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(54) Title: ISOLATED EMBRYONIC STEM CELLS THAT EXPRESS LINEAGE MARKERS AND ASSOCIATED
METHODS

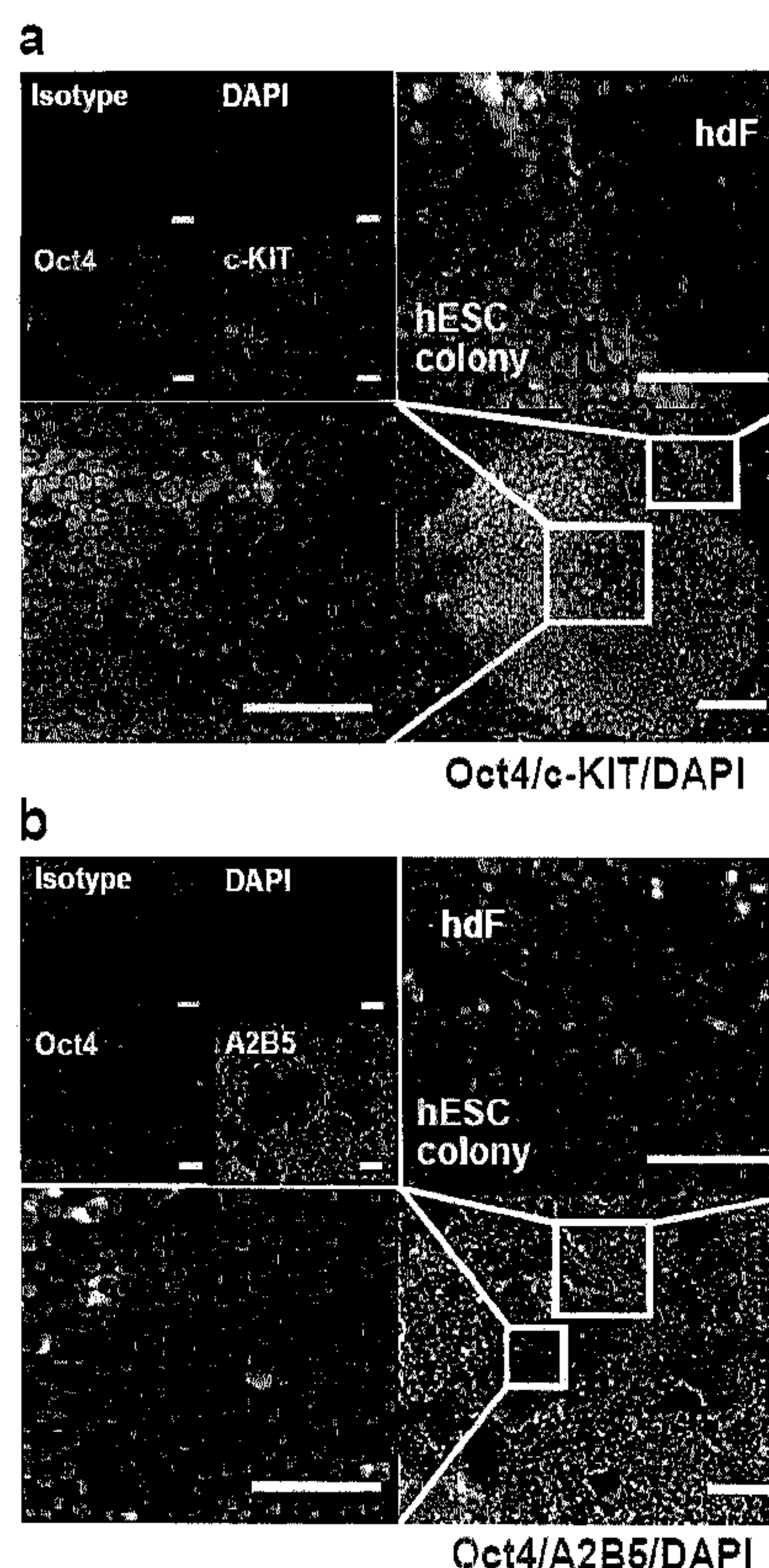


Fig. 1

(57) Abrégé/Abstract:

Described are embryonic stem cells that express one or more pluripotency markers and a lineage marker such as c-KIT or A2B5. Also described are methods for predicting the differentiation potential and/or capacity for expansion or self-renewal of a stem cell.



(57) Abrégé(suite)/Abstract(continued):

Stem cells are tested for the expression of c-KIT and/or A2B5. Stem cells that express c-KIT are predisposed to differentiate into mesodermal lineages. Stem cells that express A2B5 are predisposed to differentiate into neural lineages. Stem cells that do not express A2B5 exhibit higher expansion and self-renewal. Also provided are methods for producing populations of cells of a particular lineage and methods for identifying an effect of culture media on the differentiation potential of stem cells.

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(54) Title: ISOLATED EMBRYONIC STEM CELLS THAT EXPRESS LINEAGE MARKERS AND ASSOCIATED METHODS

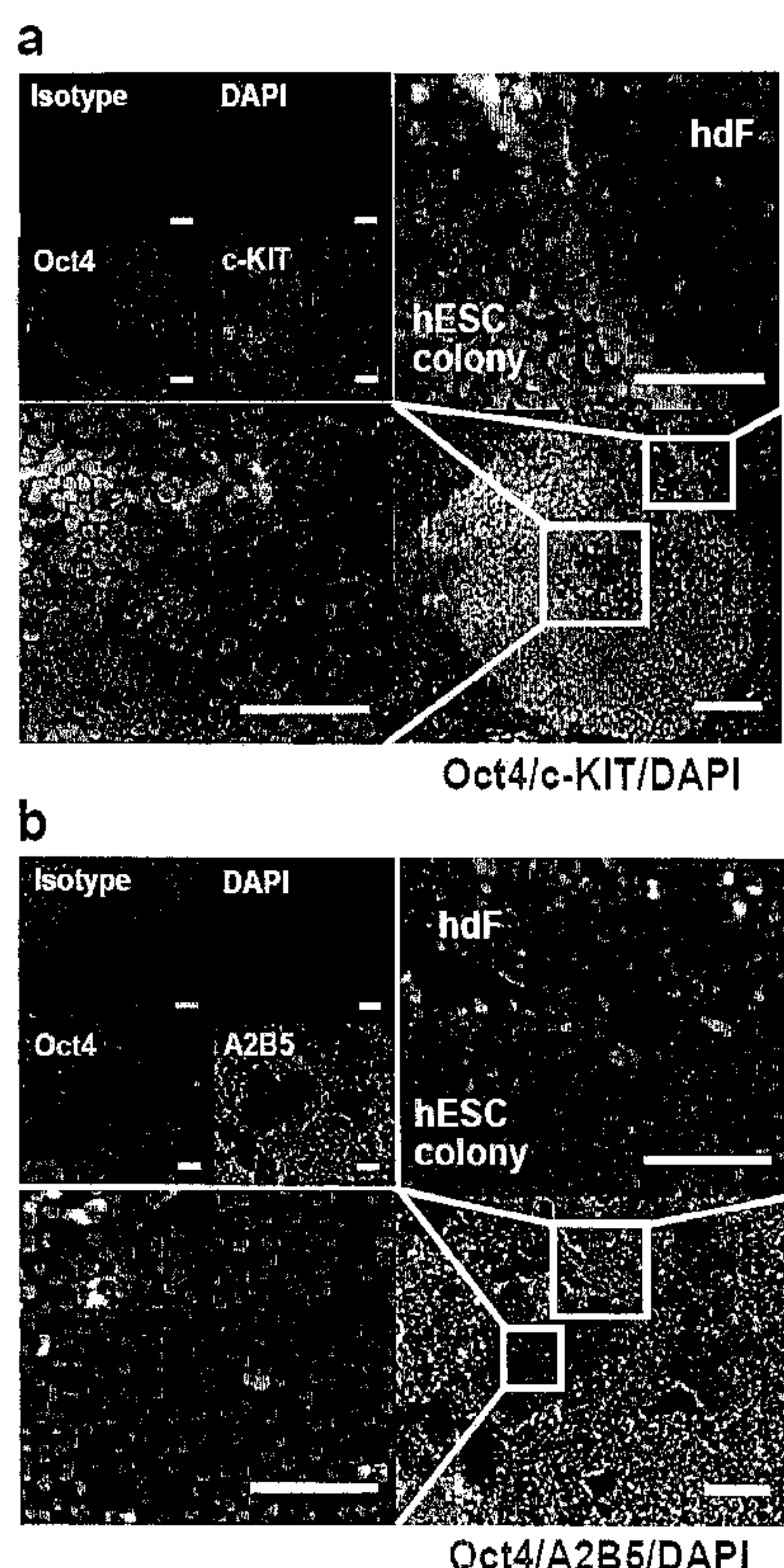


Fig. 1

(57) Abstract: Described are embryonic stem cells that express one or more pluripotency markers and a lineage marker such as c-KIT or A2B5. Also described are methods for predicting the differentiation potential and/or capacity for expansion or self-renewal of a stem cell. Stem cells are tested for the expression of c-KIT and/or A2B5. Stem cells that express c-KIT are predisposed to differentiate into mesodermal lineages. Stem cells that express A2B5 are predisposed to differentiate into neural lineages. Stem cells that do not express A2B5 exhibit higher expansion and self-renewal. Also provided are methods for producing populations of cells of a particular lineage and methods for identifying an effect of culture media on the differentiation potential of stem cells.

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**ISOLATED EMBRYONIC STEM CELLS THAT EXPRESS LINEAGE
MARKERS AND ASSOCIATED METHODS**

5

10 **Field of the Disclosure**

[0002] The disclosure relates to stem cells and in particular to embryonic stem cells that express lineage markers, methods for identifying the differentiation and/or expansion potential of embryonic stem cells as well as associated methods and screening assays.

