 1B, BM, LIF and ERKi). As the transition from compact and dome-shaped colonies to flattened monolayer colonies is indicative of the differentiation of inner cell mass (ICM) to epiblast (Tesar et al., 2007), a previously characterized set of markers was employed to confirm this

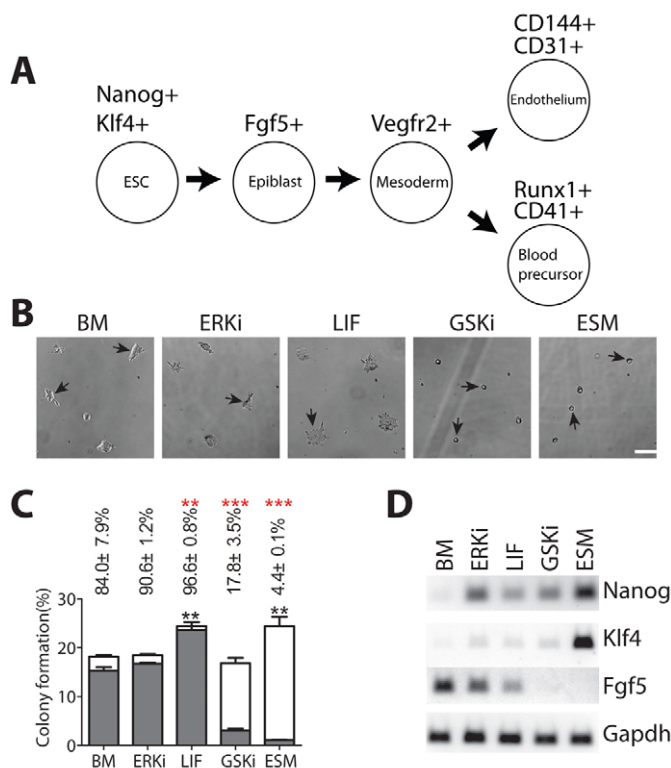


Fig. 1. Transition of ES cells into $Fgf5^+$ epiblast cells. (A) Cell types involved in the differentiation from ES cells to endothelial/blood cells. The markers commonly used for identification were shown on top of each cell type. (B) Phase-contrast morphology (arrows) of ES cells incubated in basal medium (BM) or BM plus different factors indicated for 48 hours. Dissociated ES cells (500) were seeded into each well of a 24-well plate, and colonies were counted and photographed 48 hours later. Factors used were: extracellular-signal-regulated kinase inhibitor (1 μ M ERKi), glycogen synthase kinase inhibitor (3 μ M GSKi), leukemia inhibitory factor (LIF, 10 ng/ml) and ESM (ERKi plus GSKi plus LIF). Scale bar: 100 μ m. (C) Quantification of number of colonies formed under various culture conditions shown in A (mean \pm s.e.m., $n=3$; ** $P<0.01$, *** $P<0.001$). Percentage above each bar denotes the ratio of the number of flat colonies (gray column) to the total number of colonies (gray+white columns). (D) ES cells (4000) were seeded into each well of a 12-well plate, and cells were harvested 48 hours later for semi-quantitative RT-PCR for levels of Nanog, Klf4 and Fgf5. Gapdh served as a control.

differentiation in our culture system by semi-quantitative RT-PCR. Consistent with morphological changes, ES cells differentiated in the presence of GSKi expressed a low level of $Fgf5$ (Brons et al., 2007) (an epiblast marker), while cells cultured in BM or in BM supplemented with LIF or ERKi expressed higher levels of $Fgf5$ (Fig. 1D, GSKi versus BM, LIF, or ERKi). The presence of $Fgf5$ indicates that the ES cells have undergone differentiation into cells with epiblast features, and the presence of GSKi, a canonical Wnt activator (Bennett et al., 2002; Ying et al., 2008), blocked this step of differentiation. Although GSKi was efficient in blocking epiblast differentiation, the observation of decreased expression of Klf4 (Guo et al., 2009) (a marker for ES or ICM cells; Fig. 1D, GSKi versus ESM) coupled with the concomitant reduction in number of ES colonies on the second day of differentiation (Fig. 1C; $16.8 \pm 1.1\%$ versus $24.4 \pm 1.9\%$; GSKi versus ESM, Dunnett's test, $P<0.01$) indicated that GSKi alone was insufficient to maintain ES (ICM) cell growth.

Consistent with the absence of epiblast morphology and a low level of $Fgf5$ expression, ES cells differentiated in the presence of GSKi (GSKi only or ESM) during the first 48 hours (days 0-2) failed to subsequently form typical mesodermal colonies when challenged with a mesodermal inducer (Mi, see below; see Fig. S1 in the supplementary material, compare GSKi or ESM with BM). This finding suggests that it is crucial to exclude GSKi from the culture medium during the initial 2 days (days 0-2). As expected with the retention of Nanog, a marker for pluripotency (Silva et al., 2009) (Fig. 1D, LIF and ERKi), and with the inhibitory roles ERKi (Fig. 2) and LIF (Pruitt and Natoli, 2006) play during mesoderm differentiation, the colonies that formed on day 4 were tight and lacked single cell outgrowth when cultured in the presence of either LIF or ERKi during the first 48 hours (see Fig. S1 in the supplementary material, LIF and ERKi). The presence of either LIF or ERKi did, however, allow the emergence of $Fgf5^+$ cells. Taken together, our findings indicate that it is essential to exclude all three factors (ERKi, LIF and GSKi) during the first 48 hours of ES cell culture (days 0-2) for efficient mesodermal transition.

Canonical Wnt agonist, activin and FGF are minimal but essential factors for mesodermal transition

We next determined the requirements to establish the differentiation of $Fgf5^+$ epiblast cells into mesodermal cells (Fig. 1A, epiblast to mesoderm). $Fgf5^+$ cells formed in BM on day 2 were incubated further with a combination of canonical Wnt agonist GSKi (Lindsley et al., 2006), TGF β agonist activin (Jones et al., 1995) and FGF2 (Cornell and Kimelman, 1994), factors that are suggested to be involved in the mesoderm transition. Morphologically, cultured in the presence of a combination of GSKi, activin and FGF2 (termed mesodermal inducer, Mi), most of the colonies transformed into colonies with cells dispersed in the periphery, indicating a loss of cell adhesion and mesodermal differentiation (Fig. 2A, Mi, top panel). Interestingly, these colonies were brachyury positive (Fig. 2D, Mi) with E-cadherin (Ecd)-positive cells at the center and, importantly, Vegfr2-positive (Flk1⁺) cells, the precursors of endothelial cells (Yamashita et al., 2000) and hematopoietic precursors (Nishikawa et al., 1998), in the periphery (Fig. 2A, Mi, lower 2 panels); around 30% of the population were positive for Vegfr2 on day 4 in the presence of Mi during day 2-4 (Fig. 2C, Mi, $31.2 \pm 1.1\%$, $n=3$).

However, the colonies remained 'tight' (Fig. 2A, Mi-GSKi, top panel) and small (see Fig. S2A in the supplementary material, Mi-GSKi), and lacked spindle cell outgrowth in the absence of GSKi. Furthermore, most of the cells were still expressing Ecd (Fig. 2A, Mi-GSKi, middle panel), but Vegfr2 (Fig. 2A, Mi-GSKi, bottom panel; Fig. 2D, Mi-GSKi) was downregulated, indicating a failure of these cells to be fated to the mesoderm lineage. Nevertheless, this persistence of both Ecd and brachyury (Fig. 2D, Mi-GSKi) expression strongly suggests that these colonies were still retained at the stage of primitive streak with minimal transition to mesodermal cells (Nakanishi et al., 2009).

Despite no reduction in cell number and colony size in the absence of activin (Fig. 2B; see Fig. S2A in the supplementary material, Mi-activin), the colonies remained tight with minimal spindle cell outgrowth in the periphery (Fig. 2A, Mi-activin, top panel). In addition, colonies were negative for both Ecd (Fig. 2A, Mi-activin, middle panel) and Vegfr2 (Fig. 2A, bottom panel and 2D, Mi-activin). The loss of Ecd and presence of brachyury indicate that those cells had undergone transformation into

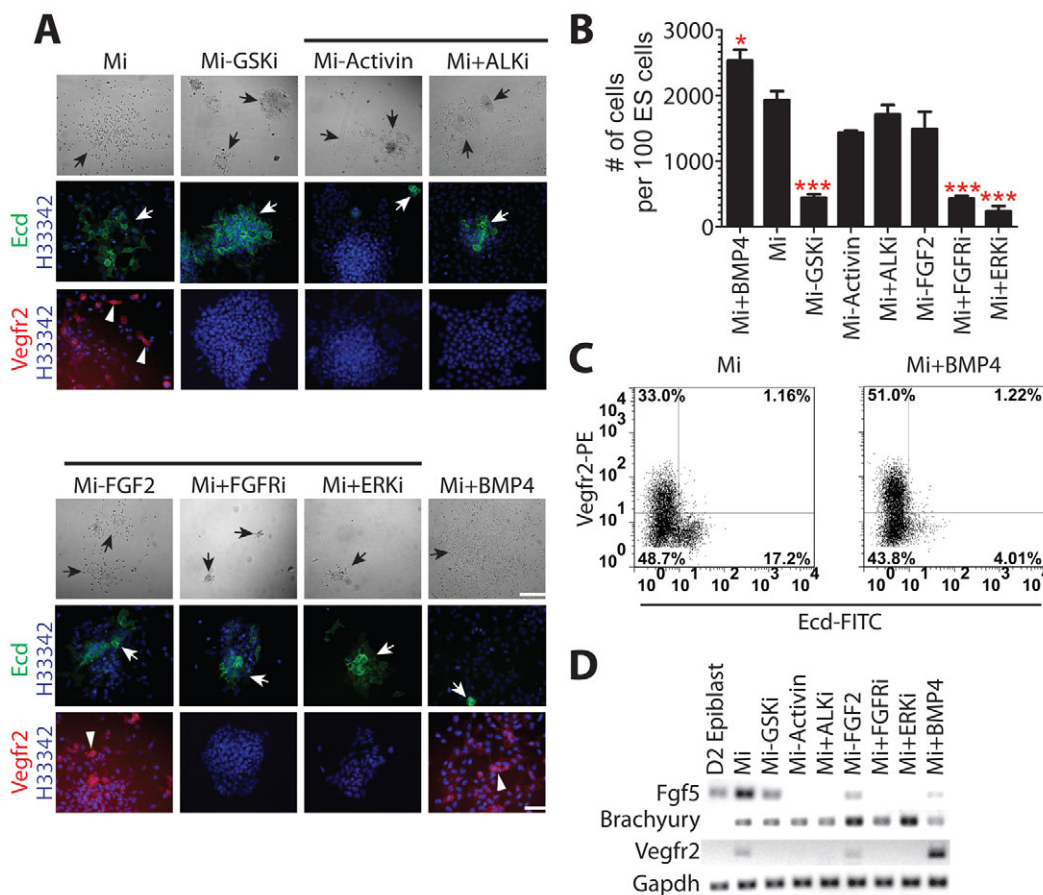


Fig. 2. Mesodermal inducer (Mi) induced mesodermal cells to express Vegfr2. (A) Phase-contrast morphology (upper panel; low-power view) of epiblast-like cells incubated in basal medium (BM) under different conditions for 48 hours as indicated (arrows). Conditions were: Mi (BM plus 3 μ M GSKi, 4 ng/ml activin and 12.5 ng/ml FGF2), Mi-GSKi (Mi minus GSKi), Mi-activin (Mi minus activin), Mi+ALKi (Mi plus activin receptor-like kinase receptor inhibitor, 4 μ M ALKi), Mi-FGF2 (Mi minus FGF2), Mi+FGFRi (Mi plus FGF receptor antagonist (Mi plus FGFRi, 100 nM), Mi+ERKi (Mi plus 1 μ M ERKi) and Mi+BMP4 (Mi plus BMP4 5 ng/ml). Representative Ecd-immunopositive (arrows, green, middle panels) or Vegfr2-immunopositive (arrowheads, red, lower panels) cells were identified for each of the culturing conditions. ES cells (500) were incubated in BM alone for 48 hours and subsequently replaced with BM containing various factors as indicated for an additional 48 hours. Scale bars: 300 μ m and 50 μ m for phase-contrast and immunofluorescence, respectively. H333342 served as the nuclear counterstain. (B) Quantification (mean \pm s.e.m., $n=3$; * $P<0.05$, *** $P<0.001$) of increase in number of cells of day 4 differentiated cells under conditions indicated in A. The number of cells under the Mi condition served as the baseline for comparison with other groups by Dunnett's post-ANOVA test. (C) Representative flow cytometry analyses for Vegfr2⁺ and Ecd⁺ expression in the mesodermal cells on day 4 induced with Mi only (left) or Mi plus BMP4 (right) during days 2-4. (D) Gene expression profile of day 4 differentiated cells, as judged by levels of Fgf5, brachyury and Vegfr2 mRNAs using RT-PCR analysis; Gapdh is used as a loading control.

mesoderm, while the absence of Vegfr2 suggests that the activation of TGF β pathway by activin is essential for the emergence of Vegfr2⁺ cells. In support of the essential role of the TGF β pathway, even in the presence of activin, the blockage of Vegfr2⁺ cell formation was also observed in the presence of an activin receptor-like kinase receptor inhibitor (ALKi, SB431542) (Inman et al., 2002) during the second to fourth day of culture (days 2-4; Fig. 2A,D, Mi+ALKi).

Ecd⁺ and Vegfr2⁺ colonies with similar morphology to Mi-induced colonies could be observed in the absence of FGF (Fig. 2A,D, Mi-FGF2), despite a minor reduction in the number of mesodermal colonies appearing on day 4 (see Fig. S2B in the supplementary material). As the secretion of FGF4 by ES cells (Ying et al., 2008) and FGF5 by their differentiated progenies (Fig. 1D and Fig. 2D, Fgf5) could result in an autocrine effect in culture, we tested the influence of FGF/ERK signaling. Inclusion of either an FGF receptor inhibitor (FGFRi; 100 nM PD173074) or an ERKi (Ying et al., 2008) (1 μ M) in Mi-containing medium during days

2-4 of culture resulted in small, tight colonies that were Ecd positive and Vegfr2 negative (Fig. 2A,D, Mi+FGFRi and ERKi) on day 4. Thus, these results demonstrate the essential role of FGF-ERK pathway in mesodermal transition.

Although inclusion of BMP4 increased total cell number, colony size and ratio of Vegfr2-positive cells (52.4 \pm 0.5%, Mi+BMP4 versus Mi, t -test=0.0001) on day 4 (Fig. 2B, see Fig. S2A in the supplementary material, and Fig. 2C, respectively, Mi+BMP4), exogenous BMP4 was not required for the induction of Vegfr2-positive cells (Fig. 2A, bottom panel, and 2D, Mi+BMP4).

To visualize directly the necessity and sufficiency of both the withdrawal of LIF, GSKi and ERKi during days 0-2, and inclusion of GSKi, activin and FGF2 during days 2-4 to form a mesodermal colony under low-density, adherent culture conditions, we used time-lapse video microscopy to capture these events (see Movie 1 in the supplementary material, note the dispersed nature of the resultant colonies, indicating loss of cell adhesion and formation of mesodermal colony).

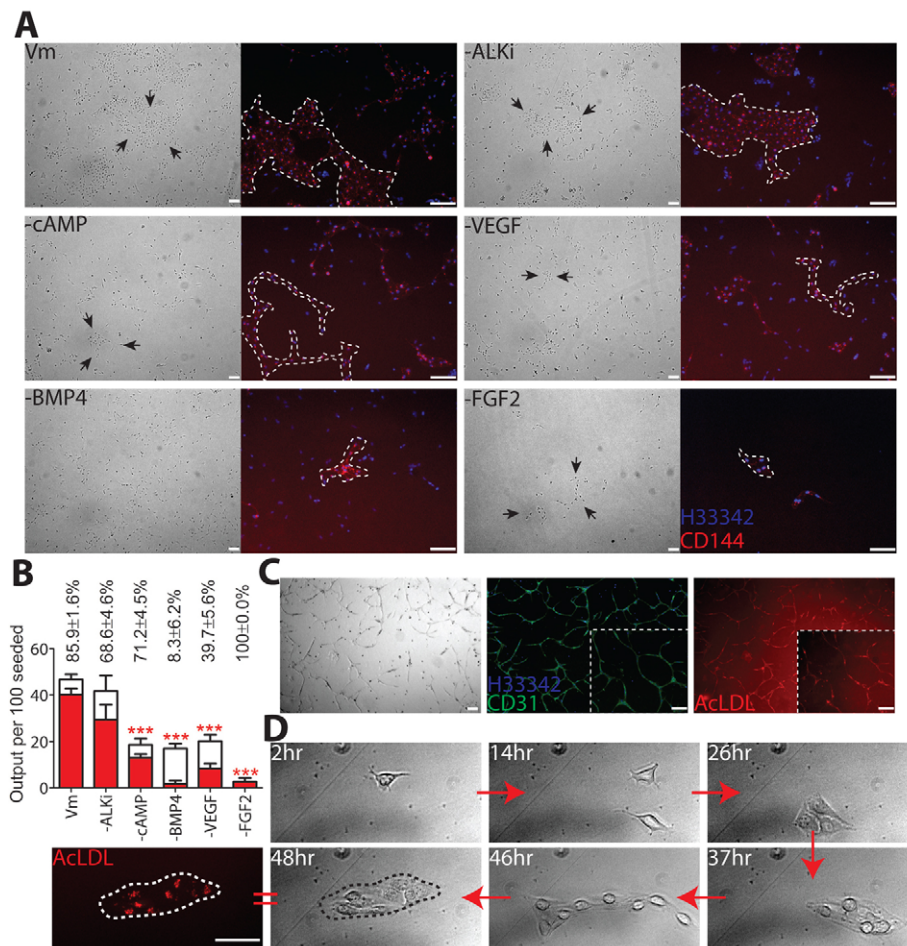


Fig. 3. Mesodermal cells replated in vasculogenic mixture (Vm) differentiated into endothelial cells in high purity. (A) ES cells (500 in each well of a 24-well plate) cultured in basal medium (BM) for 48 hours then in mesodermal inducer (Mi; BM plus 3 μ M GSKi, 4 ng/ml activin and 12.5 ng/ml FGF2) for an additional 48 hours and replated (20,000 cells in each well of a 24-well plate) into either Vm (BM plus FGF2 12.5 ng/ml, BMP4 20 ng/ml, VEGF 20 ng/ml, BrcAMP 0.25 mM and ALKi 4 μ M), Vm-FGF2 (Vm minus FGF2), Vm-BMP4 (Vm minus BMP4), Vm-VEGF (Vm minus VEGF), Vm-cAMP (Vm minus BrcAMP) or Vm-ALKi (Vm minus ALKi) for an additional 48 hours. Phase-contrast morphology (low-power view; arrows) and representative VE-cadherin (CD144) expression (higher-power view; encircled) of day 6 endothelial cells. (B) Quantification (mean \pm s.e.m., $n=4$) of the number of CD31⁺ cells (red columns) and the number of total cell (red plus white) on day 6 under low-density culture (500 day 4 mesodermal cells formed as described in A were inoculated into each well of a 24-well plate for 48 hours). Percentages (mean \pm s.e.m.) above each bar represent the ratios of CD31⁺ cell number (red column) relative to the total cell number (red+white). *A significant difference in CD31⁺ cell number when compared with the number of Vm by Dunnett's post-ANOVA test (***) $P<0.001$. (C) Low- and higher-power views (insets) of CD31⁺ (green) capillary-like structure capable of taking up AcLDL (red) formed in Matrigel assay. (D) Fate tracing of a pure endothelial colony (outlined) differentiated from the only cell in a well of a 96-well plate. AcLDL Alexa 594 (red) was added 1 hour before photographing. H333342 served as the nuclear counterstaining in Fig. 3A,C. Scale bars: 100 μ m.

FGF2, BMP4, cAMP, VEGF and ALK receptor antagonist support colonial differentiation of endothelial cells

To minimize the cell-to-cell interactions within colonies, we dissociated the mesodermal colonies, re-plated the cells on day 4 (96 hours after initiation of differentiation) and tested the effect of various factors in BM for the formation of endothelial cells (Fig. 1A, mesoderm to endothelium).

The crucial role of FGF2 for survival of endothelial precursors and efficient transition into endothelial cells was shown by dramatic loss of viable precursor cells in the absence of FGF2 24 hours later (D5 BM versus BM+bFGF=4.0 \pm 2.0 versus 36.2 \pm 2.0 per 100 mesodermal cells seeded, t -test $P=0.01$) and by the low number of endothelial colonies formed 48 hours later, respectively (Fig. 3A,B, see Figs S3 and S4 in the supplementary material,

-FGF2). Although the inclusion of BMP4 was not required for the formation of Vegfr2-positive endothelial precursors (Fig. 2A,C,D, Mi versus Mi+BMP4), we asked whether BMP4 plays an important role for transition into endothelial cells. Interestingly, we observed a dramatic reduction of VE (vascular endothelial)-cadherin (CD144)⁺ (Cdh5⁺ – Mouse Genome Informatics) and CD31⁺ (Hba-a2⁺ – Mouse Genome Informatics) endothelial sheet formation (Fig. 3A; see Fig. S3 in the supplementary material, -BMP4). Only a minority (5.5 \pm 3.6%) of colonies (pure CD31⁺ colony/total colony number; see Fig. S4 in the supplementary material) and small number (8.3 \pm 6.2%) of cells (CD31⁺ cell/total cell number, Fig. 3B) were endothelial in nature in the absence of BMP4 during days 4-6 (compare 69.8 \pm 3.3% and 85.9 \pm 1.6%, respectively, in the presence of BMP4). These findings strongly support the hypothesis that BMP4 is essential for the endothelial

fate. To a lesser degree, the number of CD31⁺ cells was reduced in the absence of VEGF (8.4±2.2 per 100 mesodermal cells seeded, Dunnett's, $P<0.001$) or in the absence of a cAMP agonist (BrcAMP, 13.1±1.6 per 100 mesodermal cells seeded, Dunnett's, $P<0.001$) when compared with the control (CD31⁺ cell: 40.2±2.8 per 100 mesodermal cells seeded) (Fig. 3A,B; see Figs S3 and S4 in the supplementary material). In addition, consistent with the previous report that TGFβ pathway inhibition enhanced the production of endothelial cells (James et al., 2010), the inclusion of ALKi during the 48-hour differentiation process increased the percentage of CD31⁺ endothelial cells (85.9±1.6% versus 68.6±4.6%, *t*-test, $P<0.05$).

To establish the functionality of endothelial cells generated by our defined conditions, we assessed the ability of these cells to form capillary-like networks. Using a standard Matrigel assay, we showed that differentiated endothelial cells formed capillary-like networks capable of taking up AcLDL (Fig. 3C). Furthermore, using a single mesodermal precursor cell cultured within a single well of a 96-well plate, we showed that the combination of the five factors was sufficient to allow the colonial growth of endothelium from a single mesodermal precursor (Fig. 3D). In summary, using our vasculogenic mixture (Vm) composed of FGF2 (12.5 ng/ml), BMP4 (20 ng/ml), VEGF (20 ng/ml), BrcAMP (0.25 mM) and ALKi (4 μM) in basal medium, we obtained 40 CD31⁺ endothelial cells (~85% purity) for every 100 day 4 mesodermal cells seeded at low concentration (~500 cells per well of a 24-well plate) in a colonial growth manner 48 hours later, as demonstrated by time-lapse microscopy (see Movie 2 in the supplementary material, showing colonial differentiation into endothelium from a single mesoderm cell).

Colonies exhibiting a tight, three-dimensional morphology prohibit direct observation with high resolution and create confounding cellular context, including intercellular communication. To avoid cell aggregation during days 2-4 in the presence of Mi (see Movie 1 in the supplementary material after replacing with Mi), epiblast equivalent was dissociated and replated in Mi-containing medium for 48 hours and subsequently replaced with Vm-containing medium and cultured for an additional 48 hours (see Movie 3 in the supplementary material, single cell and part of its progeny were pseudocolored blue). The capability to form endothelial cells under these conditions indicates that Mi is sufficient to support colonial differentiation into endothelial precursors in a cell-autonomous manner, where the aggregation observed during days 2-4 (see Movie 1 in the supplementary material) was not an essential step for the formation of endothelial cells.

Inclusion of BMP4 during mesodermal differentiation triggers formation of hemogenic endothelium

The emergence of definitive hematopoietic precursors from endothelium has been shown both in vivo (Bertrand et al., 2010; Boisset et al., 2010; Kissa and Herbomel, 2010) and in vitro (Eilken et al., 2009; Lancrin et al., 2009). Here, we define the definitive hematopoietic precursors as cells that retain the potential to differentiate into multiple blood lineages in addition to yolk sac erythroid cells in a Runx1-dependent manner (Okuda et al., 1996). On day 6 of our endothelial differentiation protocol, we rarely observed CD31⁺ and Runx1⁺ round budding cells on top of the endothelial sheets (Fig. 4A, top panel). As Runx1 has been shown to be a crucial trigger for the precursors of definitive hematopoiesis (Chen et al., 2009; Lancrin et al., 2009), the presence of Runx1⁺ budding cells suggests that our endothelial differentiation protocol

could serve as the framework for more efficient production of hematopoietic precursors (Fig. 1A, mesoderm to blood precursor). By including BMP4 (5 ng/ml) in the Mi during days 2-4 (Fig. 2A,C, Mi+BMP4), we observed that the Runx1⁺ budding cells were dramatically increased in number both on top of endothelial cells (Fig. 4A, middle panel) and in the floating fractions (see Fig. S5C in the supplementary material). The crucial role for BMP4 in this process was further verified by the inhibitory effect of Runx1-associated budding by an inhibitor BMP type I receptor (BMPRI; 250 nM LDN193189) (Yu et al., 2008) in the Mi+BMP4-containing medium during days 2-4 (Fig. 4A, bottom panel). In addition to being Runx1⁺, those budding cells were also positive for CD41 (see Fig. S5B,C in the supplementary material), an early hematopoietic marker (Mikkola et al., 2003), as well as hematopoiesis markers including PU.1 (Sfpi1 – Mouse Genome Informatics) (Okada et al., 1998), CD117 (Kit – Mouse Genome Informatics) and Tie2 (Tek – Mouse Genome Informatics) (Lancrin et al., 2009) (see Fig. S5B in the supplementary material). The crucial role of BMP4/BMP type I receptor pathway during mesodermal transition from epiblast cells was further supported by marked increase of Myb (Mucenski et al., 1991) expression only in the presence of unperturbed BMP4 activity (Fig. 4C, Mi+BMP4 versus Mi or Mi+BMP4+BMPRI; see Fig. S5A in the supplementary material, Mi+BMP4 versus Mi or Mi+BMP4+noggin). The deterministic effect of BMP4 during mesoderm differentiation is consistent with hemangioblast commitment in the early primitive streak stage and the highest amount of hemangioblasts found in the posterior/proximal region (Huber et al., 2004) where BMP4 activity is highest at the gastrula stage (Robertson et al., 2003).

Quantitatively, for every 100 precursor cells from day 4 cultured in BM containing BMP4+Mi, we obtained 165.0±15.3 live floating cells (see Fig. S5A in the supplementary material, left, Mi+BMP4) 48 hours later, a high proportion of which (84.9±0.5%, $n=3$) were CD41 positive (Fig. 4B, floating fraction). The purity of CD41-positive fraction could be maintained by replating cells onto adhesive-coated substrate (90.8±1.4%, $n=3$) for further live imaging analysis (see Fig. S5C and Movie 7 in the supplementary material). By contrast, for every 100 cell input, only about 10 (11.0±1.3) floating cells were obtained in the absence of BMP4 during days 2-4 (see Fig. S5A in the supplementary material, left, Mi, 83.3±0.7% cells were CD41+ by replating onto adhesive-coated plates). The influence of BMP4 on such greater yield during days 2-4 can also be measured by the higher percentage of floating cells relative to the total number of cells (see Fig. S5A in the supplementary material, right, Mi+BMP4 versus Mi or Mi+BMP4+noggin).

However, the increase observed in the floating fraction might simply indicate that exposure of cells to BMP4 during days 2-4 enhanced the proliferation of floating cells, rather than increasing the hemogenic potential of the day 4 mesodermal precursors. To address this issue, we showed there was a higher percentage of hemogenic colonies relative to the total number of colonies assayed by limiting dilution on 96-well plates (Table 1, Mi+BMP4 versus Mi=21.8±3.9% versus 2.3±2.3%, $P=0.0049$, $n=4$). The greater number of hemogenic colonies induced by BMP4 exposure during days 2-4 indicates that BMP4 enhanced the number of floating cells not simply by enhancing proliferation of the floating cells during days 4-6. Importantly, the results of the limiting diluting assay also implies that the higher number of floating CD41⁺, Runx1⁺ cells was not simply due to a higher input of number of hemangioblast during replating on day 4. Moreover, extended incubation (96 hours) in Vm-containing medium failed to induce Runx1⁺ buddings (see Fig. S6 in the supplementary material), suggesting that the

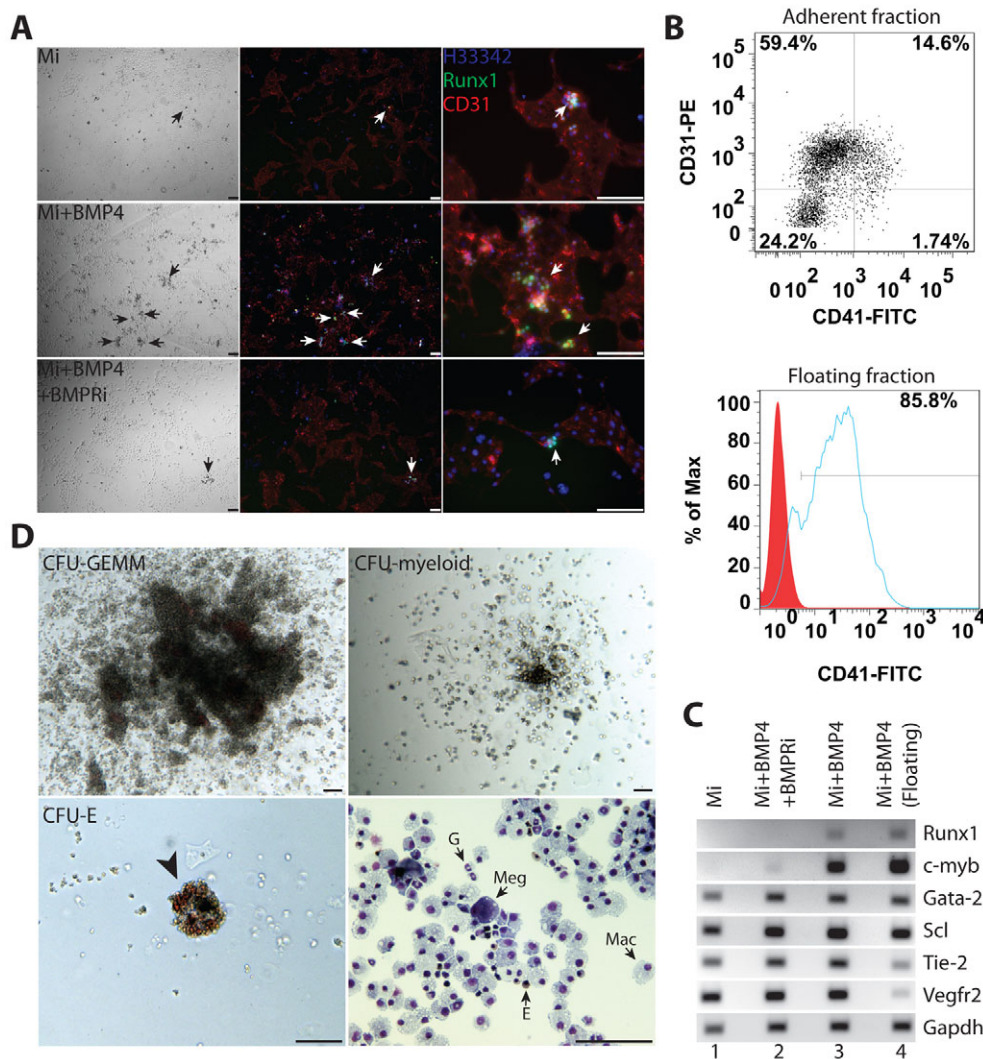


Fig. 4. Inclusion of BMP4 during days 2–4 renders endothelial cells hemogenic. (A) ES cells (500 in each well of a 24-well plate) cultured in basal medium (BM) for 48 hours, then cultured with mesodermal inducer (Mi, BM plus 3 μ M GSKi, 4 ng/ml activin and 12.5 ng/ml FGF2), Mi plus BMP4 (Mi+BMP4) or Mi+BMP4 plus LDN193189 (250 nM) (Mi+BMP4+BMPRI) for an additional 48 hours, and replated (20,000 cells in each well of a 24-well plate) into Vm (BM plus FGF2 12.5 ng/ml, BMP4 20 ng/ml, VEGF 20 ng/ml, BrcAMP 0.25 mM and ALKi 4 μ M) for an additional 60 hours. Left column shows the difference in the number budding cells (arrows), as judged by phase-contrast microscopy. Middle and right columns show representative CD31 (red) and Runx1 (green) immunoreactivity of the budding cells (higher-power view; arrows). H333342 served as the nuclear counterstain. Scale bars: 100 μ m. (B) Representative dot plot for CD31 and CD41 expression in the adherent fraction (upper) and histogram for CD41⁺ fraction in the floating fraction. Red area and blue curve represent unstained control and floating fraction, respectively, stained with FITC-labeled CD41. ES cells were differentiated in BM during days 0–2, Mi+BMP4 during days 2–4 and Vm during days 4–6 and harvested for flow cytometry on day 6. (C) Semi-quantitative RT-PCR of endothelial (Vegfr2 and Tie2) and hematopoietic markers (Scl, Myb and Runx1) from cells differentiated in identical conditions to those indicated in B. ‘Floating’ (lane 4) represents cells from floating fraction alone of identical culture condition as in lane 3. Forty-thousand cells were seeded into each well of a 12-well plate and harvested 60 hours later. Gapdh served as the control. (D) Representative pictures of CFU-GEMM, CFU-myeloid and CFU-E (arrowhead) of cells (150,000) seeded in serum containing methylcellulose medium for 8 days. Lower right panel: cytopsin preparation and Giemsa-Wright staining from a mixed colony showing the presence of megakaryocytes (Meg), granulocytes (G), erythroid cells (E) and macrophages (Mac) as indicated by arrows. Scale bars: 100 μ m.

emergence of hematopoietic precursors was due to sensitivity to BMP4 during days 2–4 rather than simply prolonged exposure to BMP4 in the cultured medium.

Although endothelial and hematopoietic precursor cells were able to form in the same medium during days 5–6, it is possible that cultures grown at high density during days 4–6 encouraged the formation of hematopoietic precursors due to non-cell autonomous paracrine induction by bystanders formed in the presence of BMP4 during days 2–4. To minimize this issue, we tested the effect of

precursors grown in low cell-density cultures. Time-lapse video imaging revealed that the hemato-endothelial colonies were still able to form from a single precursor even seeded under low-cell density conditions during days 4–6 (see Movies 4 and 5 in the supplementary material, input of 500 cells per well of a 12-well plate). In addition, time-lapse microscopy showed evidence of CD144⁺ cells transiting into CD41⁺ hematopoietic cells in day 5–6 cultures (see Movie 6 in the supplementary material), supporting the notion that CD41⁺ hematopoietic cells arose from endothelial

Table 1. Quantification of hemogenic differentiation program from three independent ES cell lines

Types of ES cell	Number of mesodermal cells on day 4 per 100 ESCs	Percentage of ES cells forming colony on day 4	Number of CD31+ cells on day 6 per 100 mesodermal cells	Purity of CD31+ cells on day 6 (%)	Percentage of CD31+ colonies on day 6	Number of floating cells on day 6 per 100 mesodermal cells induced by BMP4 during days 2-4	Percentage of floating/total cells on day 6	Percentage of hematopoietic colonies on day 6 in the absence (presence) of BMP4 during days 2-4
C57BL/6j (v26.2)	1928±138	11.3±0.8	40.2±2.7	85.9±1.6	69.8±3.2	165.0±15.3	22.4±2.3	2.3±2.3 (21.8±3.9***)
BALB/c 3 129/sv (v17.2)	895±39	9.8±0.4	127.7±13.0	89.8±0.4	82.9±0.5	175.0±6.4	29.6±1.8	9.3±2.4 (50.3±0.3***)
129S6/SvEvTac	2160±151	20.3±0.6	206.3±11.4	82.9±1.8	73.7±0.1	171.3±24.9	26.6±0.8	14.3±0.5 (68.9±3.2****)

Three ES cells lines were differentiated in our defined system and quantitated for output and purity in each major step. Data are mean±s.e.m. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

precursors. Similar capability was also observed by a limiting dilution study showing formation of a single hemogenic colony per well (see Fig. S7 in the supplementary material). These findings strongly support the view that the fate to form hematopoietic precursors is determined during mesoderm differentiation on days 2-4. However, inclusion of BMP4 during days 2-4 induced only a small fraction of colonies to be hemogenic (21.8±3.9% of colonies by limiting dilution), whereas most of the colonies remained vasculogenic (62.1±6.0% of colonies by limiting dilution assay). Even in the hemogenic colonies, endothelial morphology or markers were still retained in a subset of cells (see Movie 4 in the supplementary material). This finding indicates that the 'positional' cue exerted by early BMP4 activation had incomplete imprinting effects on the differential fates of day 4 precursors to hemogenic colonies on day 6. Interestingly, within 2-4 days of harvesting and replating in BM containing various combinations of cytokines, the cells derived from the floating fraction were able to form: TER-119⁺ (Ly76 – Mouse Genome Informatics) (see Fig. S8A in the supplementary material, bottom panel) erythroid cells that are capable of expressing both embryonic (see Fig. S8C in the supplementary material, Hbb-bh1) and adult-type (see Fig. S8C in the supplementary material, Hba-a1 and Hbb-b1) hemoglobin; multi-lobulated nucleated PU.1⁺ (see Fig. S8B3 in the supplementary material) or CD11b⁺ (Itgam⁺ – Mouse Genome Informatics) (see Fig. S8A in the supplementary material, top panel), CD45⁺ (Ptpcr⁺ – Mouse Genome Informatics) myeloid cells [see Fig. S8B4,C in the supplementary material CD45, CD115 (Csf1r – Mouse Genome Informatics), lysozyme]; and CD41⁺ (see Fig. S8A in the supplementary material, middle panel) megakaryocytes with pro-platelet protrusions (see Fig. S8B1 and Movie 7 in the supplementary material). Using serum-containing methylcellulose culture, cells from the floating fraction were also able to form colonies of erythroid, myeloid and mixed series (Fig. 4D; see Fig. S8D in the supplementary material), confirming that budding cells were multi-lineage hematopoietic precursors.