15

Background of the Disclosure

[0003] The self-renewal and differentiation conditions deployed for embryonic stem cell (ESC) propagation have traditionally assumed that a homogeneous population of cells is present within the culture. Recent studies
20 have challenged this dogma, indicating that ESC cultures are heterogeneous with individual cells displaying dynamic phenotypes (Hayashi et al., 2008; Stewart et al., 2006). In addition, to phenotype, heterogeneity occurs at the level of ESC state, notably manifested in the routine culture of ESCs where cell-to-cell variance in the levels of pluripotency-associated transcription
25 factors like Nanog, Stella, and Rex1 can be observed despite the maintenance of consistent levels of other pluripotency markers, eg. Oct4 (Chambers et al., 2007; Hayashi et al., 2008; Toyooka et al., 2008). The presence of Rex1 and Oct4 expression is a prerequisite for ESC contribution in chimeric animal assays and describes an inner cell mass phenotype largely
30 bereft of differentiation marker expression. ESCs lacking Rex1, but expressing Oct4, do not contribute to the same chimera assays and express markers of the epiblast (Toyooka et al., 2008). Such observations have

illuminated the diversity present within ESC populations and contested the preconception that expression of pluripotent markers is a molecular and cell fate surrogate of homogeneous pluripotent potential. As such, whether heterogeneity affects self-renewal of human pluripotent stem cells (hPSCs) or
5 developmental potential remains to be determined.

[0004] Despite the use of directed differentiation protocols, stimulation of human ESCs (hESCs) generates a spectrum of differentiated cell types (D'Amour et al., 2006; Lee et al., 2007). This overall result underscores the low efficiency and yield of potentially therapeutically useful cell types for cell
10 replacement therapies or *in vitro* drug interaction studies. Surprisingly, the mechanism behind this phenomenon has received little attention, but the apparent presence of competing histone modifications at genes that are known to influence lineage decisions may provide a rationale for understanding the propensity of ESC cultures to differentiate precociously
15 (Mikkelsen et al., 2007). Chromatin modifications are pivotal in the transmission of cell fate information during stem cell proliferation and differentiation (Kouzarides, 2007; Surani et al., 2007). Activating (H3K4me3) and repressive (H3K27me3) histone methylations are associated with the transcription and repression of gene expression, respectively (Kouzarides,
20 2007). Recent studies have demonstrated that both human and mouse ESCs display notably high levels of concomitant H3K4me3 and H3K27me3 modifications, termed bivalent domains, at lineage-specific gene loci (Bernstein et al., 2006). These bivalent marks are reasoned to poise ESCs for fate specification (Pietersen and van Lohuizen, 2008), despite the lack of
25 functional demonstrations to support this idea.

[0005] Accordingly, there is a need for improved markers of stem cell differentiation potential and for methods for predicting or selecting stem cells with a propensity for differentiation towards specific lineages.

Summary of the Disclosure

[0006] In one aspect, the present disclosure provides methods for predicting the differentiation potential of a stem cell. The applicants have shown that the cell surface markers stem cell factor receptor (c-KIT) and
5 A2B5 allow subfractionation of hESCs expressing equivalent levels of the pluripotency markers Oct4 and Nanog. Determining the expression of these markers allows for the prediction of the differentiation potential of the respective stem cells subsets into mesodermal or neural lineages in addition to the prediction of expansion capacity. Direct *de novo* isolation of these
10 hESC subsets demonstrated propensities for the hematopoietic and neural lineage differentiation that were concomitant with reduced self-renewal ability. Histone modification marks of gene loci associated with pluripotency and lineage specificity strongly predicated cell fate potential. The results presented herein indicate that cell fate potential is encoded within functionally
15 heterogeneous hESCs, thereby providing a means to better understand the fundamental processes that underlie cell fate initiation and lineage-priming towards enhancing lineage-specific differentiation from human pluripotent stem cells.

[0007] The present disclosure also provides isolated embryonic stem
20 cells (ESCs) that express one or more pluripotency markers and one or more lineage markers. In one embodiment, the lineage markers are c-KIT, A2B5 or both c-KIT and A2B5. Previously it was thought that ESC cultures represented a homogenous population of pluripotent stem cells possessing equipotent developmental potential. The present disclosure describes the
25 isolation and characterization of ESC sub-populations that express one or more pluripotency markers, such as OCT4 or nanog, and lineage markers such as A2B5 or c-KIT. Furthermore, ESCs that express A2B5 are demonstrated to be predisposed towards neural lineages, while ESCs that express c-KIT are demonstrated to be predisposed towards mesodermal
30 lineages. The present disclosure also describes isolated pluripotent stem

cells that express one or more pluripotency markers and are devoid of A2B5 expression and exhibit an increased capacity for expansion and self-renewal.

[0008] Accordingly, in one aspect there is provided an isolated embryonic stem cell (ESC) that expresses i) one or more pluripotency markers and ii) c-KIT or A2B5 or both c-KIT and A2B5. Also provided are isolated ESCs that express one or more pluripotency markers and do not express A2B5. In one embodiment, the ESC expresses one or more pluripotency markers selected from OCT4, SSEA3, SSEA4, nanog, TRA-1-60, TRA-1-81, Sox2, Rex1, GCTM-2 and CD-9. In one embodiment, the isolated ESCs described herein are pluripotent stem cells capable of self-renewal. In one embodiment, the isolated ESCs described herein are mammalian ESCs. In one embodiment, the ESCs are human ESCs.

[0009] In one embodiment, the isolated ESCs described herein that express c-KIT and are predisposed to differentiate into mesodermal lineages, such as hematopoietic cells. In one embodiment, the ESCs that express c-KIT are predisposed to differentiate into mesodermal lineages relative to a control population of cells such as ESCs that do not express c-KIT.

[0010] In another embodiment, the isolated ESCs described herein that express A2B5 are predisposed to differentiate into neural lineages. In one embodiment, ESCs that express A2B5 are predisposed to differentiate into neural lineages relative to a control population of cells such as ESCs that do not express A2B5.

[0011] In one embodiment, the isolated ESCs described herein that do not express A2B5 exhibit an increased capacity for expansion and self-renewal relative to cells that express A2B5.

[0012] The methods described herein are useful for producing populations of stem cells predisposed to differentiate into specific lineages. Also provided are methods for producing a single isolated ESC that is predisposed to differentiate into specific lineages. For example, in one embodiment there is provided a method of producing a population of ESCs

predisposed to differentiate into mesodermal lineages comprising separating ESCs that express c-KIT from ESCs that do not express c-KIT. Optionally, the method includes culturing a single c-KIT positive ESC or a separated population of c-KIT positive stem cells. In one embodiment, the method
5 includes differentiating a single positive c-KIT cell or a population of c-KIT positive stem cells to produce a population of mesodermal cells such as hematopoietic cells.

[0013] In another embodiment, there is provided a method for producing a single stem cell or a population of stem cells predisposed to
10 differentiate into neural lineages comprising separating ESCs that express A2B5 from ESCs that do not express A2B5. Optionally, the method includes culturing a single A2B5 positive stem cell or the separated population of A2B5 positive stem cells. In one embodiment, the method includes differentiating a single stem cell or a population of A2B5 positive stem cells to produce a
15 population of neural cells such as neurons and cells of the glia lineage.

[0014] In one embodiment, one or more stem cells that express A2B5 or c-KIT are separated from a population of cells using positive or negative selection techniques. In one embodiment, the stem cells are separated using Fluorescence Activated Cell Sorting (FACS) or other techniques for
20 separating cells based on the expression of specific markers known in the art. In one embodiment, the stem cells are separated from a population of stem cells that express one or more pluripotency markers selected from OCT4, SSEA3, SSEA4, nanog, TRA-1-60, TRA-1-81, Sox2, Rex1, GCTM-2 and CD-9.

[0015] In one aspect there is provided a method for predicting the differentiation potential of a stem cell. In one embodiment, the method comprises testing the stem cell for the expression of c-KIT or A2B5. In one
25 embodiment, stem cells that express c-KIT are identified as being predisposed to differentiate into mesodermal lineages, such as hematopoietic
30 lineages. In one embodiment, stem cells that express A2B5 are identified as being predisposed to differentiate into neural lineages. In one embodiment,

stem cells that express c-KIT are predisposed to differentiate into mesodermal lineages relative to a control population of stem cells such as stem cells that do not express c-KIT. In one embodiment, stem cells that express A2B5 are predisposed to differentiate into neural lineages relative to a control
5 population of stem cells such as stem cells that do not express A2B5. In one embodiment, the stem cells are embryonic stem cells (ESCs), optionally mammalian ESCs such as human ESCs. In one embodiment, the ESCs express one or more pluripotency markers selected from markers selected from OCT4, SSEA3, SSEA4, nanog, TRA-1-60, TRA-1-81, Sox2, Rex1,
10 GCTM-2 and CD-9. Optionally, the stem cells are tested for the expression of c-KIT or A2B5 using methods known in the art such as with antibodies specific for c-KIT or A2B5, or PCR based methods such RT-PCR.

[0016] In one embodiment, the stem cells that express c-KIT are tested for the relative enrichment of H3K4me3 histone methylation for one or more
15 mesodermal markers selected from Brachyury, MIXL1, Meox1, Eomes and TBx6. In one embodiment, the stem cells that express A2B5 are tested for the relative enrichment of H3K4me3 histone methylation for one or more neural markers selected from Pax6, NF-68, Mash1, Nestin and Sox1. Optionally, the stem cells are tested for the relative enrichment of H3K4me3 histone
20 methylation using sequential ChIP analysis. In one embodiment, cells that express c-KIT and exhibit a relative enrichment of H3K4me3 histone methylation for one or more mesodermal markers have an increased disposition towards mesodermal lineages. In one embodiment, cells that express A2B5 and exhibit a relative enrichment of H3K4me3 histone
25 methylation for one or more neural markers have an increased disposition towards neural lineages.

[0017] In another aspect, the Applicants have determined that expression of A2B5 is a marker for the capacity of a stem cell for expansion and self-renewal (clonogenic ability). In one embodiment, stem cells that do
30 not express A2B5 have a higher capacity for expansion and self-renewal relative to stem cells that do express A2B5. The higher expansion and self-

renewal capacity translates into an increase in proliferation of stem cells allowing for greater expansion prior to differentiation. Accordingly, in one embodiment, there is provided a method of screening stem cells comprising testing the stem cells for expression of A2B5, wherein cells that do not
5 express A2B5 have a higher capacity for expansion and self-renewal. Also provided are isolated populations of embryonic stem cells that do not express A2B5 as described herein. In one embodiment, populations of cells with a higher frequency of cells that express A2B5 exhibit a higher capacity for expansion and self-renewal. In one embodiment, ESCs that do not express
10 A2B5 have an increased capacity for expansion and self-renewal relative to a control population of stem cells such as stem cells that express A2B5. In one embodiment, the stem cells are embryonic stem cells (ESCs), optionally mammalian ESCs such as human ESCs. In one embodiment, the ESCs express one or more pluripotency markers selected from OCT4, SSEA3,
15 SSEA4, nanog, TRA-1-60, TRA-1-80, TRA-1-81, Sox2, Rex1, GCTM-2 and CD-9. In one embodiment, the methods described herein further comprise separating ESCs that do not express A2B5 from ESCs that express A2B5 to produce a population of ESCs with an increased capacity for expansion and self-renewal.