To establish the reproducibility of the differentiation process, we have consistently converted C57BL/6 ES cells (v26.2) to endothelial and hematopoietic precursors using this set of defined factors in over 50 independent experiments. Moreover, the generality of the differentiation process was verified by differentiation of two additional independent germline competent murine cell lines, v17.2 ES cells and an in-house strain of 129S6/SvEvTac ES cells (Table 1).

DISCUSSION

A simple and efficient method to convert ES cells to endothelial cells and blood precursors

Previous methods designed to convert ES cells to hematopoietic precursors would be problematic for clinical applications. As these strategies rely principally on serum or feeder layer (Eilken et al., 2009; Lancrin et al., 2009), significant concerns regarding pathogen contamination and allergens or passage limitation and senescence of feeder cell lines are raised. To overcome these limitations, we have developed a differentiation strategy that employs low cellular density in defined media that would minimize the exposure of cells to complex and unknown confounders. This protocol allowed us to show the sequential conversion from Klf4⁺ ES cells to Fgf5⁺ epiblasts, followed by Vegfr⁺ mesoderm, and finally to CD31⁺, CD144⁺ endothelial or CD41⁺; Runx1⁺ definitive hematopoietic precursors in a consistent, controlled environment (Fig. 5). In addition, we were able to trace the differentiation process from a single starting cell, such that cell differentiation occurs in an adherent, low-density condition, which could be followed by time-lapse video microscopy from ES cells to hemogenic endothelium without the formation of complex embryoid bodies. Importantly, our approach will allow a high percentage yield (~80%) for both endothelial cells and blood precursors (Table 1, Fig. 4B) without the use of sorting processes between differentiation steps required in previous methods (Eilken et al., 2009).

Canonical Wnt is essential for transition into mesoderm

Although BMP4 is hypothesized to be essential for the formation of mesoderm and Vegfr2⁺ angioblasts (Park et al., 2004; Winnier et al., 1995), the presence of BMP4 is not essential for the emergence of the hemangioblasts in our defined culture system (Fig. 2): the presence of BMP4 during the crucial period for the generation of hemangioblasts (days 2-4) severely hampered the hematopoiesis potential, whereas the endothelial potential is preserved in our system (Fig. 4A). The nonessential role of BMP4 in our system suggests that BMP4 exerts its mesoderm-inducing role indirectly, possibly through Wnt signaling. More specifically, that mesoderm specification can be simply induced by glycogen synthase kinase inhibitor confirms that canonical Wnt signaling through activation of β -catenin is the principal factor among other Wnt signaling pathways required for this transition step (Kühl et al., 2000; Veeman et al., 2003; Wodarz and Nusse, 1998).

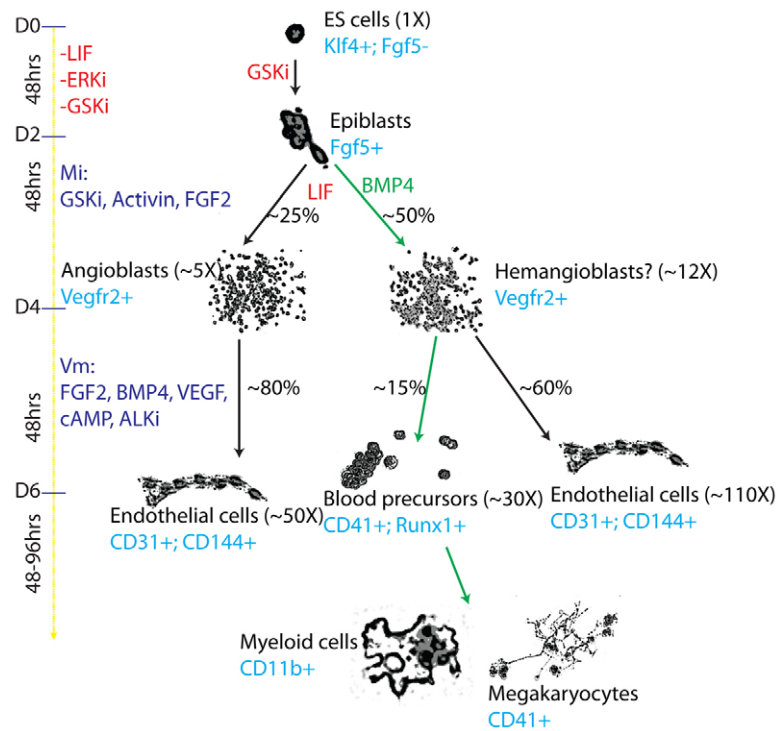


Fig. 5. Summary of the stepwise differentiation and factors sufficient for fating ES cells into hematopoietic precursors. Numbers in parentheses indicate calculated fold increase from ES cells. Percentages next to arrows demonstrate approximate efficiencies of fate determination in the resultant population. Factors in red inhibit differentiation. Presence of BMP4 (green) dramatically enhances formation of hemogenic endothelium on day (D) 6. Note the cells are not drawn to the same scale.

Colonial differentiation of single endothelial/blood precursor

Although it is known that the Vegfr2-positive mesoderm is the precursor of endothelial cells, a defined condition to maintain the survival of angioblasts and their subsequent differentiation into endothelial cells remains elusive: serum is currently the only way to demonstrate the colonial differentiation of angioblasts to endothelial cells (Yamashita et al., 2000). Here, we demonstrate that FGF2 is an essential factor for the survival of endothelial precursor, as most of the replated mesodermal precursors were lost and very few endothelial colonies were formed in the absence of FGF2 (Fig. 3). The existence of FGF2 in fetal calf serum explains the requirement of serum for the survival of angioblast (Zheng et al., 2006). Another active component in serum is BMP4 (Herrera and Inman, 2009), which is the key factor required for transition from loosely interacting angioblasts to tightly aggregated endothelium in our defined differentiation condition (Fig. 3A). Thus, the inclusion of serum mitigates the need of both factors for single colonial differentiation of angioblast. However, TGF β , a factor that also commonly exists in serum (Zheng et al., 2006), drives formation of mural cells at the expense of endothelial cells (Watabe et al., 2003). This explains why the inclusion of TGF β receptor antagonist enhances the purity of endothelial population (Fig. 3). Furthermore, this also suggests that there is still an autocrine or juxtacrine effect of TGF β in our differentiation system.

Nevertheless, we could not exclude the possibility that there are still other essential factors for the differentiation provided by the few progeny cells in the colony. However, our defined culture system for the conversion to a highly pure endothelial population from one single progenitor is the highest resolution currently

achievable. Thus, our results indicate that we have defined the minimal set of factors sufficient for angioblast survival and endothelial transition. Although it may be possible to improve the resolution with more frequent passage of cells or by addition of cell division inhibitors, these harsh manipulations could be detrimental to cell survival or even alter the cell fate determination. In any case, resolving how the downstream signaling of VEGF, BMP4 and cAMP work together to trigger the transcriptome changes to determine the endothelial fate will be an important question to pursue.

Angioblast and hemangioblast?

The dramatic difference in hemogenic potential induced by BMP4 of day 4 mesodermal population indicates the existence of two unique populations of endothelial precursors, angioblasts and hemangioblasts (Fig. 5). Whereas angioblasts formed in the absence of sufficient BMP4 activity has angiogenesis potential, only hemangioblasts, induced by BMP4, can act as both endothelial and blood precursors. Clarification of whether angioblasts and hemangioblasts are formed independently from the primitive streak, or that the angioblast was triggered to form the hemangioblast during the limited very early time window of its generation will be of interest. As Vegfr2 is expressed in both populations, it would be crucial to identify specific markers of the hemangioblast to distinguish these two populations. Although the limiting dilution colony formation assay suggests only some of the Vegfr2+ cells are hemogenic (20-50%, depending on the cell strain used), our defined system would be instrumental for comparative analysis of these two populations as the hemogenic potential is much greater in the BMP4-induced population. Through

transcriptome analysis, we envision that it would be possible to identify the early distinct markers in the hemogenic module in the Vegfr2⁺ cells.

Although BMP4, potentially through Gata2, is known to enhance both hemogenic and angiogenic fate during differentiation in a time-dependent manner (Lugus et al., 2007; Pearson et al., 2008), the methods of analysis was confounded by the use of high density culture after the induction, making it difficult to assess whether the fate change induced by BMP4 is cell autonomous or caused by a secondary bystander cell after BMP4 induction. By colonial differentiation in limiting dilution conditions, we demonstrated clearly that the fate to hemogenicity is actually established early in a cell-autonomous manner (see Fig. S7 in the supplementary material). Although BMP4 dramatically improved the generation of hemogenic colonies, the effect of induction was not complete: a large proportion of the colonies remained nonhemogenic and endothelial cells were still intermixed with blood precursors in the hemogenic colonies (see Movie 4 in the supplementary material). This phenomenon suggests that there are still unidentified exogenous signals or endogenous factors that control the blood/endothelial decision if the choice of fates is not completely stochastic.

Significance and mechanistic implications of time-sensitive fate determination

That the identical factor, BMP4, is crucial for both angiogenesis and hemogenesis raises the interesting possibility that the time-dependent responsiveness may be one effective mechanism for differential regulation by BMP4. It is possible that continuous budding of the blood precursor is energetically unfavorable or that the budding process itself prohibits efficient formation of vascular tube. Angiogenesis is a continuous and essential process during embryogenesis throughout the whole organism, whereas hematopoiesis is required only locally due to the circulation and motile property of blood cells. That both processes be tightly and independently regulated necessitates the early departure of angioblast and hemangioblast fates.

Although the exact mechanism governing the difference in the time-sensitive responsiveness remains to be determined, the differential response to the same factor would suggest that there are two independent set of transcriptional modules, angiogenic and hemogenic, that appear in a time-dependent manner. For example, whereas the hemogenic module is active during only days 2-4, the angiogenic module is active after day 4 of our differentiation program, such that the delayed exposure to BMP4 during days 4-6 fails to trigger hemogenicity because the required set of hemogenic transcription factors are no longer present. Identification of the factors and the upstream pathways responsible for the switch in responsiveness would be important towards enabling the conversion of somatic cells directly into blood precursors without relying on transfection or viral integration methods to introduce potentially hazardous DNA into cells (Szabo et al., 2010).

Conclusion

By minimizing the factors that influence intercellular communications and use of time-lapse video microscopy at single-colony resolution, our defined differentiation process allowed clear demonstration of the two independent sets of factors in triggering differentiation of ES cells into hematopoietic/endothelial lineages (Fig. 5). Our strategy also permitted the dissection of the whole differentiation process into three distinct stages, from embryonic stem cells to endothelial or hematopoietic precursors. In addition,

this defined system allows the identification of minimal yet sufficient factors required for each step of transition, providing an excellent model with which to clarify the molecular insight on hematopoietic differentiation, particularly highlighting the differential and time-sensitive roles BMP4 plays in angiogenesis and hematopoiesis. Although the transplantability of murine hematopoietic precursors and whether this defined pathway to hemogenic endothelium can be extended to human ES/iPS cells remain to be tested, our simple in vitro protocol could serve as a framework for future studies of hematopoietic stem cell differentiation in humans.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

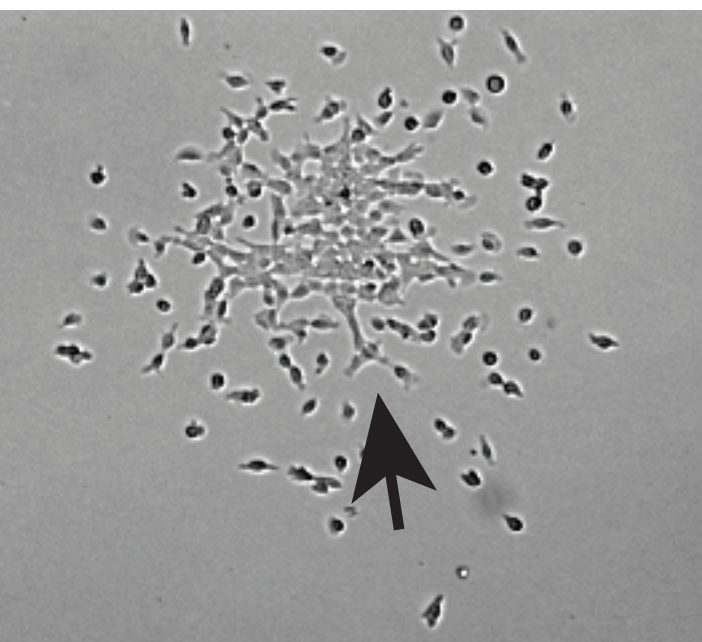
Supplementary material for this article is available at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.061440/-/DC1>

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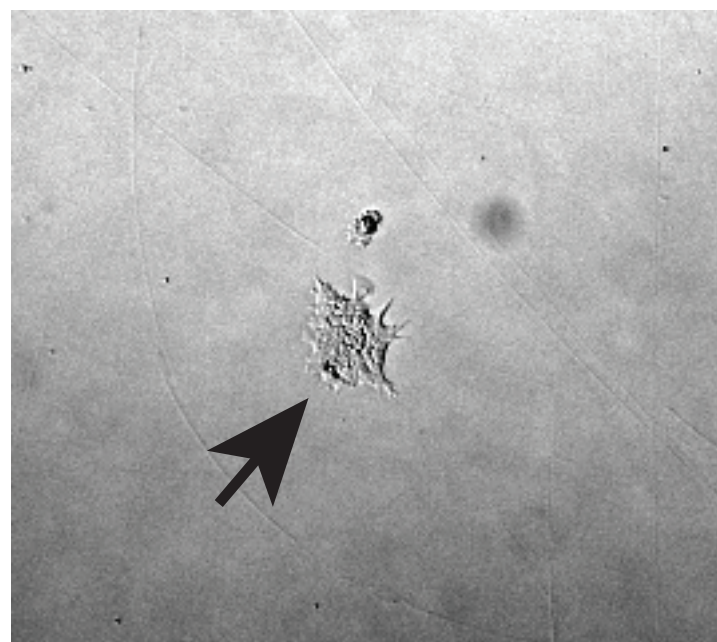
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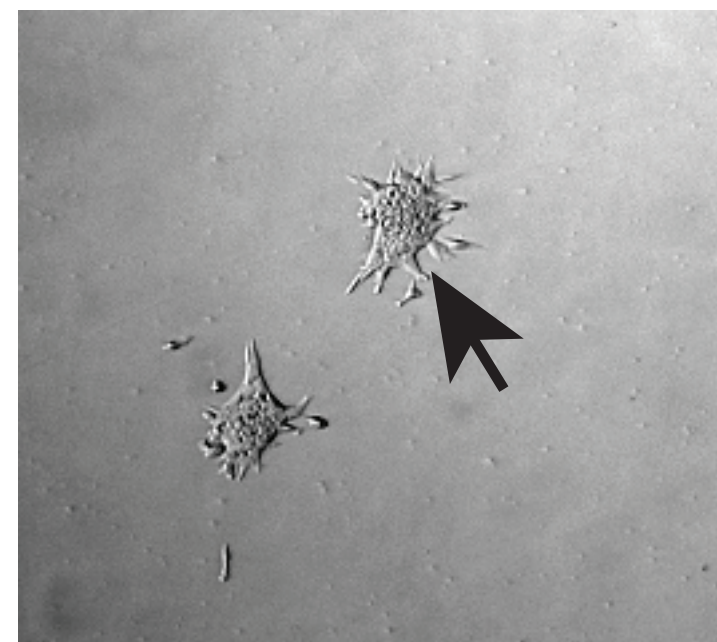
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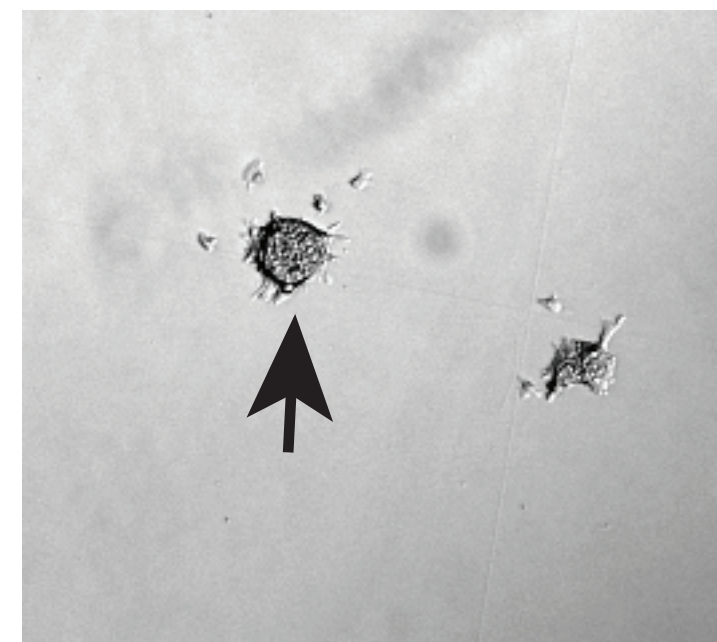
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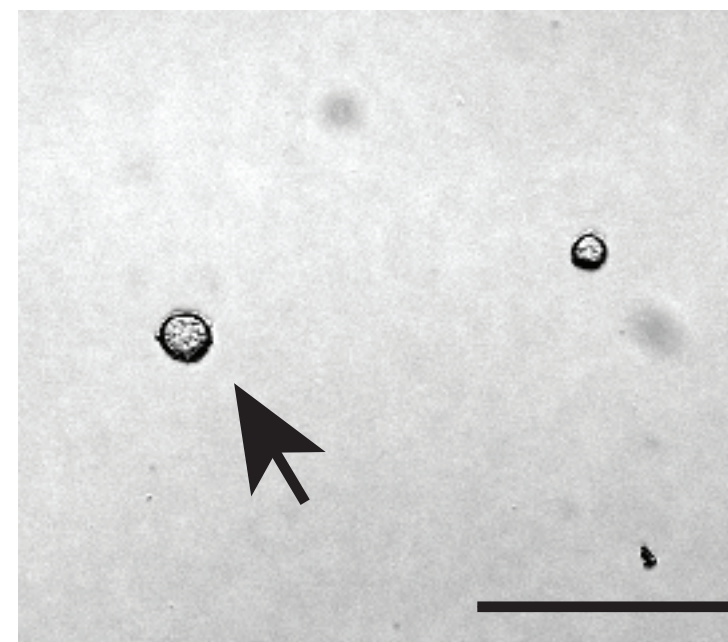
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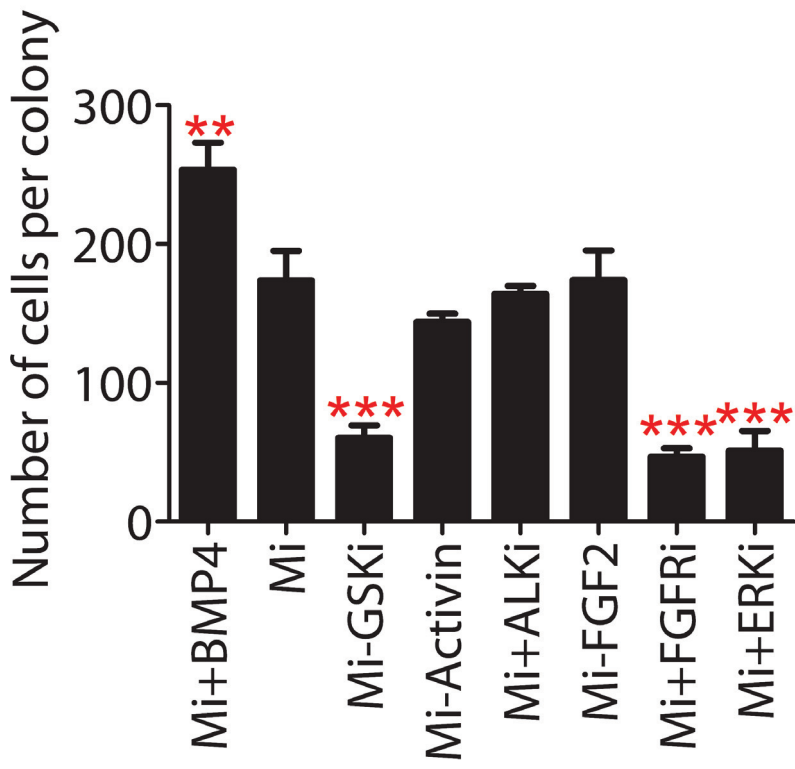
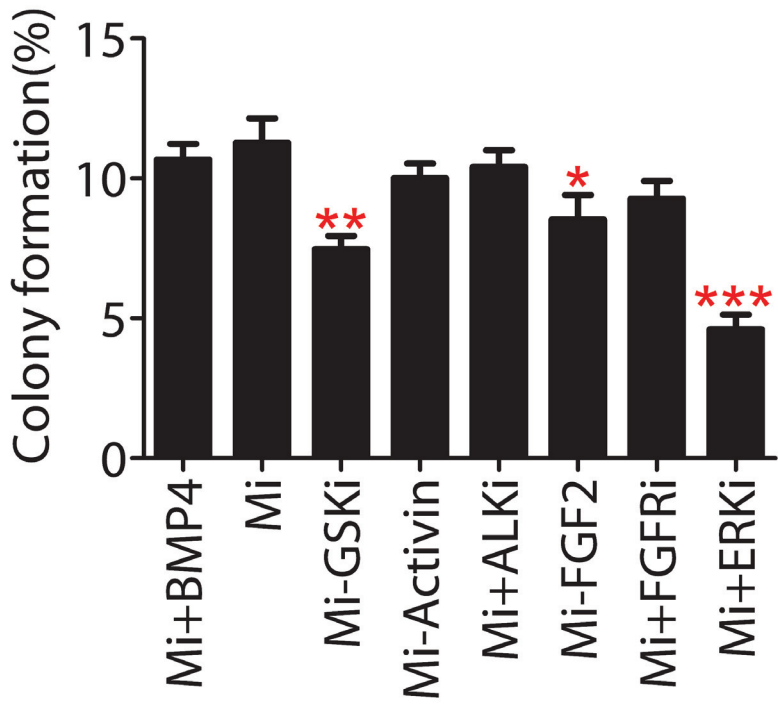