20 **[0018]** In one aspect, testing a combination of A2B5 and c-Kit allows for the assessment of the breadth of differentiation (2 out of the 3 potential lineages) and expansion potential of stem cells. The clinical application of stem cells requires high levels of expansion while maintaining the ability of the cells to differentiate. Currently there is no way to predict any of those two
25 features and systems are often compromising one feature to support another. This methodology allow for early detection of those two features saving time and resources.

[0019] In one aspect, the methods described herein can be used to monitor stem cells during culture for differentiation potential into different
30 lineages. In another aspect, the applicants have shown that culture media can influence the differentiation potential of stem cells. In one embodiment,

there is provided a method for identifying the effect of culture media on the differentiation potential of stem cells comprising culturing stem cells in the culture media and testing the stem cells for expression of markers that indicate the differentiation potential of the stem cells. In one embodiment, the stem cells are tested for the expression of A2B5 and expression of A2B5 indicates the culture media is supportive of neural differentiation of stem cells. In one embodiment, the stem cells are tested for the expression of c-KIT and expression of c-KIT indicates the culture media is supportive of mesodermal differentiation of stem cells. In one embodiment, the stem cells are embryonic stem cells, optionally mammalian ESCs or human ESCs. In one embodiment, the ESCs express one or more pluripotency markers selected from OCT4, SSEA3, SSEA4, nanog, TRA-1-60, TRA-1-81, Sox2, Rex1, GCTM-2 and CD-9.

[0020] Other features and advantages of the present disclosure will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the disclosure are given by way of illustration only.

20

Brief Description of the Drawings

[0021] **Figure 1. Distribution of c-KIT and A2B5 in Undifferentiated hESCs.** (a,b) Immunocytochemistry staining for c-KIT (a,) and A2B5 (b,) costained with Oct4 in feeder-free H1 cultures. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Scale bar, 100 μ m. Abbreviations: hdFs, hESC-derived fibroblast-like cells. (c) Percentages of C-Kit and A2B5 costained cells in H1 cultures by flow cytometry. (d) H1 cultures were costained with cKit-Oct4, A2B5-Oct4, cKit-SSEA3 and A2B5-SSEA3. Bar graphs indicate percentage of cells positive / negative for above mentioned markers. Pie charts are representative of the bar graphs. In Figure 1 (a), top

right: Oct-4 is only stained within hESC colony but c-kit stains hESC and hdF regions; Bottom left: Oct-4 and c-kit co-stain inside hESC colony. In Figure 1(b), top right: Oct-4 is only stained within hESC colony and A2B5 only stained within hdF; Bottom left: Oct-4 staining inside hESC colony but very little staining of A2B5.

[0022] Figure 2. Differential Clonogenic Capacities of c-KIT⁺/₋ and A2B5⁺/₋ Populations. (a,b) Representative FACS plots based on expression of c-KIT (a) and A2B5 (b) in feeder-free hESC cultures. Sort gate and post-sort purity are shown. (c) Frequencies shown in top histograms indicate the average and standard deviation from four independent experiments. (d) Quantitative clonogenic assay. For CIC assay, cells with >99.0% purity were seeded on irradiated hdFs (20,000 cells per well). Representative images of colonies formed from sorted c-KIT⁺/₋ (c) and A2B5⁺/₋ cells live stained with Tra 1-60 (d; arrowheads indicate day 7 colonies). (d-e) Regenerated colonies were enumerated at post-seed day 14. Scale bars, 100 μ m. Clonogenic output from c-KIT⁺/₋ (e) and A2B5⁺/₋ populations were stained with Oct4, SSEA3 and Hoest staining (f) Enlarged images of Oct4, SSEA3 and Hoechst staining. Circled areas in the bright field and stained images are enlarged images.

[0023] Figure 3. cKit and A2B5 subfractions posses gradient of pluripotent and lineage specific markers. (a-b) Q-PCR analysis for Oct4 and Nanog expression in sorted c-KIT⁺/₋ and A2B5⁺/₋ populations. The mean expression normalized against GAPDH is shown. (c-d) Q-PCR analysis for Brachyury and MIXL1 in sorted c-KIT⁺ and c-KIT⁻ populations. The mean expression normalized against GAPDH is shown. (e-f) H1 cultures were stained with either cKit and Brachury or with A2B5 and Brachury and results were analysed by flow cytometry analysis. Bar graphs indicate frequency of cKit/Brachury and A2B5/Brachury costained cells.

[0024] Figure 4. Hematopoietic Differentiation Potentials of c-KIT⁺ and c-KIT⁻ Populations. (a) Hematopoietic differentiation of c-KIT⁺/₋ cells using RA-EB assay. RA-EBs images at different stage of hematopoietic

differentiation (arrowheads indicate day 4 re-aggregates formed from sorted c-KIT⁺/ cells). Scale bars, 100 μ m. (b) Relative diameter of day 4 RA-EBs (c). Viability in day 15 RA-EBs (d-e) Schematic diagram of hematopoietic development from hESCs. Flow cytometric analysis of day 15 RA-EBs for hemogenic precursors (CD45^{neg}PFV) and committed hematopoietic (CD45⁺CD34⁺ and CD45⁺CD34⁻) cells. All bars indicate as the average and standard deviation of three independent experiments. **P<0.01.

[0025] Figure 5. Neural Differentiation Potentials of A2B5⁺ and A2B5⁻ Populations. (a) Neural differentiation of isolated A2B5⁺/ cells. While A2B5⁺ cells generated RA-EBs and then neuronal-like cells (arrowhead) when subsequently plated onto fibronectin-coated growth surface, A2B5⁻ cells failed to form RA-EBs (b). Scale bars, 100 μ m. Viability (c) and A2B5 expression at day 9 of neural differentiation. Abbreviations: ND, not detected. (d) Representative FACS plot of A2B5⁺/ fractions isolated from GFP and RFP transduced hESC lines. Mixtures of 100,000 cells for each population were re-aggregated together as follows: A2B5⁺GFP⁺ and A2B5⁻RFP⁺ or A2B5⁺GFP⁺ and A2B5⁻RFP⁺. (e-f) Fluorescent RA-EBs images and neural differentiation of re-aggregates A2B5⁺GFP⁺ and A2B5⁻RFP⁺ or A2B5⁺GFP⁺ and A2B5⁻RFP⁺. (g-h) Total percentages and relative frequencies of neural lineage markers (A2B5, GFAP and MAP2) at day 9 of neural differentiation in fluorescent RA-EBs. All bars indicate as the average and standard deviation from three independent experiments. Scale bars, 100 μ m. *P<0.05; **P<0.01.

[0026] Figure 6. Comparison of Histone Modification between Unfractionated hESC Cultures and Isolated Subpopulations. (a-c) Single ChIP analysis of histone modification on pluripotency genes (a, Oct4 and Nanog), mesodermal lineage genes (b, Brachyury, MIXL1, Meox1, Tbx6, and Eomes) and neural lineage genes (c, Pax6, NF-68, Mash1, Sox1 and Nestin) loci in unfractionated hESC cultures. (d,e) Sequential ChIP (anti-H3K4me3 ChIP followed by anti32 H3K27me3 ChIP) analysis of histone modification on Oct4 and Nanog locus in isolated c-KIT⁺/ (d) and A2B5⁺/ (e) populations. (f-h) Sequential ChIP analysis of histone modification on Mesodermal and

neural loci in unfractionated hESC cultures and isolated c-KIT⁺/₋ and isolated A2B5⁺/₋ populations. Histone modification states of target loci were determined by ChIP and Q-PCR in H1 and H9 hESCs. The error bars indicate the standard deviation of three independent experiments. *P<0.05; **P<0.01.

5 Abbreviation: UF, unfractionated culture; K4, H3K4me₃; K27, H3K27me₃; Act= activation; Biv=bivalency. (h-i) Sequential ChIP analysis of histone modification on additional meso and neural genes in isolated c-KIT⁺/₋ and A2B5⁺/₋ populations.

[0027] Figure 7. Proposed Model. (a) Single ChIP analysis of histone
10 modification on pluripotency genes (a, Oct4 and Nanog), mesodermal lineage genes (b, Brachyury, MIXL1) and neural lineage genes (c, Pax6, NF-68) loci in c-KITA2B5⁻ hESC cultures. (b) Sequential ChIP (anti-H3K27me₃ ChIP followed by anti-H3K4me₃ ChIP) analysis of histone modification on pluripotency, mesodermal and neural loci in unfractionated hESC cultures and
15 isolated c-KITA2B5⁻ populations. (d) Current reports exclusively use unfractionated cultures of hESCs. Accordingly, the experimental evidence from unfractionated hESC cultures suggests that the undifferentiated cells “straddle” the boundary of clonogenicity and specification, which is manifest as expression of pluripotent markers but concomitant bivalent H3K27me₃ and
20 H3K4me₃ marks at lineage associated genes. (e) When hESC culture heterogeneity is deconstructed by fractionating the cultures, previously described bivalent histone marks at lineage associated gene loci are resolved down to activating monovalent histone marks and a plastic gradient of clonogenicity and specification is revealed. These monovalent epigenetic
25 marks at lineage associated gene loci now provide a predictive index of eventual cell fate progression.

[0028] Figure 8. Expression of c-KIT and A2B5 in multiple hESC Lines. (a,b) Immunohistochemistry for c-KIT (a) and A2B5 (b) co-stained with Oct4 in feeder-free H1, CA2 and HES3 cultures. Nuclei are counterstained
30 with DAPI. Scale bars, 100 μm. Black and white arrowheads indicate hES colonies and hdF, respectively. (c,d) Immunocytochemistry staining for c-KIT

(c) and A2B5 (d) in H1 and H9 cultures maintained on MEFs. A2B5 is co-stained with HLA. Scale bars, 100 μ m. hESC colony and MEFs are indicated in magnified images. (e,f) Flow cytometric analysis of c-KIT and A2B5 in H1 (e) and H9 (f) cultures maintained on MEFs. The pan-human marker TRA-1-85 distinguishes MEFs from the hESCs. In Figure 8(a), Oct4 stain occurs within the ESC colonies and hDF (black and white arrows respectively). In Figure 8(b) A2B5 staining only occurs in hDFs (white arrows).

[0029] Figure 9. Phenotypic interchangeability between positive and negative fractions. (a,b) Phenotypic analysis in long-term culture of clones generated from (a) c-KIT⁺/⁻ and (b) A2B5⁺/⁻ cells. Clones derived from c-KIT⁺/⁻ and A2B5⁺/⁻ cells were expanded for 30 days. The percentages of c-KIT⁺ and A2B5⁺ cells were measured by flow cytometric analysis. The mean percentages are shown in each histogram.

[0030] Figure 10. Differential Clonogenic Capacities of c-KIT⁺/⁻ and A2B5⁺/⁻ Populations upon addition of Rock Inhibitor. (a) Quantitative clonogenic assay. For CIC assay, cells with >99.0% purity were seeded on irradiated hDFs (20,000 cells per well). Representative images of colonies formed from sorted c-KIT⁺/⁻ (c) and A2B5⁺/⁻ cells. Regenerated colonies were enumerated at post-seed day 14. Scale bars, 100 μ m. Clonogenic output from c-KIT⁺/⁻ and A2B5⁺/⁻ populations were stained with Oct4, SSEA3 and Hoechst staining (b) Frequencies shown in histograms indicate the average number of colonies generated.

[0031] Figure 11. Optimization of RA-EB protocol. (a) Optimizing steps for RA-EB formation. (b) Phase contrast images of RA-EBs formed from live cells isolated by cell sorter. Scale bars, 100 μ m. (c) Diagram depicting optimized RA-EB protocol and differentiation under hematopoietic or neural inductions.

[0032] Figure 12. Neural differentiation potentials of A2B5⁺ and A2B5⁻ populations. (a,b) Neural differentiation of re-aggregates from the mixtures of A2B5⁺GFP⁺ and A2B5⁻RFP⁺ (a) and the proportion of GFP⁺ to RFP⁺ cell number in RA-EBs used (b). (c,d) Neural differentiation of re-

aggregates from the mixtures of A2B5-GFP+ and A2B5-RFP+ (c) and the proportion of GFP+ to RFP+ cell number in RA-EBs used (d). EBs were analyzed by FACS for expression of neural lineage markers (A2B5, GFAP and MAP2) at day 9 of neural differentiation. Frequencies are shown in each of contour plot. All bars indicate as the average and standard deviation from four independent experiments.

[0033] Figure 13. Histone modification in adult fibroblast cells. (a,b) Single (a) and sequential (b) ChIP analysis of pluripotent genes (Oct4 and Nanog) and lineage specific genes (Brachyury, MIXL1 and Pax6) in adult fibroblast cells.

[0034] Figure 14. hESCs grown in mTeSR1 increased cellular expansion compared to MEF-CM (A) Morphological changes of hESCs were observed in hESCs grown under mTeSR1 medium condition compared to MEF-CM. Undifferentiated hESC colonies in mTeSR1 became progressively smaller and thicker than MEF-CM hESC colonies. Additionally, morphology of hESC-derived fibroblasts (hdFs) in mTeSR1 was distinct from MEF-CM counterparts. Scale bars, 100µm. (B to G) Quantitative analysis of hESC growth in MEF-CM and mTeSR1. Montage images (16 images each, 1C) of hESCs cultures treated with mTeSR1 showed approximately 5 times higher colony numbers (1B) of smaller area (D) than the ones grown in MEF-CM. mTeSR1 also induced an increase in cell count (E), with cells displaying smaller nuclear area (F) and overall cell area (G) in comparison to MEF-CM treated cultures. ** P<0.01. (H and I) Early apoptosis kinetic analysis showed lower cell death levels in mTeSR1 treated cells in comparison to MEF-CM hESC cultures. ** P<0.01. (J) Increased numbers of hESCs every passage in mTeSR1 resulted in greater accumulated total cell numbers compared to hESC cultures grown in MEF-CM passage by passage. Even though the frequency of SSEA3 was similar, accumulated SSEA3+ cell numbers were highly increased in hESCs expanded in mTeSR1 than MEF-CM based on increased cell numbers every passage (inset graph). This observation was consistent in three different hESC lines namely, H1, H9 and CA2. ** P<0.01.

(K) Viability of hESC exposed to mTeSR1 was slightly higher than the ones of MEF-CM treated hESC throughout the 5-week period. * $P < 0.05$; ** $P < 0.01$. (L) Relative number of cells expressing pluripotent stem cell markers, SSEA3 and Oct4 (inset graph) were similar in hESCs maintained under MEF-CM and mTeSR1 conditions.

[0035] Figure 15. Lineage-specific differentiation potential of hESCs could be controlled by culture medium conditions. Differentiation potential of hESCs grown in two different medium conditions toward either hematopoietic (A-C) or neural (D-I) lineages was examined. (A) Morphology of EBs formed from hESCs maintained either in MEF-CM or mTeSR1 was similar. Scale bars, 100 μ m. (B and C) Hematopoietic differentiation potential was higher in EBs formed with hESCs cultured in MEF-CM than mTeSR1. FACS analysis of EBs formed at passage #3 showed higher frequency of CD45+ blood cells in MEF-CM than mTeSR1 (B). Similarly in vitro functional hematopoietic colony formation assay showed higher number of colonies in EBs formed using hESCs cultured in MEFCM (C). * $P < 0.05$; ** $P < 0.01$. (D-F) Neurospheres derived from hESCs grown in MEF-CM or mTeSR1 (D). With same seeding density, higher number of neurospheres was observed in mTeSR1-derived EBs (E). Average numbers of cells per neurospheres were slightly higher in mTeSR1-derived EBs (F). Scale bars, 100 μ m. * $P < 0.05$. (G-I) mTeSR1 increased neural differentiation of hESCs in comparison to MEFCM. Together with higher total number of cells (G) in mTeSR1 derived neurospheres, frequency (H) and relative number (I) of cells expressing the neural-specific marker nestin were highly increased in mTeSR1 than MEF-CM. * $P < 0.05$. (J) Immunocytochemical staining of Oct4 (O4), neuron specific class III betatubulin (Tuj1) and glial fibrillar acidic protein (GFAP) demonstrating the neural differentiation potential of mTeSR1 treated hESCs. Scale bars, 100 μ m.

[0036] Figure 16. Changing culture medium rebalance the growth of hESCs (A) Scheme of further experiments to examine whether the growth and differentiation toward hematopoietic or neural potential of hESCs can be

reversed by the culture medium. (B and C) Cultures of hESCs newly adapted in mTeSR1 medium (M-T) showed higher total and accumulated cell numbers relatively to hESCs maintained in MEF-CM throughout the experiment (B) whereas MEF-CM treatment of cell originally expanded in mTeSR1 showed a reduced accumulated total cell number (C). * $P < 0.05$; ** $P < 0.01$. (D and E) Culture medium change did not affect the expression level of SSEA3 over 4 passages. Expression level of SSEA3 was similarly sustained in hESCs maintained in different medium conditions. Expression of SSEA3 was compared in M-M vs. M-T (D) and T-T vs. T-M (E). (F and G) hESCs grown in M-T condition showed gradual increment of accumulated SSEA3+ cells in comparison to cells maintained under MEF-CM (F). Conversely, accumulated SSEA3+ cell number gradually decreased in hESCs under T-M conditions when compared to T-T control (G). * $P < 0.05$. (H) Morphological changes of hESCs upon medium changes. hESCs maintained either in MEF-CM (M-M) or mTeSR1 (T-T) displayed significant morphological differences which were reversed upon media exchange in week 5. Scale bars, 100 μ m.

[0037] Figure 17. Differentiation potential altered by medium conditions of undifferentiated hESCs (A) Morphological observation of hematopoietic colony subtypes in semi-solid methylcellulose culture. (B and C) hESCs grown in MEF-CM generated higher numbers of CFU, which decreased after media swap into mTeSR1 (B), mTeSR1 media exchange with MEF-CM induced a recovery of CFU numbers over the 4 passages. ** $P < 0.01$; * $P < 0.05$. (D and E) A gradual decrease in accumulated CD45+ blood cell numbers indicating a reduction of hematopoietic differentiation potential of hESCs in M-T vs M-M (D). Media swap to MEF-CM reversed hematopoietic differentiation capacity loss of hESCs treated with mTeSR1 medium (T-T) (E). * $P < 0.05$; ** $P < 0.01$. (F) Flow cytometric analysis of CD45 expressions in day 20 EBs formed with hESCs grown in M-M, M-T, T-M, and T-T and harvested at passage #5+4. (G and H) Tracking the emergence of CD45+ blood cell differentiation in day 20 EBs formed from hESCs grown in various medium conditions over 4 passages. * $P < 0.05$; ** $P < 0.01$.

[0038] Figure 18. Predictive lineage-specific marker expressions in undifferentiated hESC cultures (A) Expression level of hematopoietic marker, c-kit was significantly lower in mTeSR1 treated hESCs vs MEF-CM treated hESCs whereas A2B5 expression was greater in mTeSR1 treated cells in comparison to MEF-CM controls. ** $P < 0.01$; * $P < 0.05$. (B and C) Flow cytometric analysis revealed differential expressions of lineage-specific markers in undifferentiated ESCs at passage #5+4. hESCs subjected to M-T treatment showed higher ectoderm-specific A2B5 expression and lower expression of mesoderm-specific c-kit in relation to M-M control (A). Conversely, T-M conditions increased c-kit and decreased A2B5 expression in comparison to the T-T control (B). ** $P < 0.01$.

Detailed Description of the Disclosure

[0039] The present description provides methods for predicting the differentiation potential of pluripotent stem cells. The present description also provides isolated embryonic stem cells that express both pluripotency markers and lineage markers. As shown in Example 1, stem cells that express c-KIT (also known as Mast/stem cell growth factor receptor (SCFR), proto-oncogene c-Kit, tyrosine-protein kinase Kit and CD117) are predisposed to differentiate into mesodermal lineages such as hematopoietic lineages. Furthermore, it has surprisingly been determined that stem cells that express A2B5 are predisposed to differentiate into neural lineages. While the expression of c-KIT and A2B5 has been associated with mesodermal and neural lineages, the present description provides embryonic stem cells that express c-KIT and A2B5 and demonstrates that embryonic stem cells that express c-KIT are predisposed to differentiated in mesodermal lineages, and embryonic stem cells that express A2B5 are predisposed differentiate into neural lineages. The present description also provides embryonic stem cells that do not express A2B5 that have been shown to exhibit increased an capacity for expansion and self-renewal.