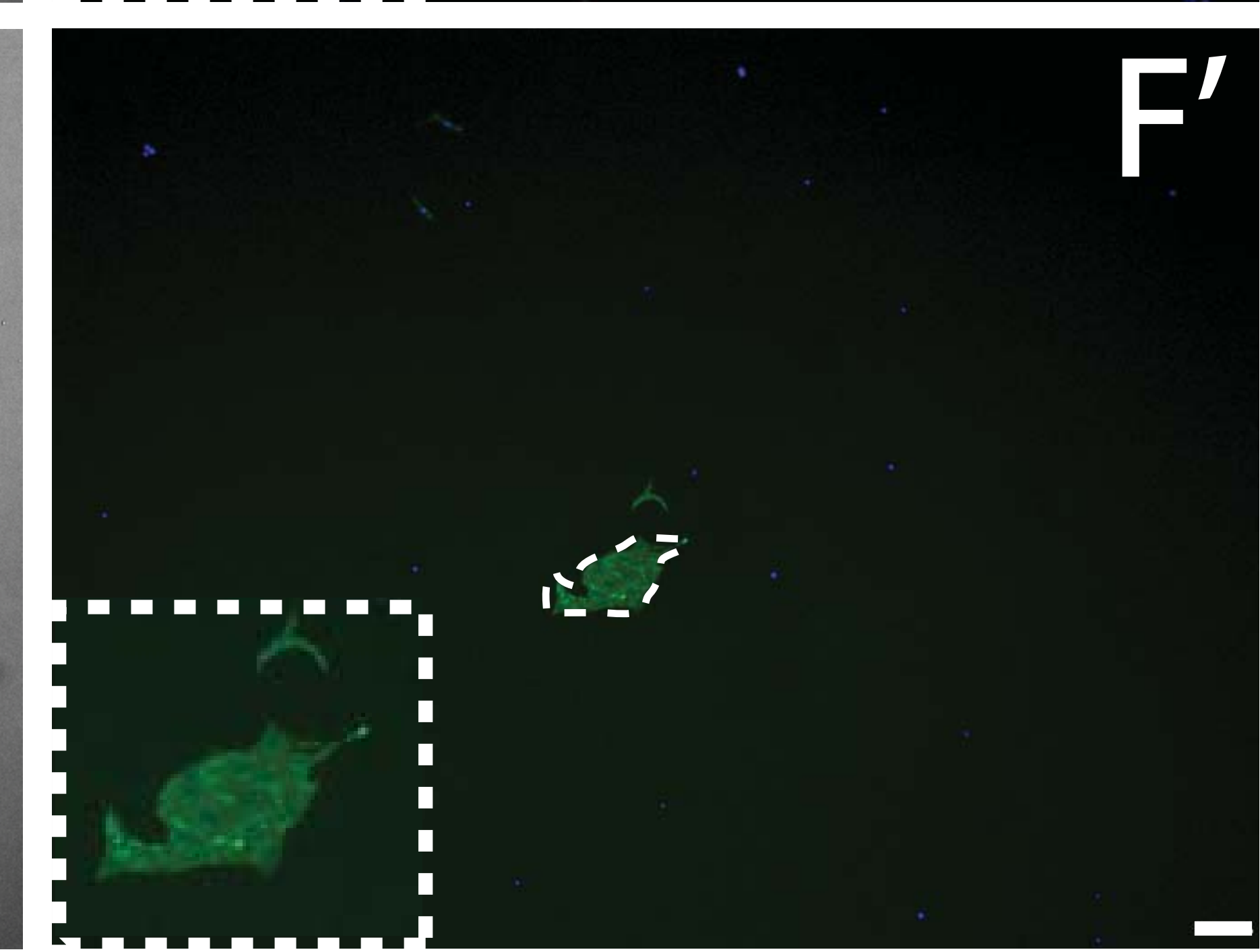
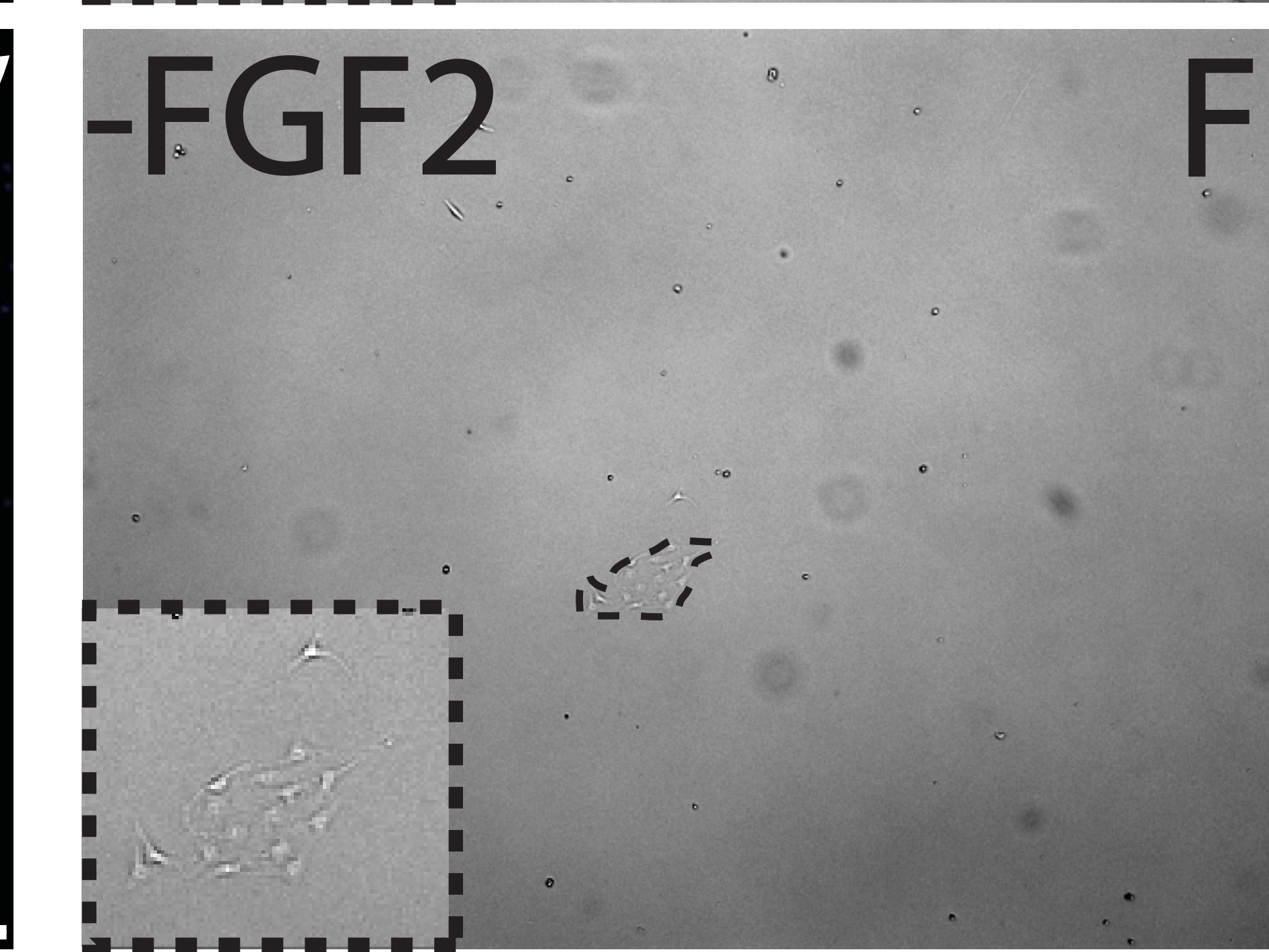
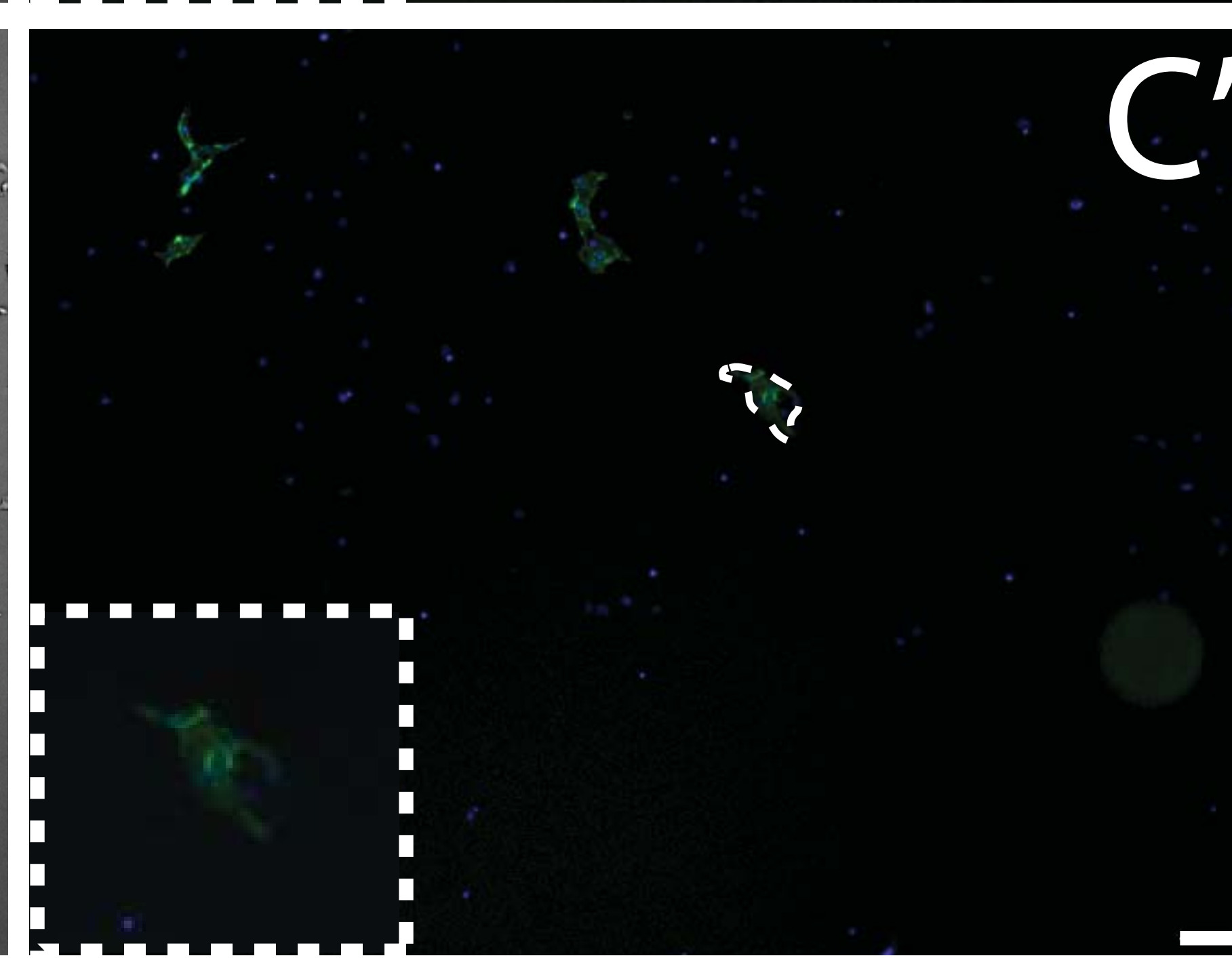
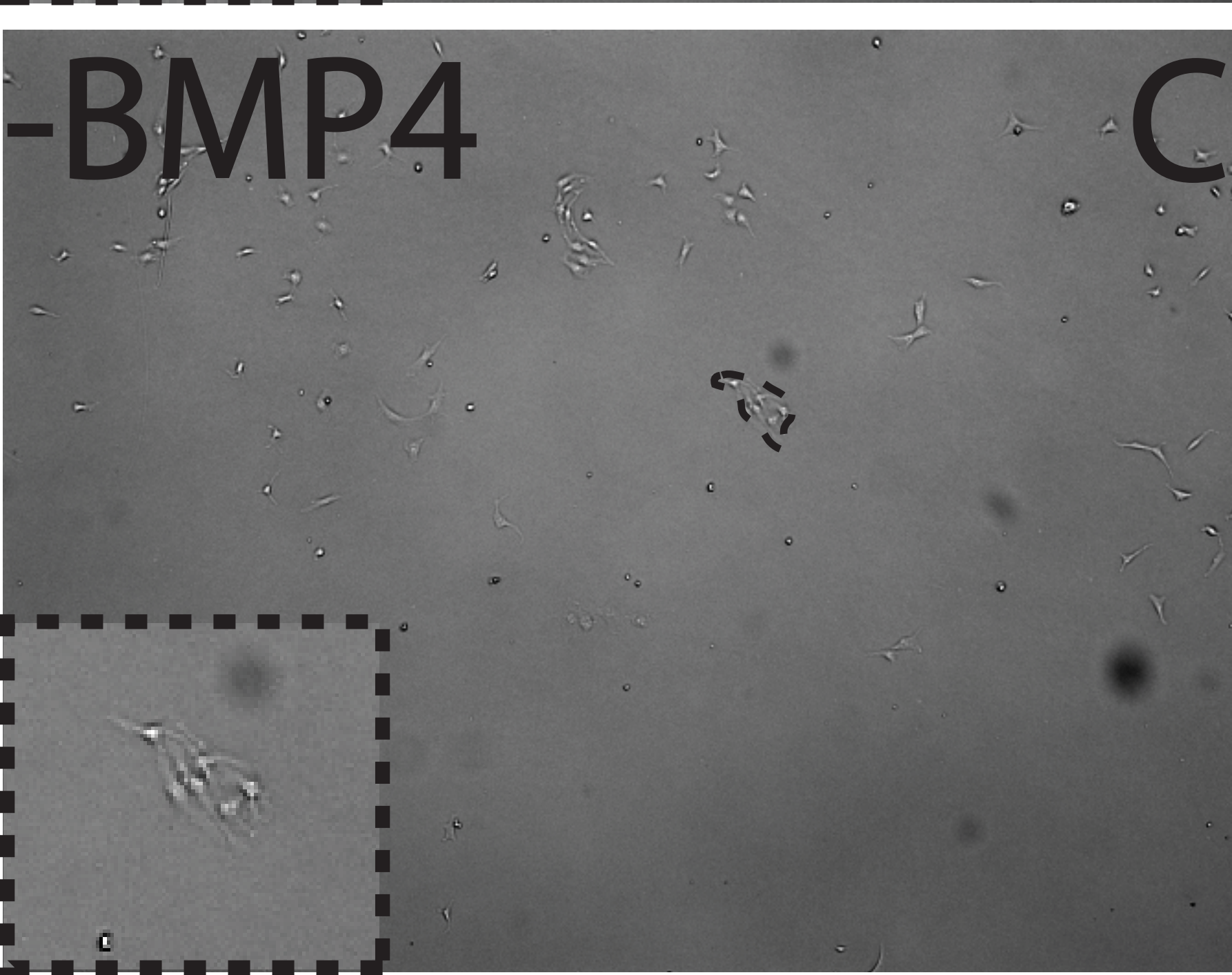
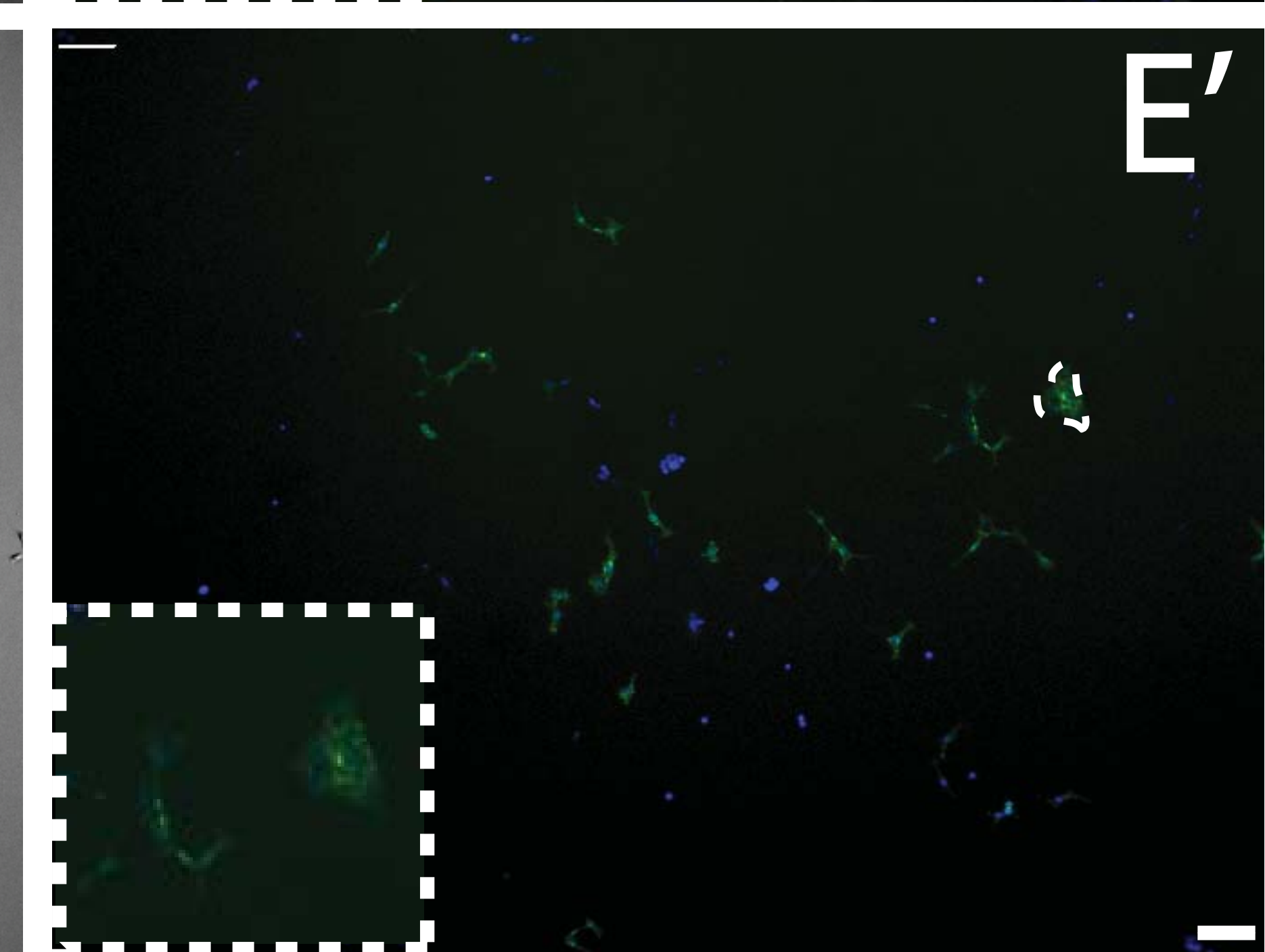
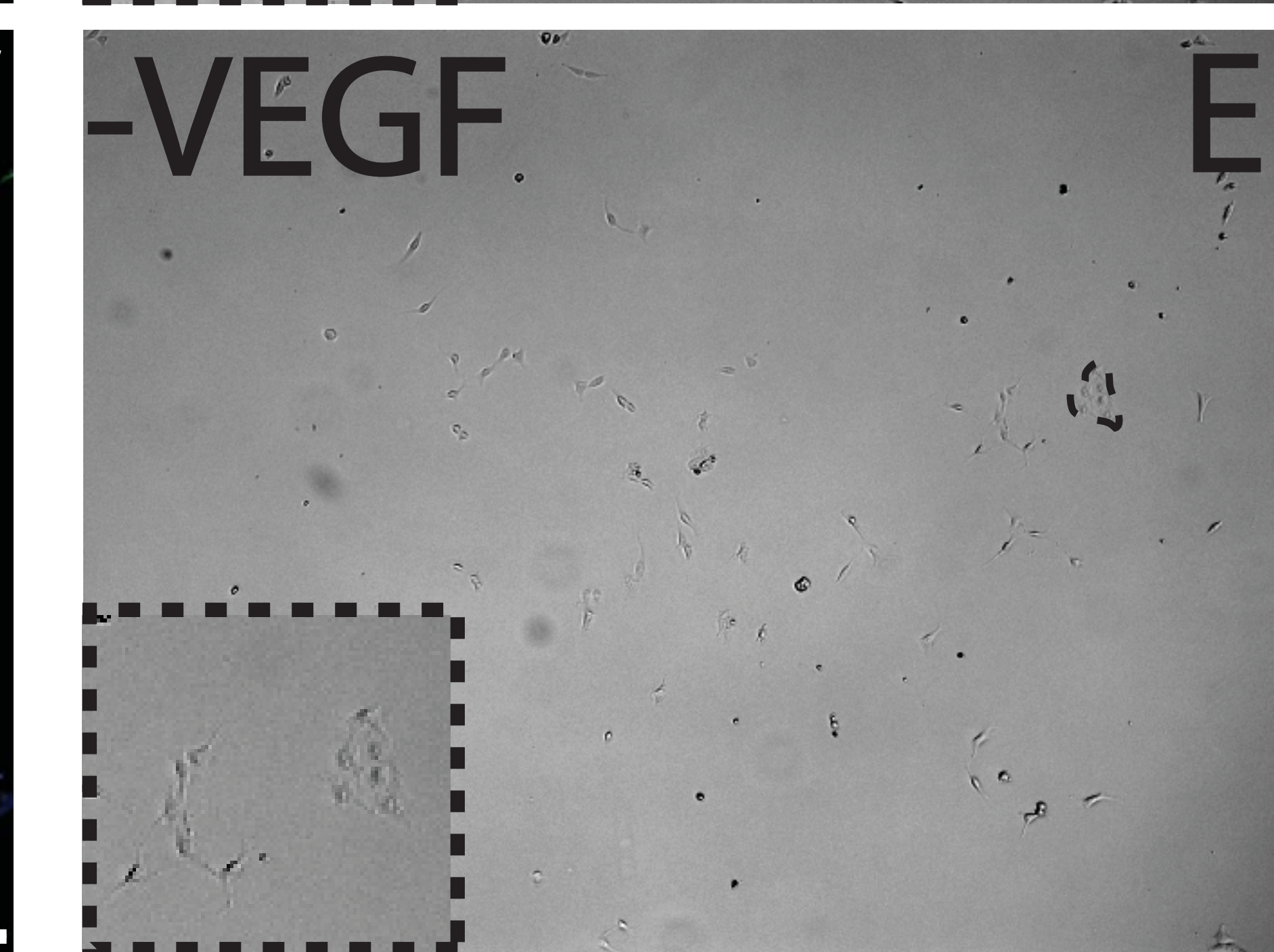
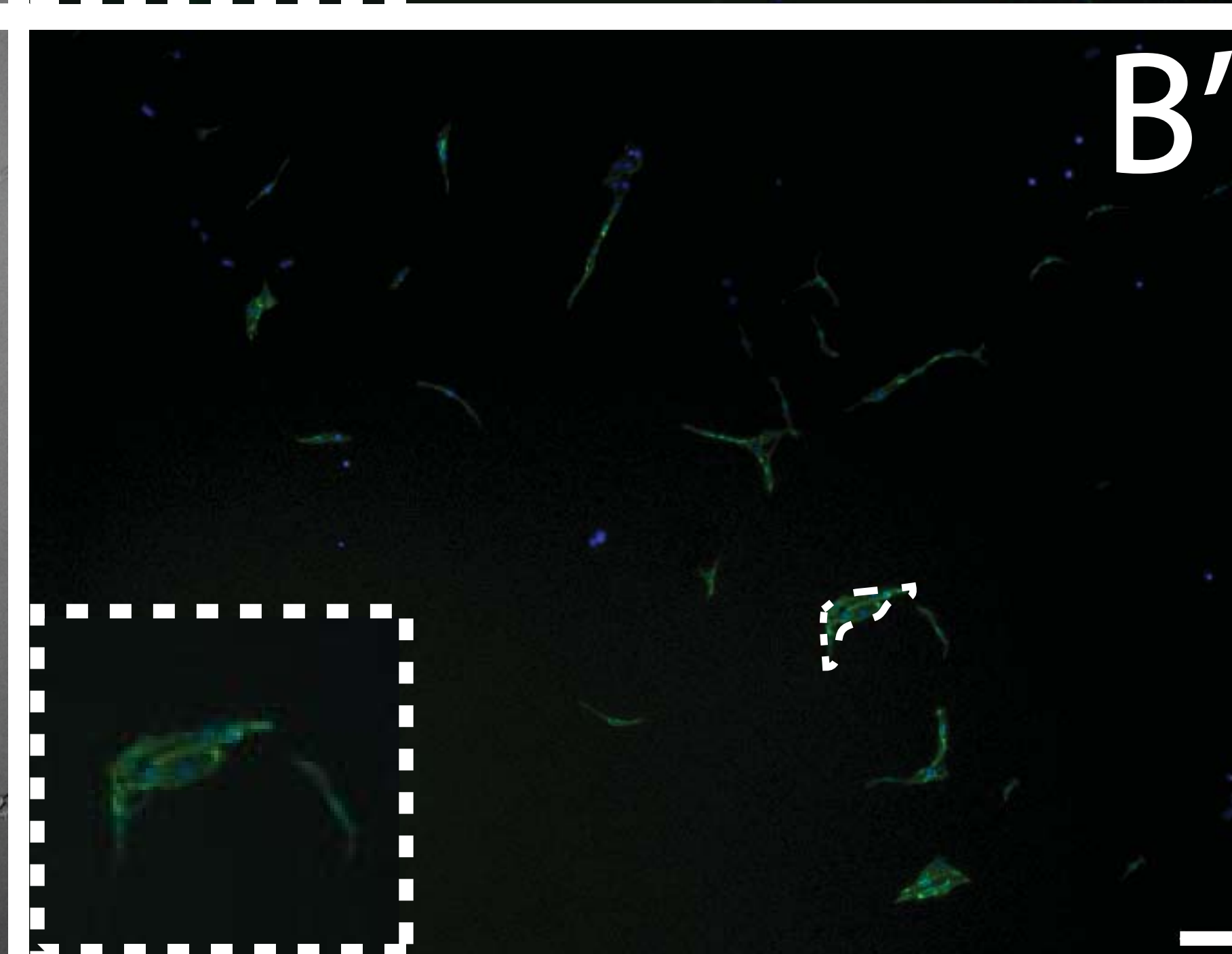