[0040] As used herein, “stem cell” refers to a cell that is capable of self-renewal and or differentiating into one or more specialized cell types. The term “stem cell” optionally includes induced pluripotent stem cells or embryonic stem cells. “Embryonic stem cell” refers to a pluripotent cell
5 derived from the inner cell mass of the blastocyst, or a cell from an embryonic stem cell line. Examples of embryonic stem cell lines include, but are not limited to, H1, H9, HES3 and CA2 cell lines. Embryonic stem cells are also characterized by the expression of pluripotency markers including OCT4, SSEA3, SSEA4, nanog, TRA-1-60, TRA-1-81, Sox2, Rex1, GCTM-2 and CD-
10 9. Optionally, the term “embryonic stem cell” includes induced pluripotent stem cells that are capable of self-renewal.

[0041] As used herein, “differentiation potential” refers to the ability of a stem cell to form specialized cell types or lineages. For example, stem cells may be predisposed to differentiate into certain lineages or cell types such as
15 hematopoietic cells or neural cells.

[0042] As used herein “mesodermal lineages” refers to cells that give rise to cells that are typically derived from the mesoderm in a developing embryo. Examples of mesodermal lineages include cells found in tissues such as bone, cartilage, muscle, adipose tissue, connective tissue (including
20 that of the dermis), blood, vascular, reproductive, excretory and urinogenital systems. In one embodiment, mesodermal lineages express one or more markers selected from Brachyury, MIXL1, Meox1, Eomes and TBx6. The term “mesodermal lineage” includes cells that differentiate into cells in the “hematopoietic lineage”.

[0043] As used herein, “hematopoietic lineage” refers to cells that give rise to cells typically found in the blood including hematopoietic stem cells, hemogenic precursors and mature cell types from the myeloid lineages (monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, dendritic cells), and lymphoid
30 lineages (T-cells, B-cells, NK-cells).

[0044] As used herein, “neural lineage” refers to cells that give rise to neural progenitors in a developing embryo. Examples of cells in the neural lineage include, but are not limited to, neural precursors, neurons and glial cells such as astrocytes or oligodendrocytes. In one embodiment, neural
5 lineages express one or more markers selected from Pax6, NF-68, Mash1, Nestin and Sox1.

[0045] As used herein, “capacity for self-renewal” refers to the ability of a single cell to grow into a colony. Optionally, self-renewal can be determined using a colony initiating cell assay by replating single cells in feeder-free
10 hESC culture conditions as described in Example 1.

[0046] As used herein “differentiation” refers to the process by which a less specialized cell such as a stem cell develops or matures to possess a more distinct form and function with a concomitant loss of potential. Cells that are less specialized can be differentiated into cells that are more specialized
15 by culturing the cells under particular conditions or in specific media as known in the art.

[0047] As used herein, “pluripotency” refers to ability of a stem cell to differentiate into any of the endoderm, mesoderm, or ectoderm germ layers and give rise to any fetal or adult cell type.

20 **[0048]** As used herein, “pluripotency marker” refers to a detectable characteristic or expression product of a cell that is associated with a cell that is pluripotent. Optionally, the pluripotency marker that is an expression product is an mRNA, cDNA or protein, or fragment thereof. In one embodiment, the pluripotency marker is selected from OCT4, SSEA3, SSEA4,
25 nanog, TRA-1-60, TRA-1-81, Sox2, Rex1, GCTM-2 and CD-9.

[0049] As used herein “predisposed to differentiate” refers to a cell or cells that are more likely to differentiate into a particular cell lineage or type relative to a control group of cells. For example, ESCs that express A2B5 are more likely to differentiate into cells of the neural lineage cells relative to
30 ESCs that do not express A2B5 or to a general population of ESCs.

[0050] As used herein “culturing” refers to maintaining cells in media with or without cell division or differentiation for any period of time.

[0051] In one aspect of the disclosure, there is provided an isolated embryonic stem cell (ESC) that expresses i) one or more pluripotency markers and ii) c-KIT or A2B5 or both c-KIT and A2B5. In one embodiment, the isolated ESC expresses one or more pluripotency markers selected from OCT4, SSEA3, SSEA4, nanog, TRA-1-60, TRA-1-80, TRA-1-81, Sox2, Rex1, GCTM-2 and CD-9. Optionally, the isolated ESC expresses 2 or more markers, 3 or more markers, 4 or more markers or 5 or more markers selected from OCT4, SSEA3, SSEA4, nanog, TRA-1-60, TRA-1-81, Sox2, Rex1, GCTM-2 and CD-9. In one embodiment, the isolated ESC expresses OCT4 and nanog. In one embodiment, the isolated ESC expresses OCT4, nanog, SSEA3 and TRA-1-60. In one embodiment, the ESCs that express c-KIT or A2B5 described herein are mammalian ESCs, optionally human ESCs.

[0052] In one embodiment, the ESCs described herein express c-KIT. As shown in Example 1, ESCs that express c-KIT are predisposed to differentiate into mesodermal lineages relative to hESCs that do not express c-KIT. In one embodiment, the ESCs described herein that express c-KIT are predisposed to differentiate into hematopoietic cells such as hemogenic precursors, primitive hematopoietic progenitors or mature hematopoietic cells.

[0053] In one embodiment, the isolated ESCs described herein express A2B5. As shown in Example 1, ESCs that express A2B5 are predisposed to differentiate into neural lineages relative to hESCs that do not express A2B5. In one embodiment, ESCs that express A2B5 are predisposed to differentiate into neurons or glia cells, such as astrocytes or oligodendrocytes.

[0054] The ESCs of the present disclosure that can readily be isolated from populations of ESCs such as ESCs from an organism, optionally a human subject, or ESCs from a cell line. For example in one embodiment, ESCs that express c-KIT or A2B5 can be isolated or separated from a population of ESCs using Fluorescence Activated Cell Sorting (FACS) or

other methods known in the art to select and/or separate cells based on expression or the presence of cell surface markers.

[0055] The methods described herein are useful for producing a population of stem cells predisposed to differentiate into a specific lineage. For example, in one embodiment there is provided a method for producing a population of ESCs predisposed to differentiate into mesodermal lineages comprising separating ESCs that express c-KIT from ESCs that do not express c-KIT. In another embodiment, there is provided a method for producing a population of ESCs predisposed to differentiate into neural lineages comprising separating ESCs that express A2B5 from ESCs that do not express A2B5. In one embodiment, the ESCs that express c-KIT or A2B5 are separated from a population of ESCs that express one or more pluripotency markers selected from OCT4, SSEA3, SSEA4, nanog, TRA-1-60, TRA-1-81, Sox2, Rex1, GCTM-2 and CD-9. A skilled person will appreciate that the methods described herein that refer to producing a population of cells are also useful for producing a single isolated cell.

[0056] In one embodiment, the ESCs that express c-KIT or A2B5 also express one or more pluripotency markers. For example, in one embodiment, the pluripotency markers are selected from OCT4, SSEA3, SSEA4, nanog, TRA-1-60, TRA-1-81, Sox2, Rex1, GCTM-2 and CD-9. In one embodiment, the ESCs that express c-KIT or A2B5 also express 2 or more, 3 or more, 4 or more or 5 or more pluripotency markers selected from OCT4, SSEA3, SSEA4, nanog, TRA-1-60, TRA-1-81, Sox2, Rex1, GCTM-2 and CD-9. In one embodiment, the ESCs that express c-KIT or A2B5 also express OCT4 and nanog.

[0057] One or more ESCs that express c-KIT or A2B5 can be separated from cells that do not express c-KIT or A2B5 using any suitable methods for the separation of cells based on the presence or expression of c-KIT or A2B5. For example in one embodiment, the step of separating the cells that express c-KIT or A2B5 from cells that do not express c-KIT or A2B5 includes positive or negative selection steps. In one embodiment, the

methods described herein use affinity binding agents that are selective for c-KIT or A2B5 such as antibodies or fragments of antibodies. In one embodiment, the methods described herein include contacting cells with an antibody or antibody fragment that is selective for A2B5 or c-KIT. In one
5 embodiment, the step of separating the cells comprises detecting or identifying cells in a sample of cells that express c-KIT or A2B5 and separating the cells that express c-KIT or A2B5 from the remaining cells in the sample. In one embodiment, the cells are separated using Fluorescence Activated Cell Sorting (FACS). In one embodiment, the cells are separated
10 using magnetic-based separations such as by using binding agents selective for c-KIT or A2B5 that are conjugated to magnetic beads.

[0058] Antibodies useful for practicing the methods described herein are readily available to one of skill in the art. For example, antibodies for c-KIT are commercially available from Santa Cruz Biotechnology, Inc. (Santa
15 Cruz, Ca) and antibodies for A2B5 are commercially available from R&D Systems, Inc. (Minneapolis, Mn). Antibodies specific for A2B5 or c-KIT or other markers described herein can be generated using techniques known in the art. For examples of methods of the preparation and uses of monoclonal antibodies, see U.S. Pat. Nos. 5,688,681, 5,688,657, 5,683,693, 5,667,781,
20 5,665,356, 5,591,628, 5,510,241, 5,503,987, 5,501,988, 5,500,345 and 5,496,705 that are incorporated by reference in their entirety. Examples of the preparation and uses of polyclonal antibodies are disclosed in U.S. Pat. Nos. 5,512,282, 4,828,985, 5,225,331 and 5,124,147, which are incorporated by reference in their entirety. The term "antibody" as used herein is intended to
25 include monoclonal antibodies, polyclonal antibodies, and chimeric antibodies, and fragments thereof that specifically bind the markers described herein. The antibody may be from recombinant sources and/or produced in transgenic animals. The term "antibody fragment" as used herein is intended to include Fab, Fab', F(ab')₂, scFv, dsFv, ds-scFv, dimers, minibodies, diabodies, and
30 multimers thereof and bispecific antibody fragments. Antibodies can be fragmented using conventional techniques. For example, F(ab')₂ fragments can be generated by treating the antibody with pepsin. The resulting F(ab')₂

fragment can be treated to reduce disulfide bridges to produce Fab' fragments. Papain digestion can lead to the formation of Fab fragments. Fab, Fab' and F(ab')₂, scFv, dsFv, ds-scFv, dimers, minibodies, diabodies, bispecific antibody fragments and other fragments can also be synthesized by
5 recombinant techniques.