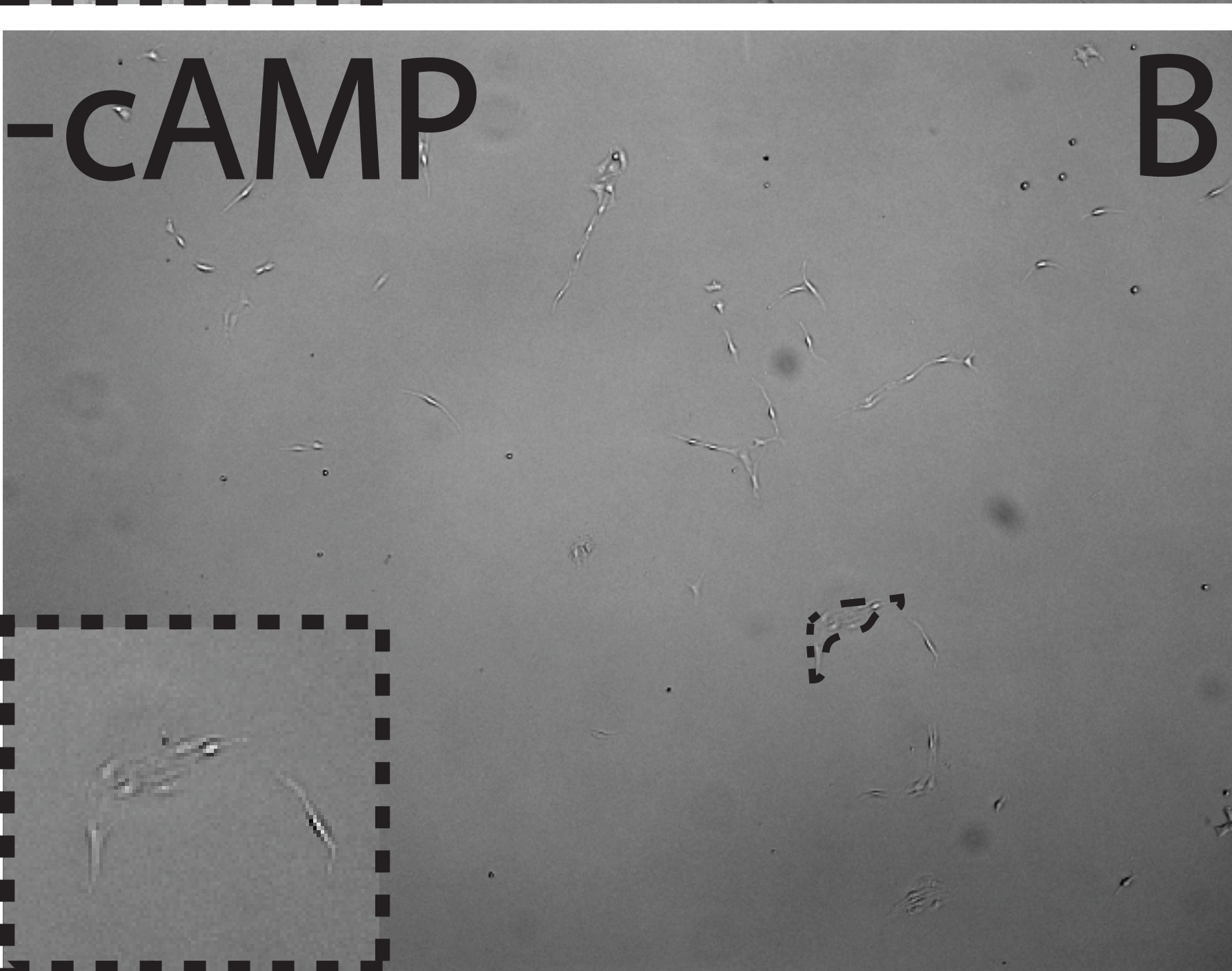
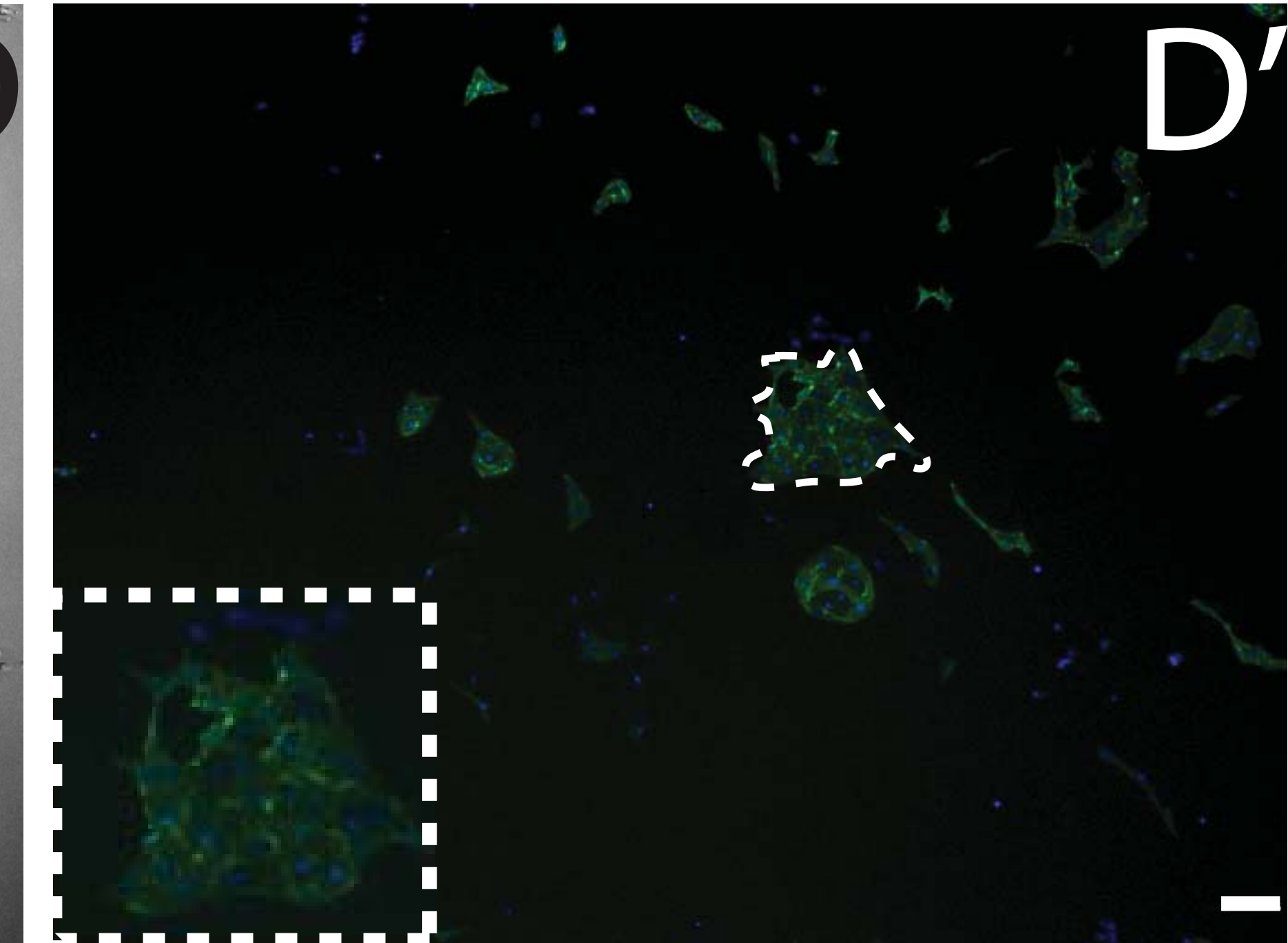
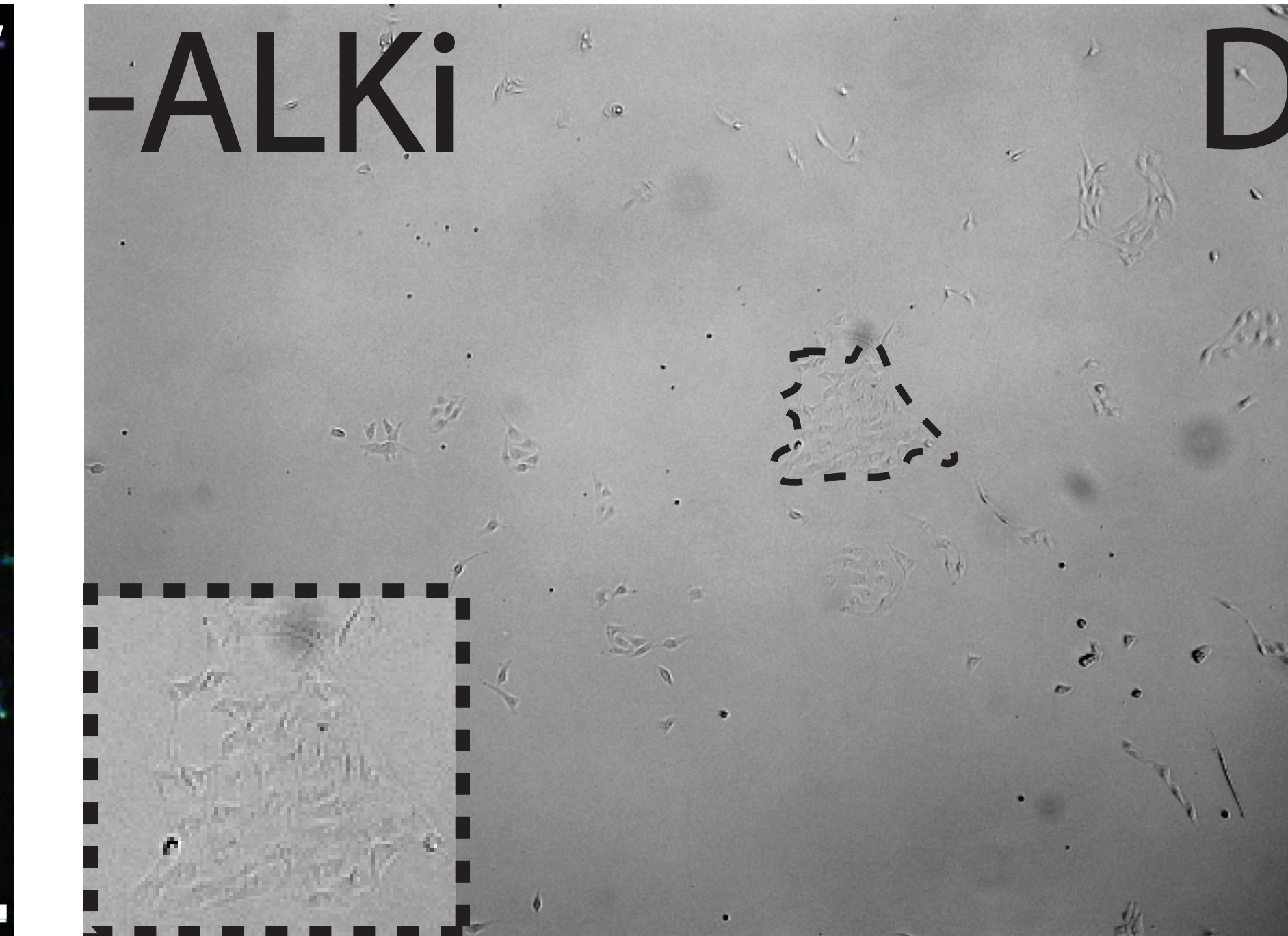
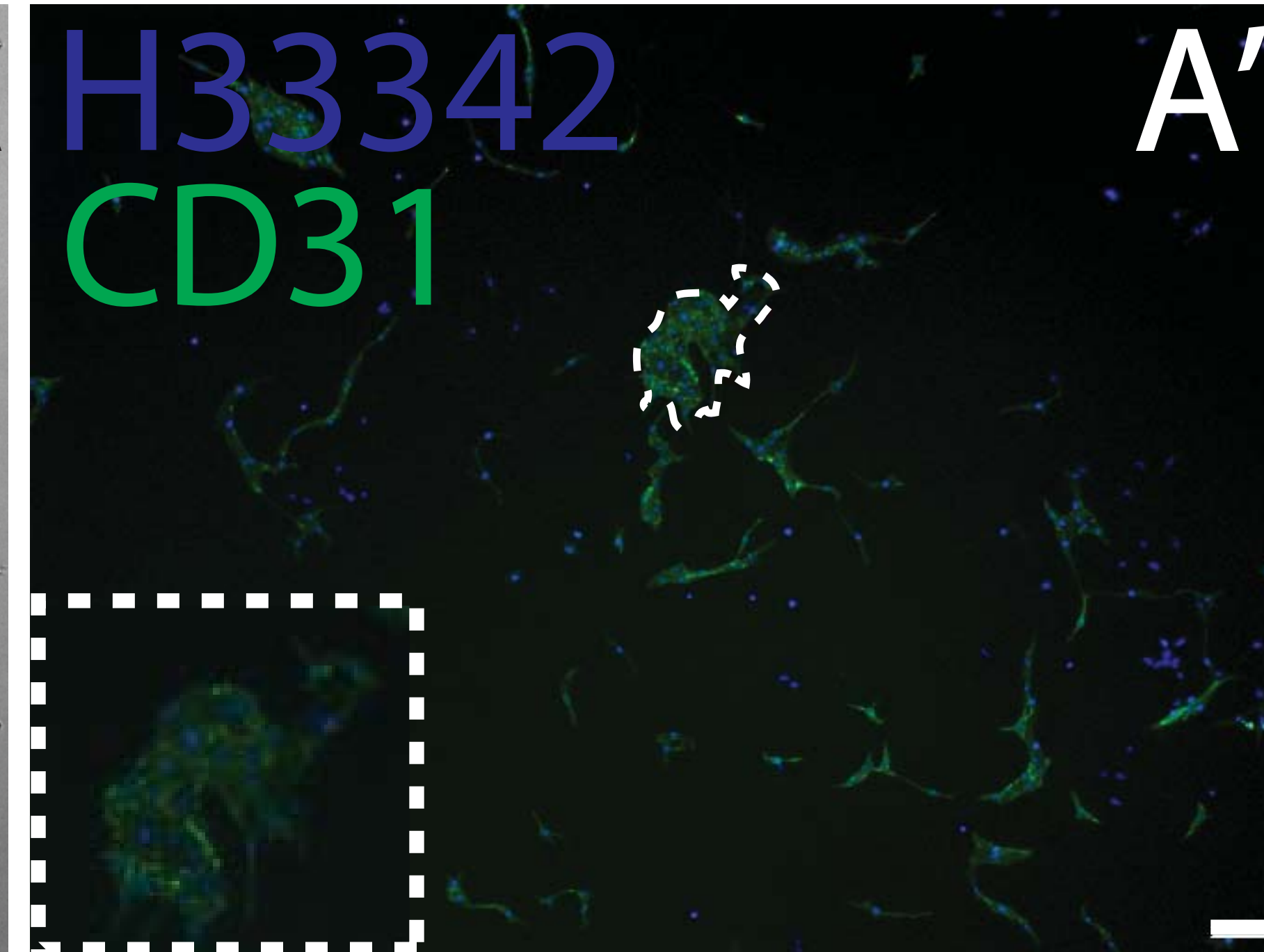
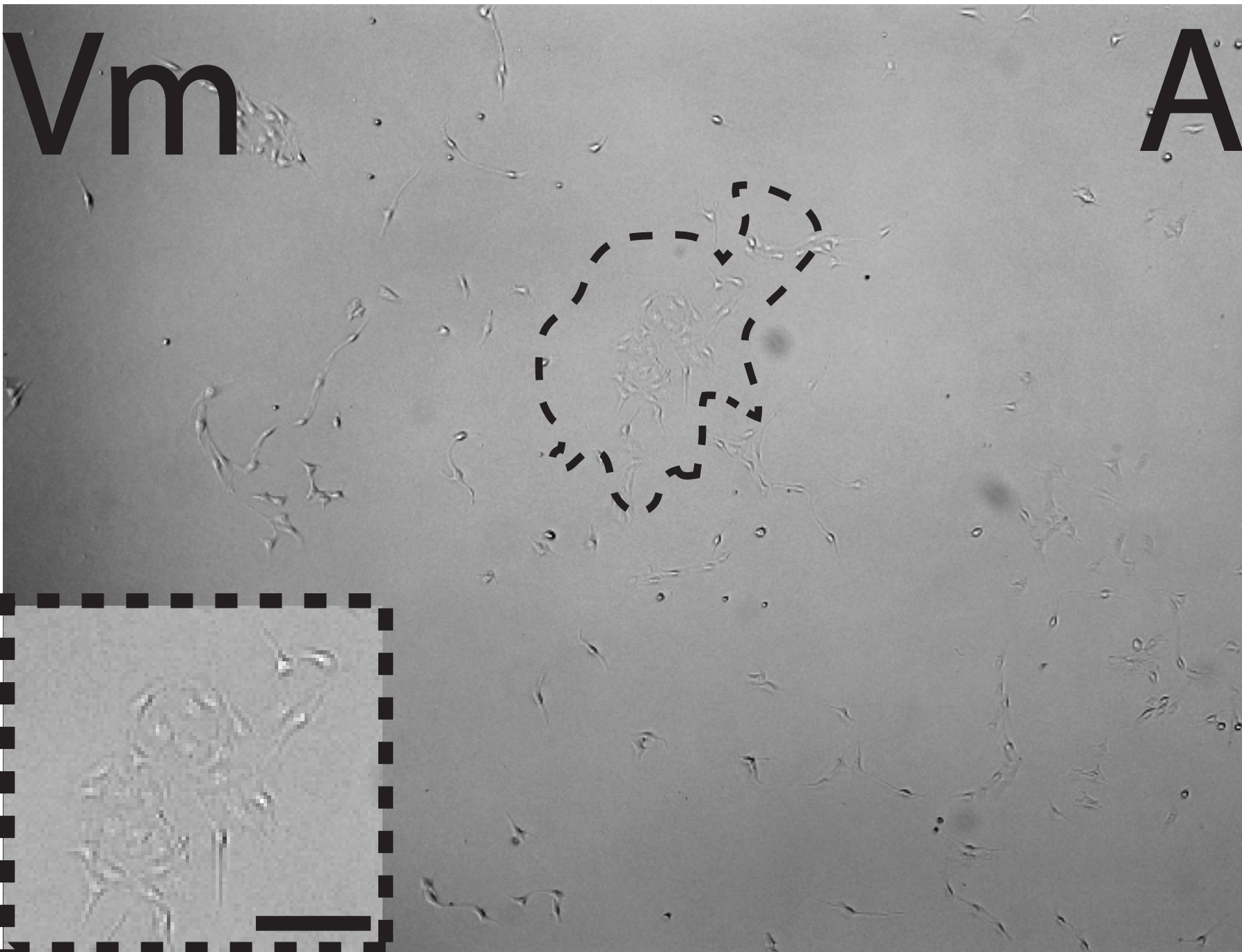
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