[0059] Suitable methods for isolating or separating cells include, but are not limited, to conventional affinity or antibody techniques known in the art. For example, flow cytometry, FACS, antibody-coated magnetic beads, affinity chromatography and "panning" with a binding reagent or antibody
10 attached to a solid matrix or solid phase capture medium are useful for separating cells or testing cells for the expression of specific markers as described herein.

[0060] In one embodiment, flow cytometry, or FACS, may be used to separate single cells or cell populations or test a cell for the expression of one
15 or more markers. In one embodiment, the cells are contacted with a binding reagent that specifically binds to a marker as described herein, such as to an antibody specific for c-KIT or A2B5 or a pluripotency marker. Different detectable labels may be used in conjunction with different antibodies, such as fluorescent labels with different emission spectra, to facilitate the
20 identification or separation of cells simultaneously based on multiple markers.

[0061] The binding reagent or antibody may be conjugated with labels to allow for ease of identification or separation of the particular cell type that expresses a particular marker such as magnetic beads, biotin, which binds with high affinity to avidin or streptavidin fluorochromes, which can be used
25 with a fluorescence activated cell sorter, haptens and the like. Optionally, primary and secondary antibodies may be used in the testing or separating methods described herein.

[0062] In one embodiment, the binding reagent may be directly or indirectly conjugated to a label or magnetic reagent, such as a super-
30 paramagnetic microparticle, fluorochromes or fluorophores, or other detectable label or agent which facilitates the separation of the cell. Direct

conjugation may be achieved by use of various chemical linking groups, as known in the art. In some embodiments, the binding reagent may be coupled to the microparticles or labels through side chain amino or sulfhydryl groups and heterofunctional cross-linking reagent as known in the art.

5 **[0063]** Optionally, the methods described herein include the step of culturing the separated cells in order to maintain the cells. In one embodiment, the step of culturing the cells includes culturing individual cells and allowing the individual cells to clonally expand into a population of ESCs. The methods described herein are also useful for producing a population of
10 cells that can then be differentiated into a specific cell type, such as mesodermal cells or neural cells.

[0064] In one embodiment, ESCs that express c-KIT produced by the methods described herein are cultured and differentiated into mesodermal cells such as hematopoietic cells. In one embodiment, ESCs that express
15 A2B5 produced by the methods described herein are cultured and differentiated into neural cells such as neurons or glial cells. Optionally the cells produced by the methods described herein can then be used in therapeutic interventions that require populations of ESCs or cells derived from ESCs.

20 **[0065]** In another aspect, there is provided a method for predicting the differentiation potential of an embryonic stem cell (ESC). In one embodiment the method comprises testing an ESC for expression of c-KIT or A2B5. As shown in Examples 1 and 2, testing of the expression of c-KIT or A2B5 in populations of ESCs allows for the identification of a subset of cells with
25 different lineage potentials. In one embodiment, ESCs that express c-KIT are identified as predisposed to differentiate into mesodermal lineages. In one embodiment, cells that express A2B5 are identified as predisposed to differentiate into neural lineages. In one embodiment, the cells are identified as predisposed to differentiate into mesodermal lineages or neural lineages
30 relative to a control group of cells, such as cells that do not express c-KIT or A2B5. In one embodiment, the ESCs express one or more pluripotency

markers selected from OCT4, SSEA3, SSEA4, nanog, TRA-1-60, TRA-1-81, Sox2, Rex1, GCTM-2 and CD-9. In one embodiment, the ESCs express one more pluripotency markers selected from OCT4, nanog, SSEA3 and TRA-1-60. In one embodiment, the ESCs express OCT4 and nanog.

5 **[0066]** The methods for predicting the differentiation potential of an ESC described herein are useful for predicting the potential of an ESC to differentiate into hematopoietic cells such as hemogenic precursors, primitive hematopoietic progenitors or mature hematopoietic cells. In one embodiment, the methods for predicting the differentiation potential of an ESC are useful for
10 predicting the potential of an ESC to differentiate into neural cells such as neurons or glial cells. In one embodiment, the glial cells are astrocytes or oligodendrocytes.

[0067] The expression of markers such as c-KIT, A2B5 or the pluripotent markers described herein can be tested using any suitable method
15 known in the art for the determining the presence or expression of the marker. For example, in one embodiment, the expression of a marker is tested using antibodies specific for that marker. In one embodiment, the expression of c-KIT or A2B5 is tested by contacting the cells with antibodies for c-KIT or A2B5 and detecting the binding of the antibodies to one or more cells, such as by
20 using fluorescently labeled antibodies. Other suitable methods for testing the expression of markers include PCR based methods such as RT-PCR or the use of expression arrays.

[0068] Optionally, the methods described herein further comprise testing stem cells for one or more additional markers of differentiation
25 potential known in the art. For example, in one embodiment, the methods further comprise testing a stem cell for one or more mesodermal markers such as Brachyury, MIXL1, Meox1, Eomes or TBx6. In one embodiment, the methods further comprise testing the stem cell for one or more neural lineage markers such as Pax6, NF-68, Mash1, Nestin or Sox1.

[0069] As shown in Example 1, stem cells that are devoid of A2B5 expression show more capacity for expansion and self-renewal compared to stem cells that are express A2B5. Accordingly, in one embodiment there is
5 provided a method of screening stem cells for capacity for expansion and self-renewal comprising testing the stem cells for expression of A2B5.

[0070] In another aspect of the present description shown in Example 2, culture media conditions may influence the differentiation potential of stem cells. The use of markers of differentiation potential such as c-KIT and A2B5
10 are useful for determining the effect of culture media on stem cells. In one embodiment, screening for the expression of the c-KIT and/or A2B5 in stem cells cultured in a particular culture media is useful for assaying the culture media for any effects predisposing the stem cells to differentiate into mesodermal lineages or neural lineages. Accordingly, in one embodiment,
15 there is provided a method for identifying an effect of culture media on the differentiation potential of stem cells comprising culturing stem cells in the culture media and testing the stem cells for expression of markers that indicate the differentiation potential of the stem cells. In one embodiment, expression of A2B5 indicates the culture media is supportive of neural
20 differentiation of stem cells. In one embodiment, expression of c-KIT indicates the culture media is supportive of mesodermal differentiation of stem cells.

[0071] The above disclosure generally describes the present disclosure. A more complete understanding can be obtained by reference to the following specific examples. These examples are described solely for the
25 purpose of illustration and are not intended to limit the scope of the disclosure. Changes in form and substitution of equivalents are contemplated as circumstances might suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitation.

30 **[0072]** The following non-limiting examples are illustrative of the present disclosure:

EXAMPLES

EXAMPLE 1: c-KIT and A2B5 as Markers of Cell Fate Potential of Human Pluripotent Stem Cells

Methods

5 **[0073]** hESC Culture

[0074] Undifferentiated hESC lines H1, H9, HES3 and CA2 were maintained in feeder-free culture as previously described (Bendall et al., 2007). Briefly, hESCs were cultured on Matrigel (BD Biosciences)-coated six-well plate with MEF-CM supplemented with 8 ng ml⁻¹ of hbFGF (Invitrogen). In order to maintain undifferentiated state, MEF-CM was changed daily and hESCs were passaged at a 1:2 split ratio every 6-7 days by enzymatic dissociation with 200 U ml⁻¹ collagenase IV (Invitrogen). Human ESCs culture was carried out at 37°C in humidified atmosphere containing 5% CO₂. H1 and H9 hESC lines were used for all experiments. H1, H9, HES3 and CA2 were used for immunofluorescence and FACS analysis.

[0075] Flow cytometry

[0076] Single cell suspensions of undifferentiated hESCs were obtained by dissociating with TrypLE (Invitrogen) or collagenase IV at 37°C. EBs were dissociated with 0.4 U/ml collagenase B (Roche Diagnostics) for 2 h in 37°C incubator, followed by treatment with cell dissociation buffer (Invitrogen) for 10 min in 37°C. They were passed through a 70 µm cell strainer (BD Biosciences). For intracellular staining of Oct4 antibody, Cytofix/Cytoperm (BD Biosciences) and Perm/Wash buffer (BD Biosciences) were used following the manufacturer's instructions. The following primary and secondary antibodies were used: mouse anti-Oct4 (BD Bioscience), mouse anti-CD117-allophycocyanin (APC) (CALTAG), mouse anti-IGF1R-phycoerythrin (PE) (BD Biosciences), mouse anti-A2B5 (R&D), mouse anti-GFAP (R&D), mouse anti-MAP2 (BD Biosciences), Rat anti-SSEA-3 (Develop Studies Hybridoma Bank), anti-brachyury (Abcam), goat anti-mouse Alexa 488 (Invitrogen) and goat anti-mouse Alexa 647 (Invitrogen). We used 7-

amino actinomycin (7-AAD, Immunotech) to eliminate dead cells. Surface marker expressions were used using FACSCalibur (BDIS) and FlowJo software (Tree Star).

[0077] Immunocytochemical analysis

5 **[0078]** Human ESCs cultured on MEFs or in Matrigel-coated 4-well chamber slide (Lab-Tek) were rinsed three times before fixation with 4% paraformaldehyde in PBS for 20 min and then permeablized with 0.5% saponin in PBS containing 1% BSA. Cells were blocked with 10% normal rabbit serum for 30 min at room temperature. The following primary and
10 secondary antibodies were used: mouse anti-A2B5 (R&D), mouse anti-c-KIT (Santa Cruz Biotechnology), goat anti-Oct3/4 (N19) (Santa Cruz Biotechnology), Rat anti-HLA-A/B/C (Abcam), rabbit antimouse Alexa 594 (Invitrogen), goat anti-mouse fluorescein isothiocyanate (FITC) (Santa Cruz Biotechnology), goat anti-rat Alexa 594 (Invitrogen) and rabbit anti-mouse
15 FITC (Zymed). Primary and secondary antibodies were diluted with PBS containing 10% rabbit or goat serum. Primaries were incubated at 4°C for 24 h, and secondaries were incubated at room temperature for 1 h. Chamber slides counterstained using VECTASHIELD Mounting Medium with DAPI (Vector Labs) and examined using an Olympus IX51 microscope.
20 Fluorescence images were captured with a Photometrix Cool Snap HQ2 camera using Image-Pro 3DA version 6.0. *Live Staining:* Tra 1-60 antibody (anti-mouse IgM; Millipore), was conjugated with secondary antibody (Alexa 647- goat anti mouse IgM). This preconjugated sterile antibody mix was added to the well containing emerging CIC's (6 days post seeding). Antibody
25 was incubated with cells for approximately 20-30 minutes. Culture medium was washed twice and cells were visualized by Montage imaging system.