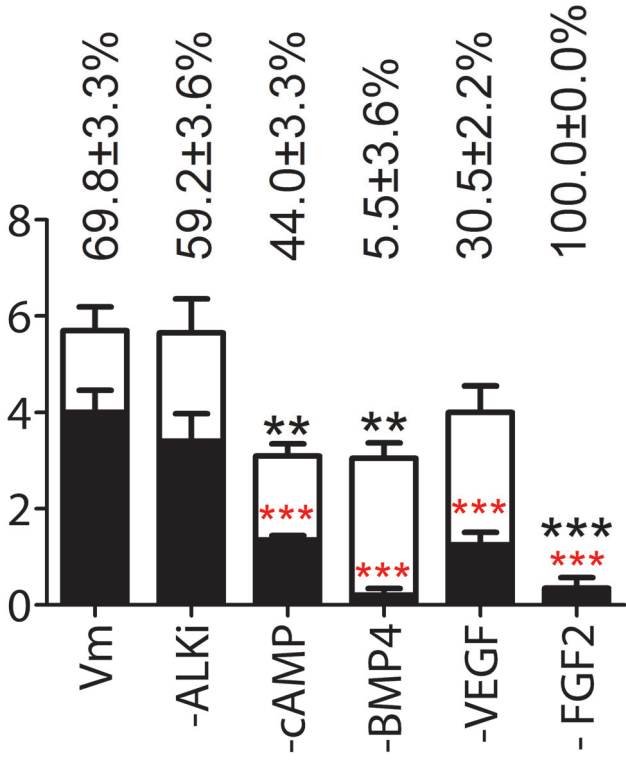
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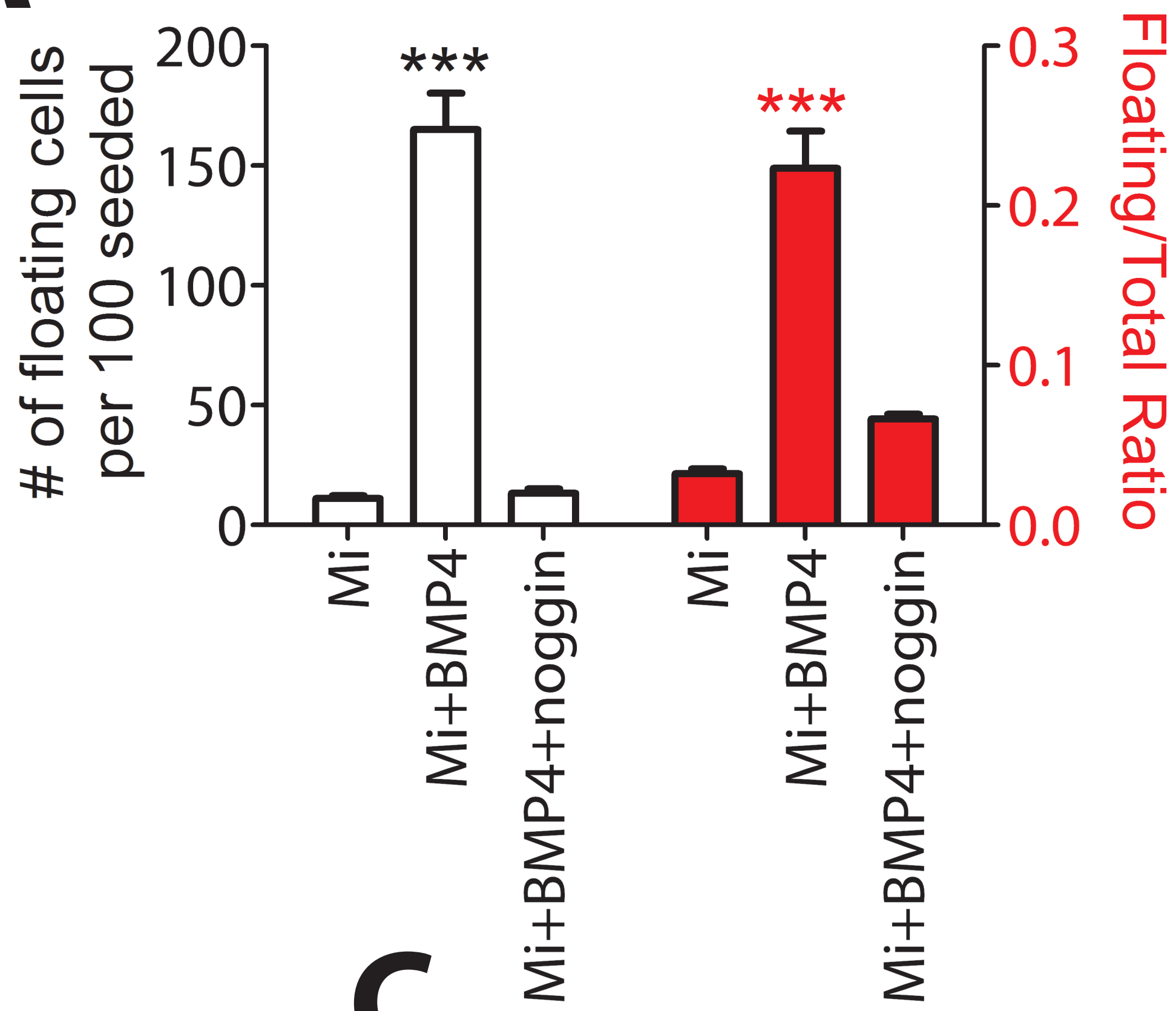
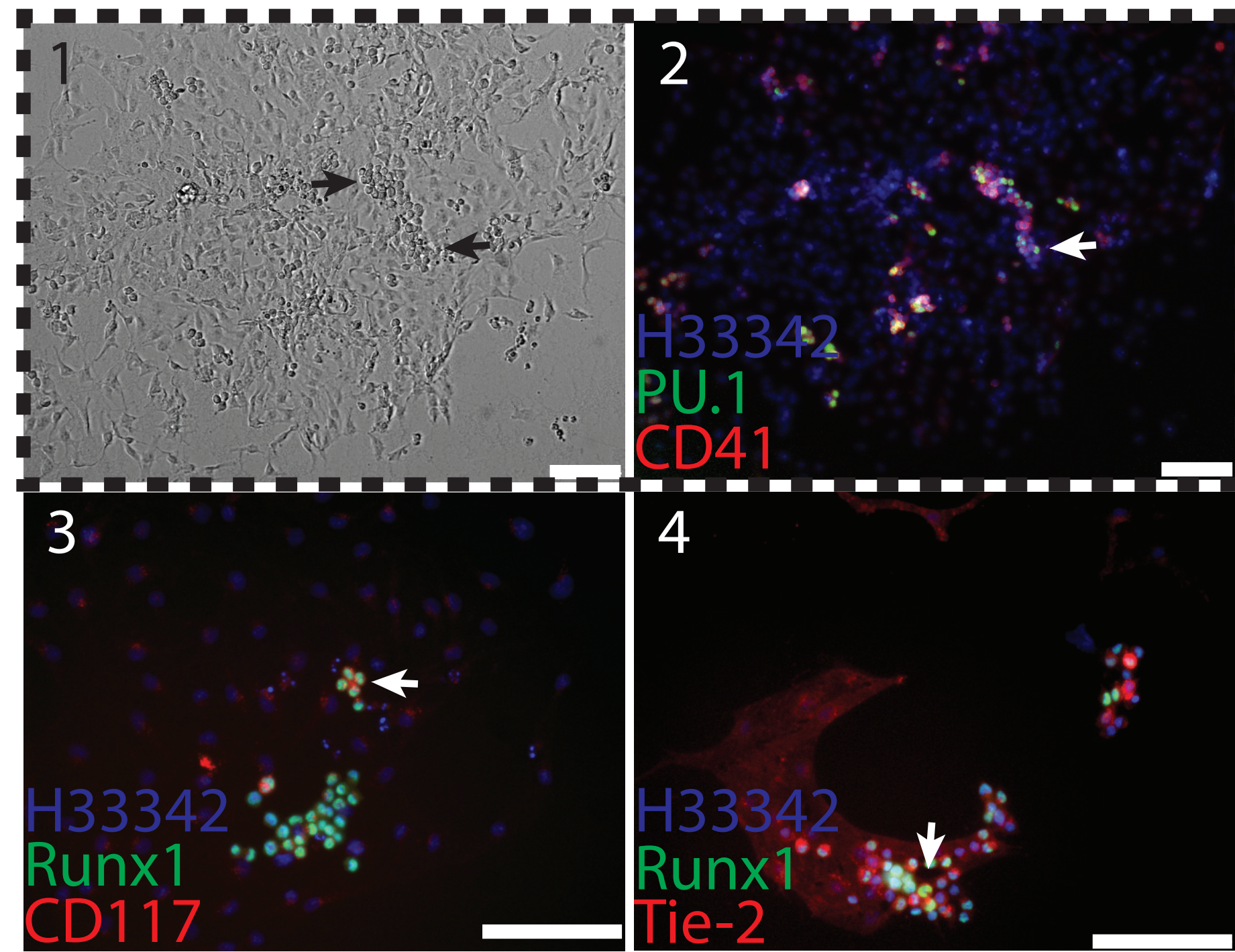
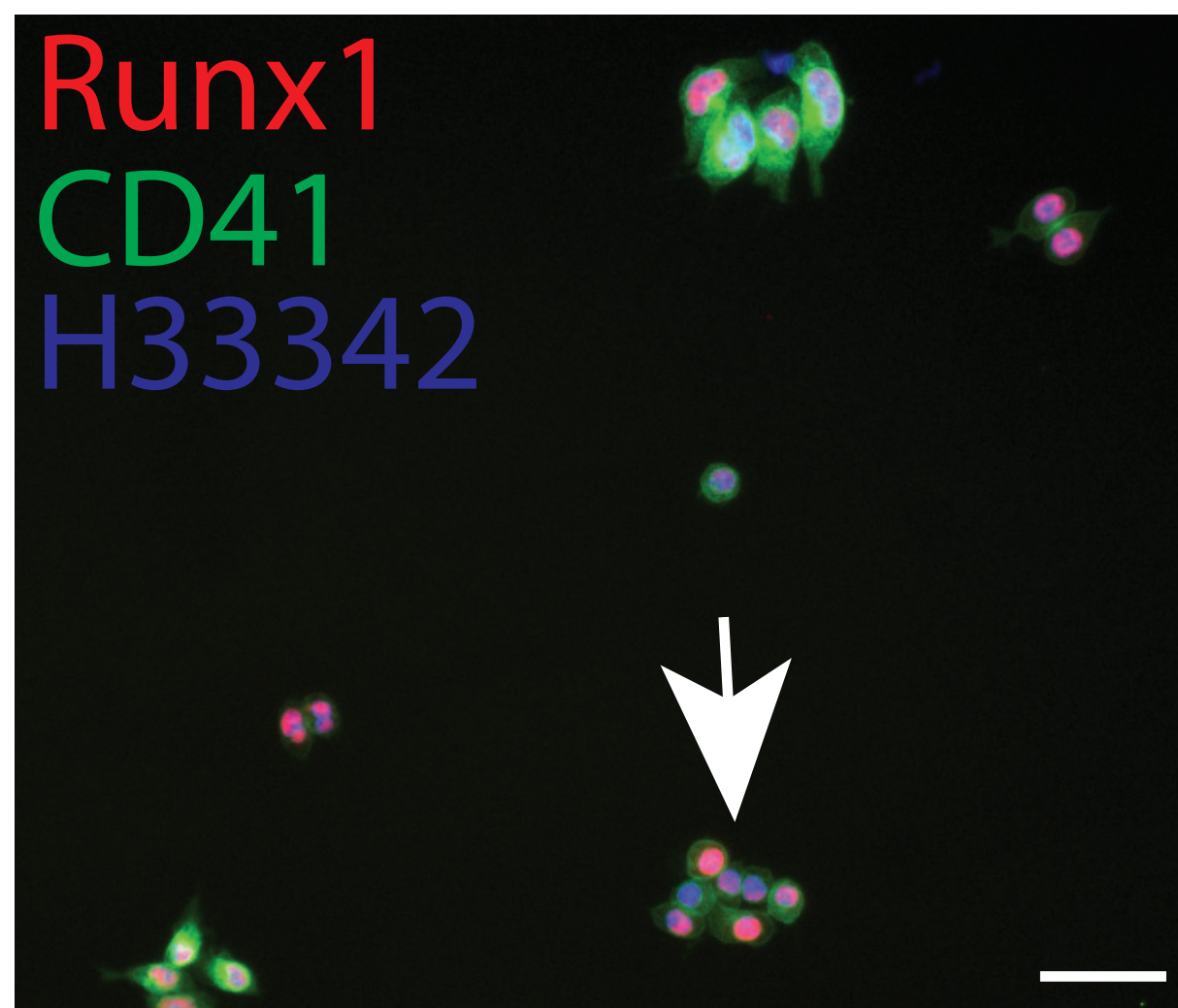
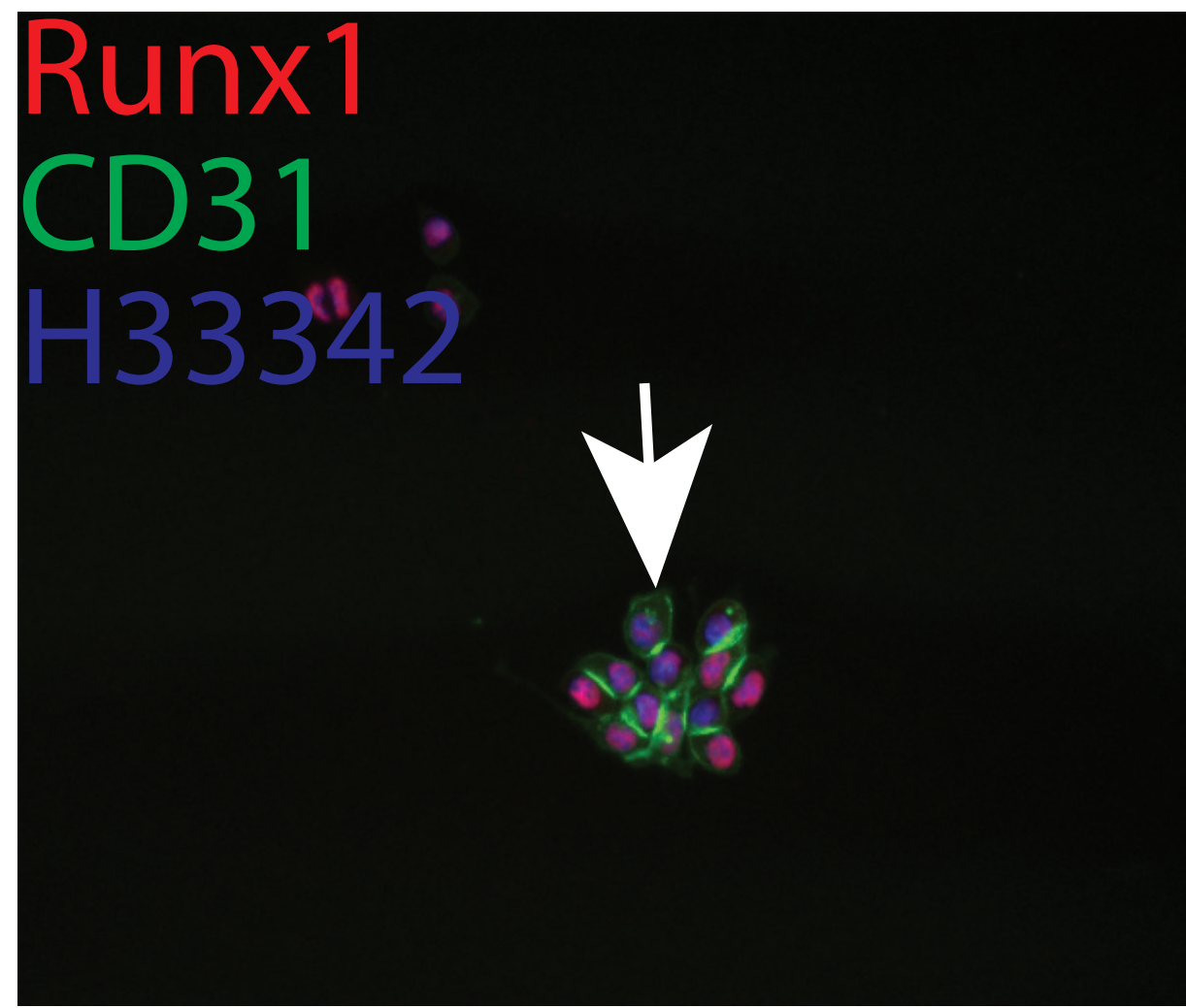