[0079] hdFs preparation

[0080] hdFs were prepared as previously described (Stewart et al., 2006). Briefly, we treated collagenase IV for 10 min to hESC culture, removed
30 collagenase IV, and then washed the wells with KO-DMEM (Invitrogen) to collect hdFs. Supernatant containing the cells was centrifuged and the cell

pellet was re-suspended in MEF-CM supplemented with 8 ng ml⁻¹ hbFGF. We transferred the cells to fresh to Matrigel-coated wells and changed the medium every other day. The confluent hdFs were passaged in the same manner as for hESCs and frozen in liquid nitrogen until use for clonogenic assay.

[0081] Reverse transcription and quantitative real-time PCR

[0082] Total RNA was extracted from sorted cells using Absolutely RNA microprep kit (Stratagene). First strand cDNA was synthesized from 1 µg of total RNA and then subjected to real-time quantitative PCR using SYBR Green PCR Master Mix and RT-PCR reagents (Applied Biosystems). Quantitative PCR was performed on an Mx3000P qPCR system (Stratagene) using the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min. Specific PCR products were detected by the fluorescence double strand DNA-binding dye, SYBR Green. PCR amplification was performed in triplicate and replicated in three independent experiments. Melting curve analyses were performed to confirm correct PCR product size and absence of nonspecific bands. The expression levels of each gene were normalized to GAPDH and the relative quantification was performed using the comparative C_T method according to manufacturer's protocols (Applied Biosystems).

[0083] hESC isolation and clonogenic assay

[0084] Undifferentiated hESCs were treated with TrypLE for 10 min to dissociate into single cells and passed through a 70 µm cell strainer for hESC clonogenic assays as shown previously (Bendall et al., 2007; Stewart et al., 2006) Single cell suspensions were stained for c-KIT and A2B5 as described above. c-KIT^{+/+} and A2B5^{+/+} subsets were selected from live cells identified by 7-AAD exclusion using FACS Aria (BD Pharmingen). For clonogenic assay, FACS isolated cells were rinsed twice and centrifuged at 450 g for 5 min. The cell pellet was re-suspended in MEF-CM supplemented with 8 ng ml⁻¹ hbFGF. For quantitative analysis of CIC assay, 20,000 cells per well were seeded containing Matrigel plus irradiated hdFs and the medium was changed every

other day. After 14 days, colonies were counted. To assess the quality of the CIC generated cells from individual subfractions, cells were seeded at following densities 1. cKit⁺ 40,000, cKit⁻ 25,000, A2B5⁺ 200,000 and cKit⁺ 10,000.

5 **[0085]** EB and RA-EB formation

[0086] For hematopoietic differentiation using the clumps of hESC colonies, EBs were generated by suspension culture methods as previously described (Cerdan et al., 2007). Briefly, on the day of passage, the confluent undifferentiated hESCs were treated with 200 U ml⁻¹ collagenase IV for 5 min
10 and then transferred to 6-well ultra low attachment (ULA) plate (Corning). These clumps were incubated overnight to allow EB formation in EB medium consisting of KO-DMEM supplemented with 20% non-heat inactivated fetal bovine serum (Hyclone), 1% nonessential amino acids, 1 mM L-Glutamine, 0.1 mM β -mercaptoethanol. For differentiation using sorted single cell
15 suspensions, EBs were generated by forced aggregation. Sorted single cell suspensions were rinsed twice with PBS and then re-suspended in EB medium. Re-aggregation was carried out by placing the defined number (200,000 cells) of sorted single cells reconstituted with 150 μ l of EB medium in 96-well round bottom plate (NUNC). After distribution, we added 150 μ l of
20 1:15 diluted Matrigel into each well to improve adhesion between cells. The plates were centrifuged at 450 g for 5 min and incubated overnight to allow re-aggregation.

[0087] Hematopoietic and Neural differentiation

[0088] For hematopoietic differentiation, EBs generated using clumps
25 and single cells were transferred to new ULA plate containing EB medium supplemented with hematopoietic growth factors (hGFs) as follows: 50 ng ml⁻¹ hG-CSF (Amgen), 300 ng ml⁻¹ hSCF (Amgen), 10 ng ml⁻¹ hIL-3 (R&D), 10 ng ml⁻¹ hIL-6 (R&D), 25 ng ml⁻¹ hBMP-4 (R&D) and 300 ng ml⁻¹ hFlt-3L (R&D). EBs were cultured for 15 days with change of fresh medium with hGFs every
30 3 days. For neural differentiation, aggregates were resuspended with EB medium and transfer to ULA plate. After 4 days in suspension culture, EB

were plated onto fibronectin-coated plate (Falcon) in neural differentiation medium composed of DMEM/F12 (Gibco) with B27 and N2 supplements (Gibco), 25 ng ml⁻¹ hEFG (R&D), 2.5 ng ml⁻¹ hIGF (R&D), 25 ng ml⁻¹ hPDGF-AA (R&D) and 8 ng ml⁻¹ hbFGF. After 5 days under this condition, EBs were
5 collected for FACS analysis.

[0089] Hematopoietic Colony forming assay

[0090] Colony forming assay was performed by plating single cell suspensions of dissociated EB into methylcellulose H4230 (Stem Cell Technologies) as previously described (Chadwick et al., 2003). Briefly, EBs
10 were dissociated with collagenase B and cell dissociation buffer and then filtered with 40-µm cell strainer. Dissociated EBs were counted and plated 10,000 cells into methylcellulose H4230 supplemented with recombinant human growth factors as follows: 50 ng ml⁻¹ hSCF, 3 units ml⁻¹ hEPO (Amgen), 10 ng ml⁻¹ hGM-CSF (Novartis) and 10 ng ml⁻¹ hIL-3. Cells were
15 incubated at 37°C and 5% CO₂ in humidified atmosphere. After incubation for 14 days, colonies were counted based on morphological characteristics.

[0091] Chromatin immunoprecipitation analysis

[0092] A2B5^{+/+} and c-KIT^{+/+} subsets were sorted from H1 and H9 hESCs. Approximately 80,000 sorted cells were crosslinked using 1%
20 formaldehyde. Chromatin was digested in buffer containing 0.1 % SDS to obtain fragments of approximately of 500 bp length. Sonicated DNA was subjected to immunoprecipitation using anti-trimethyl H3K4 (Abcam), anti trimethyl-H3K9 (Abcam), anti trimethyl-H3K27 (Abcam), anti rabbit IgG and anti-mouse IgG antibodies. Immunoprecipitated DNA was further reverse
25 crosslinked, purified and subjected to Q-PCR analysis using Cyber Green dye. To calculate the relative enrichment, signals observed in control antibody were subtracted from signals of specific antibody and then divided the resulting difference by signals observed from one fiftieth of ChIP input material.

[0093] Data analysis

[0094] Data are presented as mean $\pm$ standard deviation (s.d.). p values were calculated using Student t -test. p values <0.05 were considered as statistically significant. $p<0.05$ (asterisk) and $p<0.01$ (double asterisk).

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Results**[0095]** Identification of developmentally distinct hESCs expressing c-KIT and A2B5

[0096] hESC culture conditions favor survival of self-renewing cells (Thomson et al., 1998; Xu et al., 2001). Using immunohistochemistry screens two markers, c-KIT and A2B5, were detected in embryonic stem cells that have previously been associated with mesoderm progenitors and neural precursors, respectively (Carpenter et al., 2001; Yang et al., 2008). Cells expressing c-KIT were located within and outside morphologically-identified hESC colonies (Fig. 1a), whereas A2B5 expression was restricted to regions outside colonies previously associated with supportive cells termed hESC-derived "fibroblasts" (hdFs) (Fig. 1b). These subfractions were present in four independent hESC lines (Figs. 8a-b). In addition to feeder-free systems, we examined the expression of c-KIT and A2B5 in hESCs co-cultured with irradiated mouse embryonic fibroblasts (MEFs). The location of c-KIT and A2B5 was conserved and consistently observed in multiple hESC lines cocultured on MEFs (Figs. 8c-f). These observations suggest that expression of c-KIT and A2B5 is generalizable to self-renewing cultures of hESCs independent of growth conditions or hESC cell line.

25 **[0097]** To quantify this observation, flow cytometry analysis was used where hESC cultures revealed a composition of 35% c-KIT⁺, 10% A2B5⁺ and 50% c-KIT⁻/A2B5⁻ cells (Fig. 1c). A small fraction (5%) of cells stained positive for both c-KIT and A2B5 (Fig. 1c) and was strictly localized to the periphery of hESC colonies potentially representing a dynamic transient state of primitive to lineage-primed cells (Fig. 8d). Co-expression of the pluripotency markers

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Oct4 and SSEA3 was examined in the c-KIT and A2B5 positive subfractions identified. Oct4 and SSEA3 positive cells were equally distributed in c-KIT⁺ and c-KIT⁻ subfractions (Figs. 1d-e, respectively), while Oct4 and SSEA3 negative cells were highly enriched with c-KIT⁻ compared to the c-KIT⁺ subfraction (Figs. 1d-e). The vast majority of Oct4 and SSEA3 positive cells were observed in A2B5⁻ hESCs (Figs. 1f-g), and Oct4 and SSEA3 negative cells were predominantly found in A2B5⁺ hESCs (Figs. 1f-g). These data indicate that cells expressing pluripotent-associated markers Oct4 and SSEA3 exist at various frequencies among subfractions of c-KIT^{+/+} and A2B5^{+/+} cells identified, thus revealing a previously unappreciated level of underlying heterogeneity amongst hESCs.