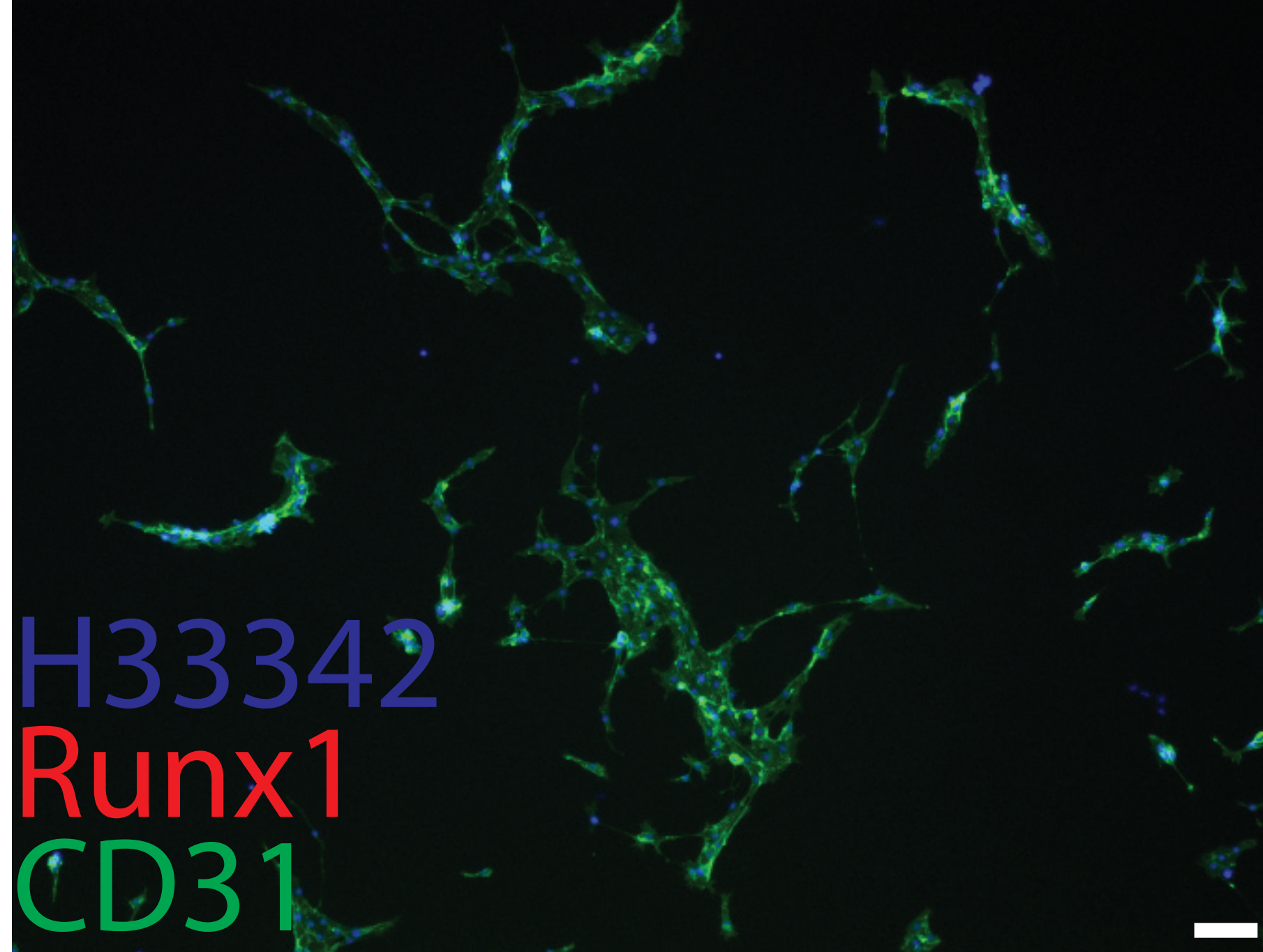
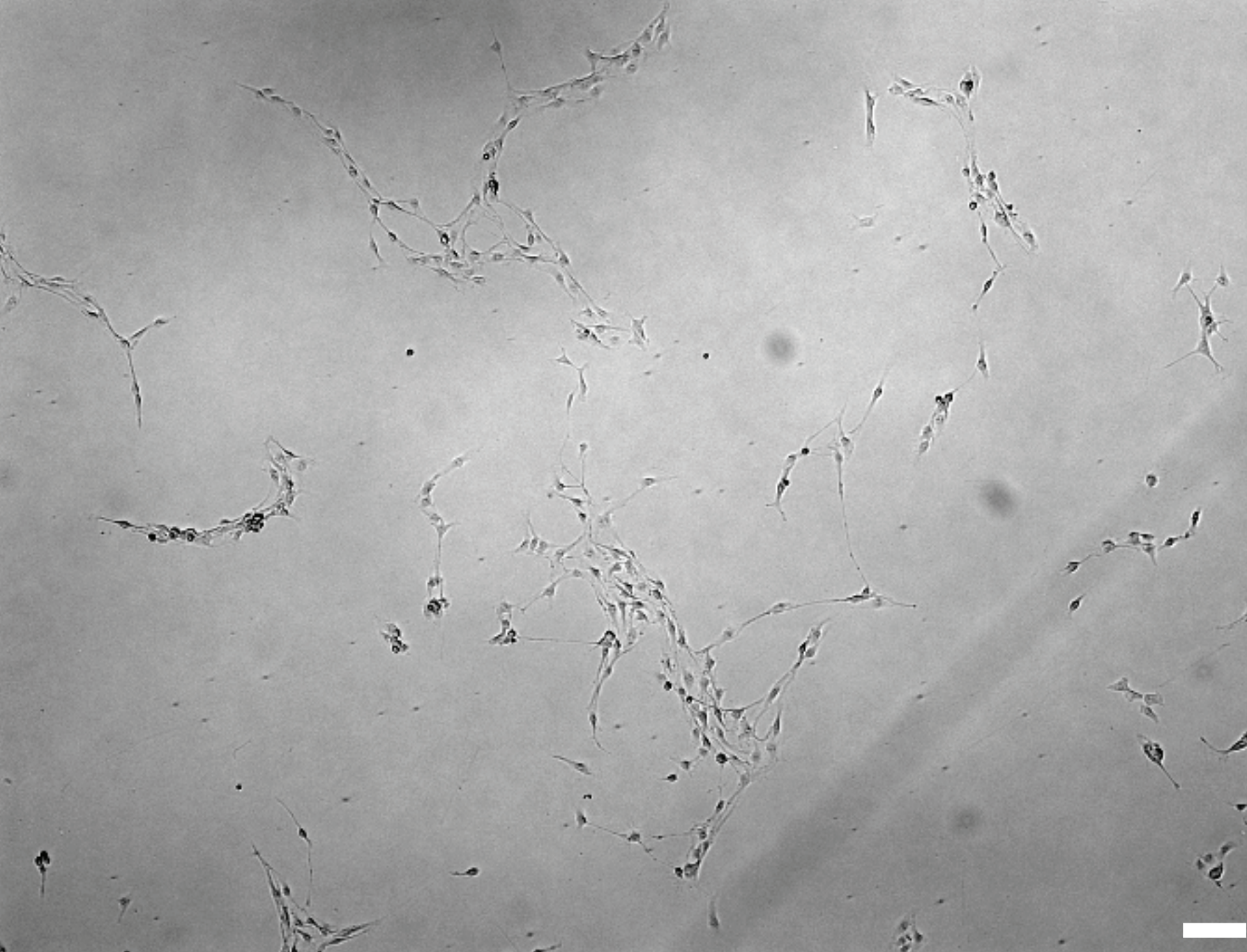
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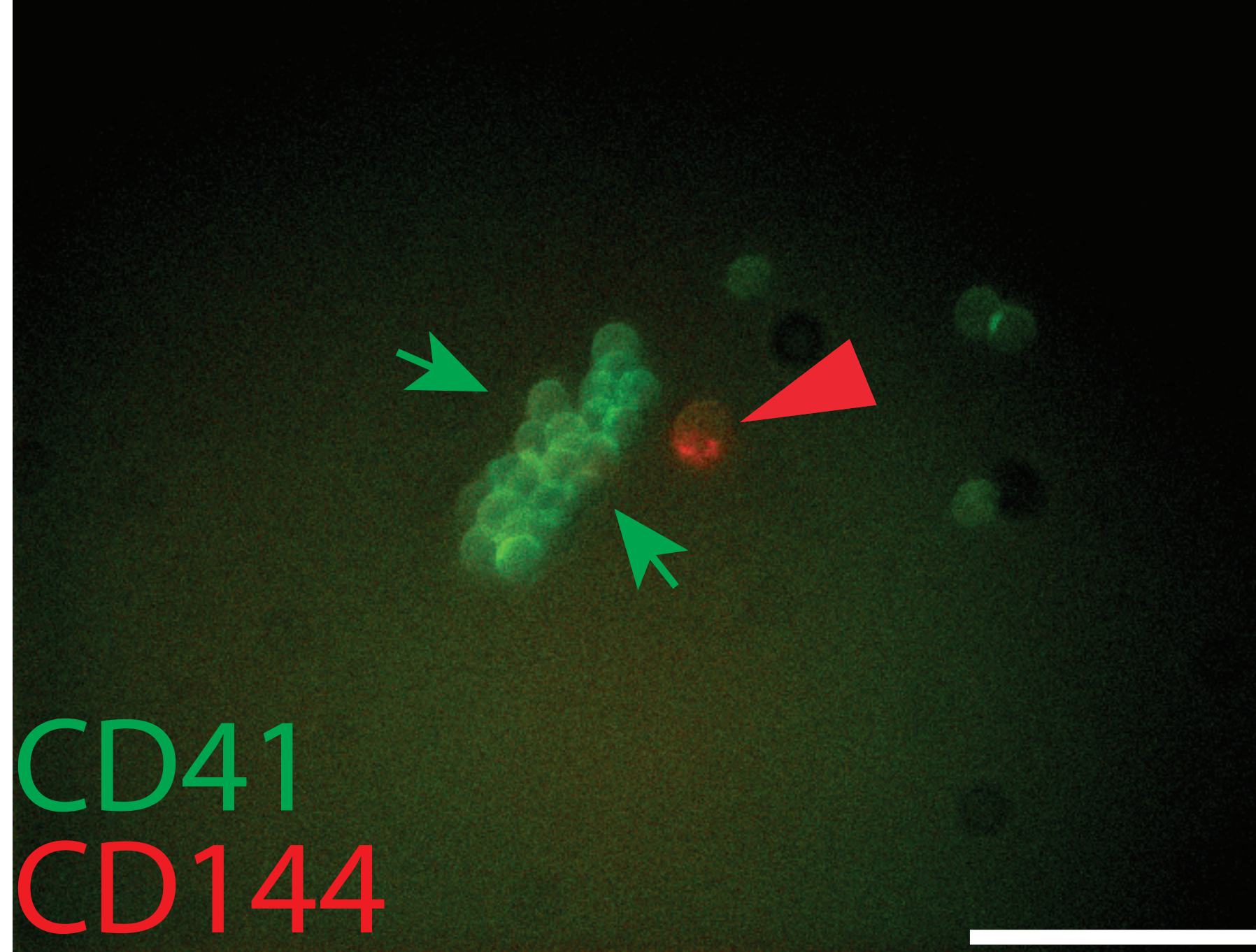
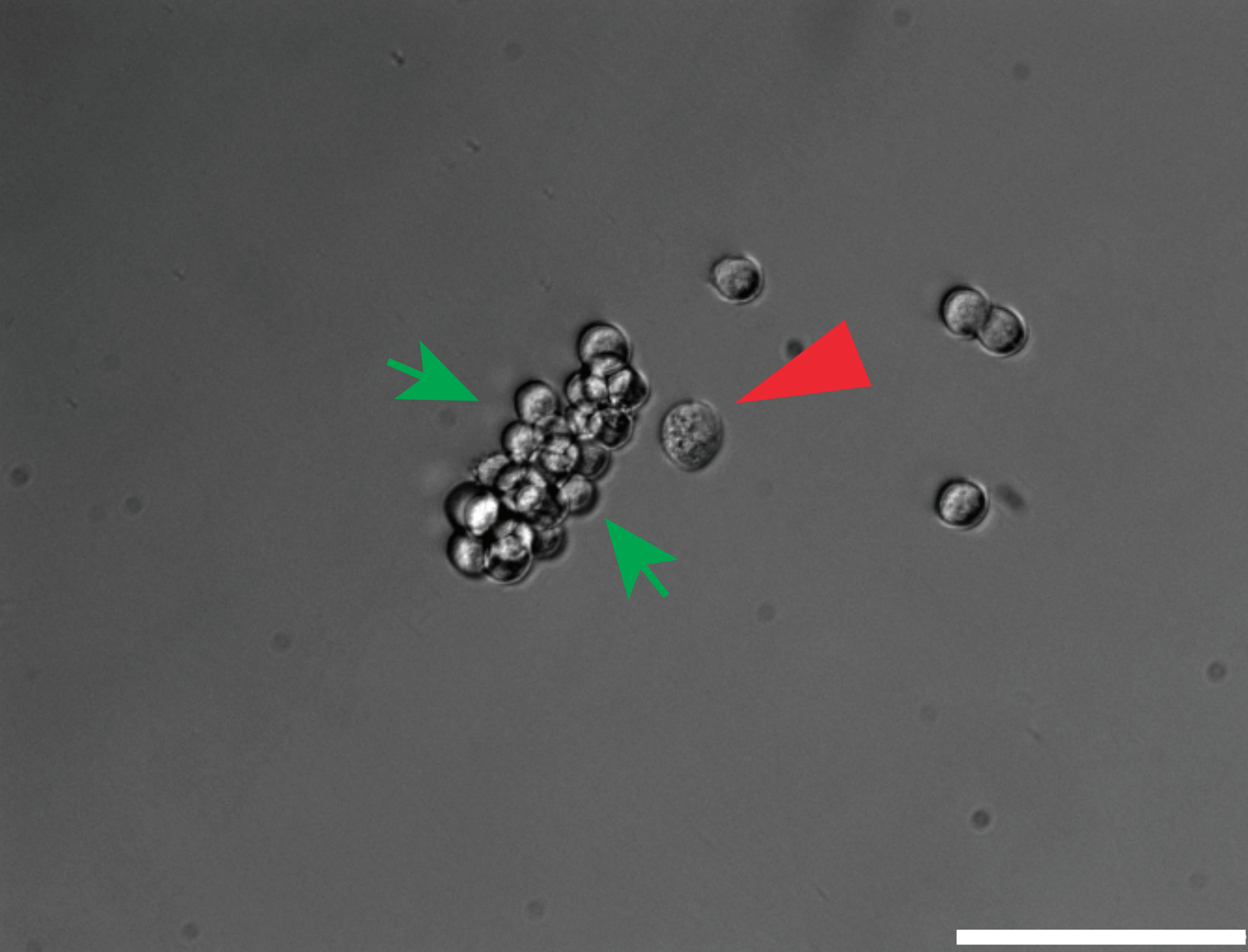


Colony formation(%)



A**B****C**





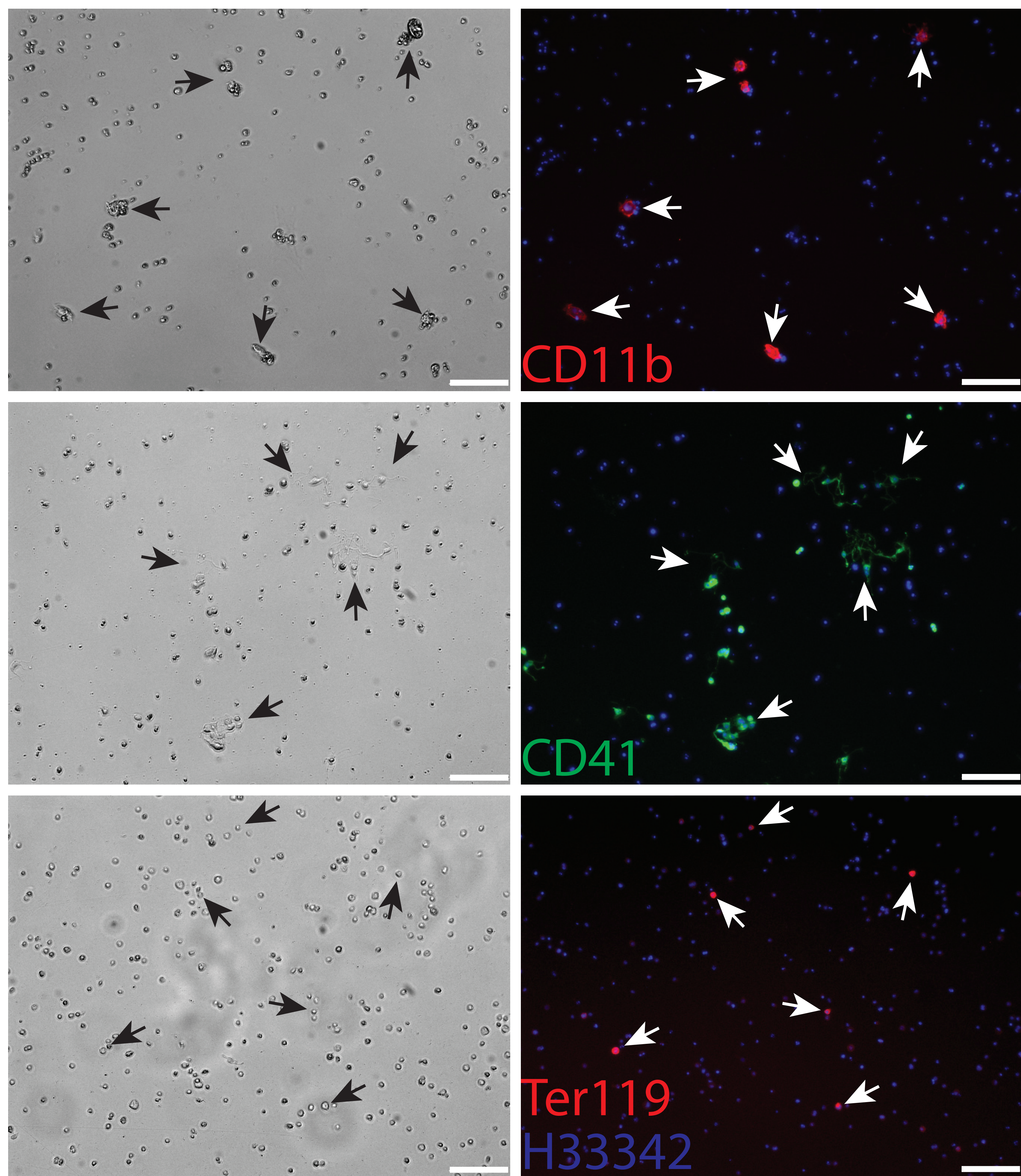
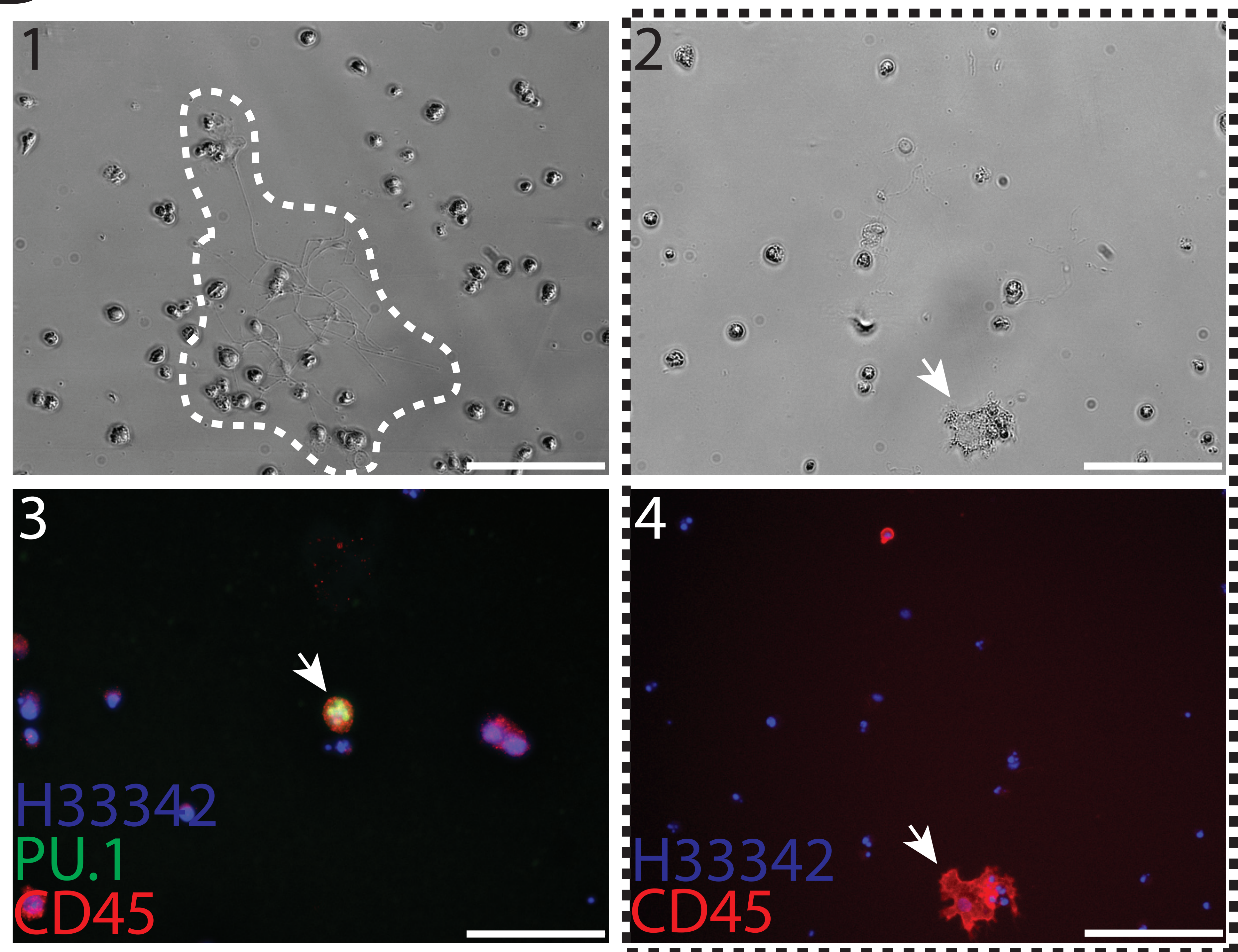
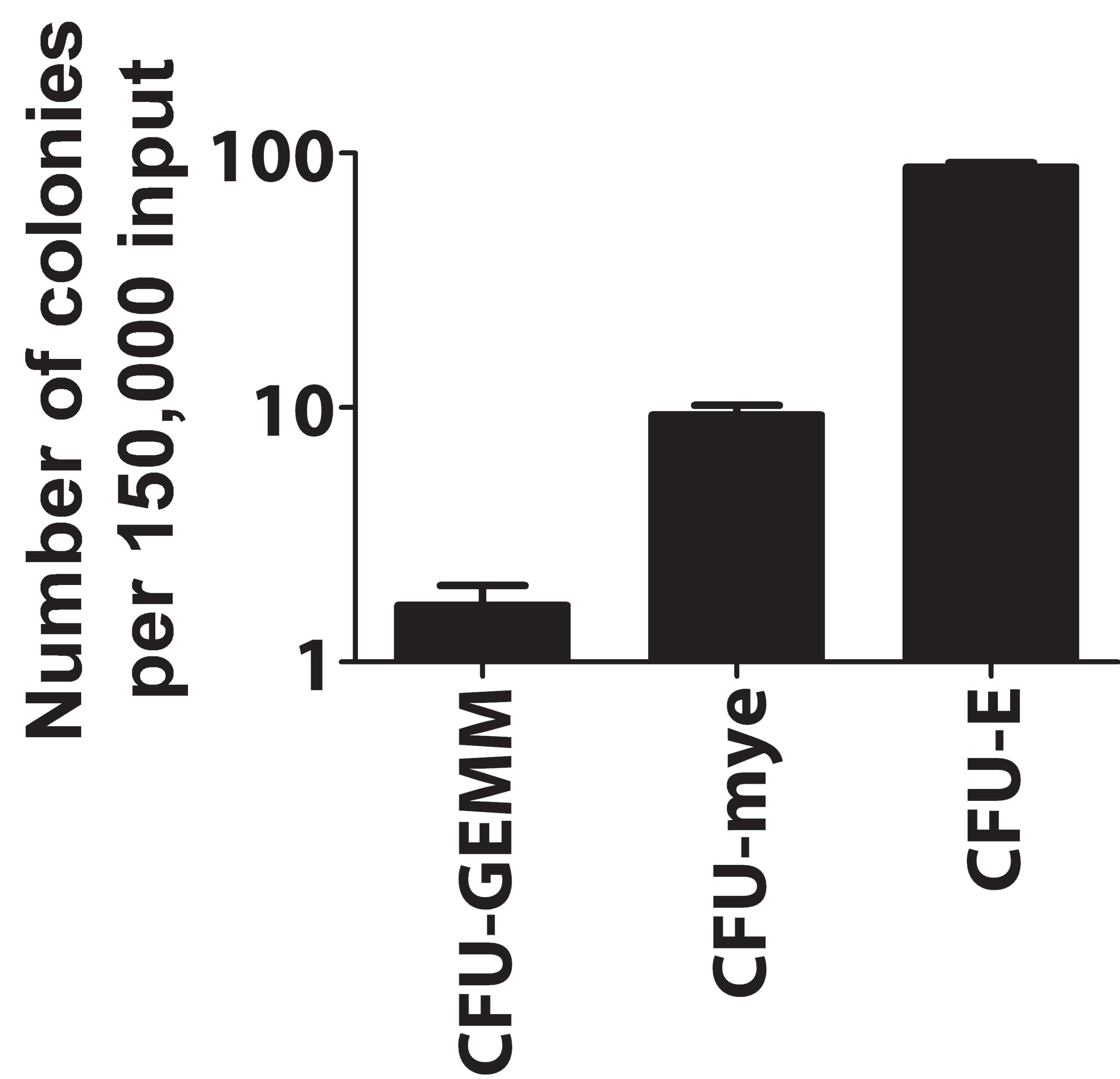
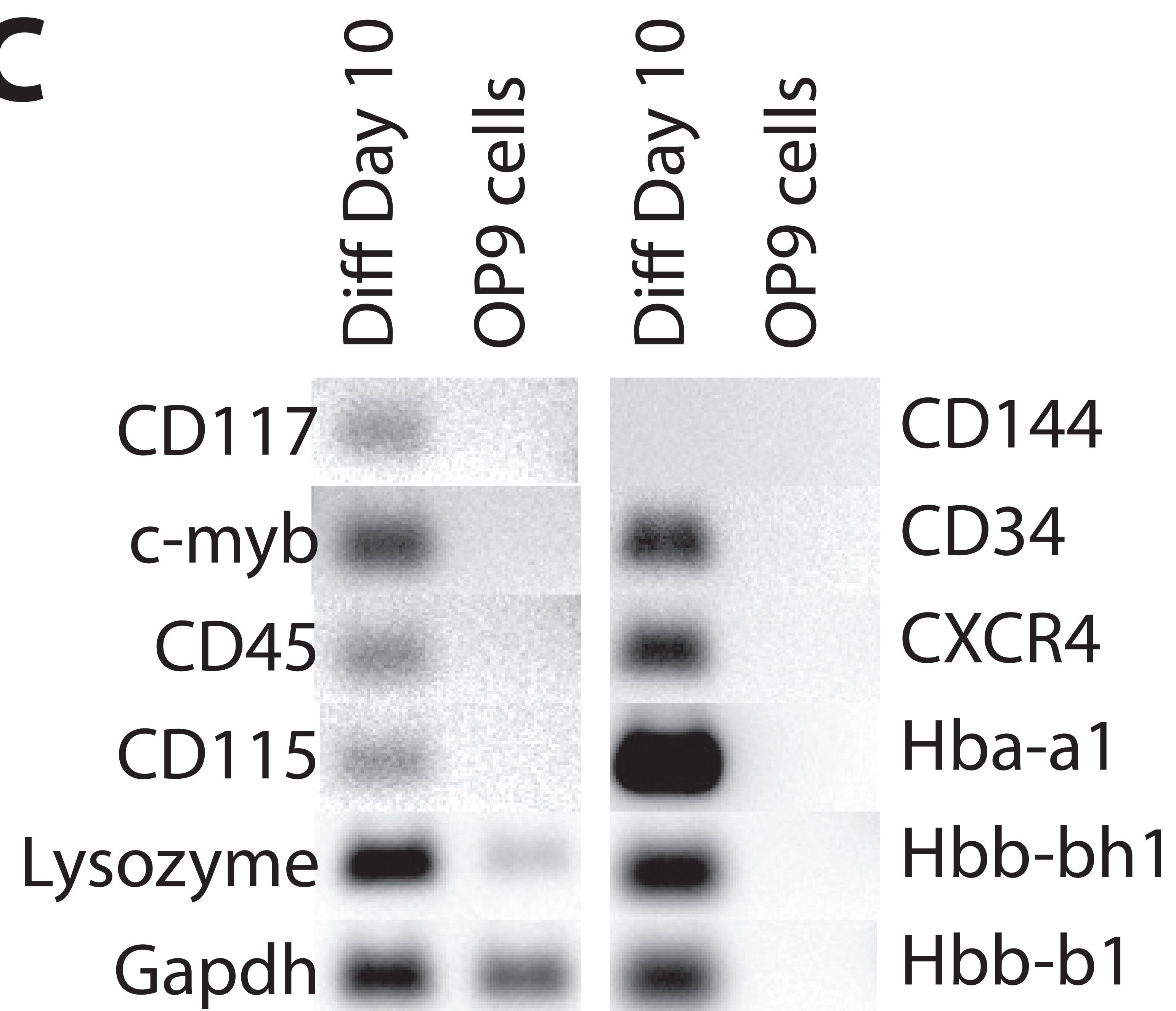
A**B****D****C**

Table S1. Primer sets for RT-PCR

Nanog	5'-AGAGGAAGGGCGAGGAGA	5'-TTCTTGCTTACAAGGGTCTGC
Klf4	5'-GTGCCCCGACTAACCCTTG	5'-GTCGTTGAACTCCTCGGTCT
Fgf5	5'-GCTGTGTCTCAGGGGATTGT	5'-ACAGTCATCCGTAATAATTTGGC
Gapdh	5'-TTGAGGTCAATGAAGGGGGT	5'-TCGTCCCGTAGACAAAATGG
Brachyury	5'-CAGCCCACCTACTGGCTCTA	5'-GAGCCTCGAAAGAACTGAGC
Flk-1	5'-TCCAGAATCCTCTTCCATGC	5'-CCAGAGACCCTCGTTTTCAG
Runx1	5'-CTGCGGGGTTTCAACAGTTC	5'-GAGCCCACTACCCTCTGTCTT
c-myb	5'-GTCCTCTGTCTTCCACAGG	5'-TGCCTCAAAGCCTTTACCG
SCL	5'-GACACAATTCAGGACTGGTAGAC	5'-GGCATGATCCACTTTCTGTTCA
Tie-2	5'-TTTCGGCATCAGACACAAGA	5'-CCGGCTTAGTTCTCTGTGGA
CD117	5'-GCCACGTCTCAGCCATCTG	5'-GTCGCCAGCTTCAACTATTAAC
CD45	5'-ATGGTCTCTGAATAAAGCCCA	5'-TCAGCACTATTGGTAGGCTCC
CD115	5'-TTGCCTTCGTATCTCTCGATG	5'-CTCTGCTGGTCTACTGCTG
Lysozyme	5'-ATGGAATGGCTGGCTACTATGG	5'-ACCAGTATCGGCTATTGATCTGA
CD144	5'-CGTTGGACTTGATCTTTCCC	5'-CGCCAAAAGAGAGACTGGAT
CD34	5'-CTGGTACTTCCAGGGATGCT	5'-TGGGTAGCTCTCTGCCTGAT
Cxcr4	5'-GAAGTGGGGTCTGGAGACTAT	5'-TTGCCGACTATGCCAGTCAAG
Hba-a1	5'-CACCACCAAGACCTACTTTCC	5'-CAGTGGCTCAGGAGCTTGA
Hbb-bh1	5'-GAAACCCCGGATTAGAGCC	5'-GAGCAAAGGTCTCCTTGAGGT
Hbb-b1	5'-GCACCTGACTGATGCTGAGAA	5'-TTCATCGGCGTTCACCTTCC