[0098] Fractionated hESC subpopulations possess clonogenic self renewal capacity at distinct frequencies

[0099] To determine developmental potential among hESCs expressing or lacking c-KIT and A2B5 in hESC cultures, cultures of hESCs were stained with c-KIT or A2B5 antibodies conjugated to flurochromes to enable direct isolation by fluorescence-activated cell sorting (FACS) (Figs. 2a-b). The self-renewal potential of purified subfractions was analyzed in a quantitative hESC-CIC (colony-initiating cell) assay by re-plating single cells in feeder-free hESC culture conditions as shown previously (Bendall et al., 2007; Stewart et al., 2008). Clonogenic regeneration capacity was equivalent among c-KIT⁺ vs. c-KIT⁻ hESCs (7.5 and 8 per 5K cells, respectively), whereas the subfraction devoid of A2B5 expression enriched for clonogenic ability at 23 colonies per 5K cells, compared to A2B5 expressing hESC subfractions that contained clonogenic self-renewal ability, albeit at 2 colonies for 5K (Fig. 2c).

[00100] Although the relative frequencies of hESC-CIC residing in these four subfractions of hESCs can be measured and compared, the quality and composition of individual colonies derived from hESC-CIC of each fraction requires further examination at the individual colony level independent of frequency. Accordingly, live staining on emerging colonies using montage confocal imaging of individual wells seeded was performed with single cells

from all four hESC subfractions. At Day 6 post single cell plating, culture were stained with the antibody against the pluripotency marker Tra 1-60 , and again via multiparameter endpoint staining using Oct4, SSEA3, Hoechst, c-KIT, and A2B5 at Day 15. The results demonstrated initiation of compact pluripotent colonies from all the subfractions occurs equally, indicating that differences in CIC frequency (Fig. 2c) was not due to kinetics of CIC emergence or potential over time amongst these subfractions of hESCs (Fig. 2d). In addition to these static time points, this equality was confirmed by time course imaging between Days 6-15 of colony emergence recorded every 8 hours for each of the 4 subfractions of hESCs (data not shown). Based on Day 15 endpoint staining, functionally defined clonogenic hESCs (Stewart et al., 2006) were capable of re-establishing complete composition of hESC cultures from which the subfractions were originally derived, including comprising hDFs and identical profile of Oct4, SSEA3, c-KIT, or A2B5 expression (Figs. 2e-f and Fig. 9). Furthermore, addition of the chemical ROCK inhibitor that has been reported to increase cloning efficiency from unfractionated hESCs by increasing survival upon disaggregation and subsequent re-plating(Watanabe et al., 2007) had no effect on cloning efficiency of CICs generated or acquisition of pluripotency markers on CICs using this assay (Figs. 10a-b). Although the ROCK inhibitor provides a survival advantage in the absence of a homologous niche support layer (Watanabe et al., 2007), inhibition of the pathway has no augmenting effect in the system of clonogenic readout (Stewart et al., 2006) that is supplemented by hDF support.

[00101] c-KIT and A2B5 hESCs possess gradient of lineage specific markers

[00102] To further examine the basis for co-expression of pluripotency and lineage-specific markers of isolated hESC subsets, gene expression analysis was performed by Q-RT-PCR. Oct4 and Nanog transcripts were equivalently detected in c-KIT⁺ and c-KIT⁻ fractions (Fig. 3a), supportive of the flow cytometry analysis (Fig. 1d) and consistent with functional clonogenic capacity (hESC-CICs) from these two subfractions of hESCs (Fig. 2c).

Consistent with the presence of clonogenic self-renewing capacity from both A2B5⁺ and A2B5⁻ cells, both subfractions expressed Oct and Nanog, however, the A2B5⁻ hESCs expressed 25-35-fold higher levels of both compared to the A2B5⁺ fraction (Fig. 3b). To determine whether c-KIT or
5 A2B5 expression on hESCs affected lineage propensity towards mesoderm or neural fates, transcript levels of lineage-associated genes were assessed; Brachyury, MIXL1 for mesoderm (Ng et al., 2005; Vijayaragavan et al., 2009) and; Pax6, NF-68 for neural (Itskovitz-Eldor et al., 2000; Wu et al., 2007), lineages. Transcript levels for Brachyury and MIXL1 showed approximately 2-
10 fold increase of in c-KIT⁺ cells compared to expression levels in c-KIT⁻ cells (Fig. 3c). Similarly, both neural genes Pax6 and NF-68 were expressed at higher levels in the A2B5⁺ fraction than in the A2B5⁻ fraction of hESCs (Fig. 3d). To verify whether the transcript expression of these lineage-associated genes correlated with protein in these subfractions of hESCs, Brachyury
15 (surrogate mesodermal marker) was used to validate via intracellular staining. The c-KIT⁺ hESC subfraction contained 3-4-fold higher Brachyury positive cells consistent with mesodermal predisposition, compared to c-KIT⁻ hESCs and A2B5⁺ cells (Figs. 3e-f). Collectively, these results demonstrate that a gradient of clonogenic self-renewal exists among hESC cultures that can be
20 further characterized by a combination of lineage-associated and pluripotent markers.

[00103] c-KIT⁺ and A2B5⁺ hESCs are predisposed for lineage-specific differentiation

[00104] Using the well-characterized hematopoietic lineage as an
25 example of mesodermal potential (Chadwick et al., 2003; Vijayaragavan et al., 2009), we fractionated c-KIT⁺ and c-KIT⁻ subpopulations and assayed for embryoid body (EB)-derived hematopoietic development using Re-Aggregated-Embryoid Bodies (RA-EBs) from FACS-isolated hESC-derived populations (Fig. 11). This direct isolation into differentiation conditions
30 prevents re-culturing of isolated hESC subsets that reset pluripotent state and hESC culture heterogeneity, as shown in Fig. 2 and Fig. 10. RA-EBs were

successfully formed from isolated c-KIT⁺ and c-KIT⁻ cells and cultured in hematopoietic-inducing conditions for 15 days (Fig. 4a). During EB hematopoietic development, both c-KIT⁺ and c-KIT⁻ RA-EBs showed no significant differences in diameter and viability (Figs. 4b-c). As previously characterized, the hematopoietic development from hESCs can be divided into two phases; bipotent hemogenic-specified phase (Days 0-7), and hematopoietic committed phase (Days 7-15) (Vijayaragavan et al., 2009; Wang et al., 2004) (Fig. 4d). The frequencies of hemogenic precursors, primitive hematopoietic progenitors, and mature hematopoietic cells were significantly enhanced in c-KIT⁺ RA-EBs compared to c-KIT⁻ RA-EBs (Fig. 4e). These data indicate a predisposition of c-KIT⁺ hESCs to give rise to mesodermal development, distinctly from remaining hESCs.

[00105] To further explore the potential differentiation bias of hESCs, we also formed RA-EBs from A2B5⁺ and A2B5⁻ hESCs. RA-EBs from the A2B5⁺ cells preferentially differentiated to neural lineages, producing extensive neurite-like outgrowths when plated back onto a fibronectin growth substrate (Fig. 5a). However, the A2B5⁻ hESCs displayed extremely low viability (5.1%) upon isolation (Fig. 5b) and in contrast to A2B5⁺ hESCs failed to form RA-EBs, thereby preventing side-by-side comparison of neural differentiation capacity to A2B5⁺ hESCs (Figs. 5a-c). This inability for A2B5⁻ hESCs to form EBs raised the possibility of a unique relationship between A2B5⁻ and A2B5⁺ cells for lineage development. To explore this idea, A2B5⁺ and A2B5⁻ subpopulations from either GFP⁺ or RFP⁺ marked hESC lines were prospectively isolated. A2B5⁺GFP⁺ cells were then mixed with A2B5⁻RFP⁺ cells, or in the reverse A2B5⁻GFP⁺ cells were mixed with the A2B5⁺RFP⁺ subfractions (Fig. 5d). Using these mixtures, RA-EBs were successfully formed from both combinations (Figs. 5e-f). Under culture conditions conducive to neural differentiation, A2B5⁺ cells (green in Fig. 5e, red in Fig. 5f) were present in greater numbers than A2B5⁻ cells (red in Fig. 5e, green in Fig. 5f). Notably, higher frequencies of neural-lineage markers such as A2B5, glial fibrillary acidic protein (GFAP) indicative of astrocytes (Shin et al., 2006), and microtubule-associated protein 2 (MAP2) indicating neurons (Jang et al.),

were observed in A2B5⁺ RA-EBs (80.3%, 68.2%, and 71.1%) compared to that of A2B5⁻ RA-EBs (11.5%, 13.4%, and 14.5%) (Figs. 5g-h and Fig. 12). Total frequencies of neural lineage markers and the proportion of GFP⁺ to RFP⁺ cells in RA-EBs at Day 9 of neural differentiation are shown in Figs. 5g-h, indicating that these effects were not related to the use of either GFP or RFP cell lines. These results indicate that A2B5⁺ hESCs have a propensity for neural development, but also are capable of rescuing survival and neural capacity of purified A2B5⁻ hESCs incapable of participating in EB development alone.

10 **[00106]** Overall, these studies demonstrate that c-KIT⁺ and A2B5⁺ hESCs are lineage-biased towards hematopoietic and neural cell fate, respectively. In addition, the present observations demonstrate that a functional dependency among subfractions within hESC cultures extends beyond niche requirements to support hESC self-renewal (Bendall et al., 15 2008; Bendall et al., 2007; Stewart et al., 2008), but is equally critical for lineage development in the case of neural differentiation shown here.

[00107] c-KIT⁺ and A2B5⁺ hESCs are epigenetically primed for differentiation

[00108] To determine the molecular basis for functional predisposition of 20 c-KIT or A2B5 hESC subsets to differentiation towards a hematopoietic or neural cell fate, whether there is an epigenetic mechanism underlying these lineage biases was investigated. As shown previously (Pan et al., 2007), using antibodies against H3K4me3 and H3K27me3 extensive enrichment of H3K4me3 activation mark on the Oct4 and Nanog loci in unfractionated (UF) 25 hESC cultures (Fig. 6a) was found. Consistent with previous reports (Bernstein et al., 2006; Boyer et al., 2006; Pan et al., 2007; Surface et al., 2010; Zhao et al., 2007), gene loci associated with mesodermal (Brachyury, MIXL1, Meox1, Eomes, and Tbx6) and neural (Pax6, NF-68, Mash1, Nestin, and Sox1) lineages showed a bivalent pattern (Biv) for H3K4me3 activation 30 and the H3K27me3 repression marks in UF hESC cultures (Figs. 6b-c). In total, these observations are similar to previous reports describing that

H3K4me3 activation marks are primarily associated with pluripotency markers, and bivalent marks are typically observed at developmental gene loci for hESCs (Bernstein et al., 2006; Pan et al., 2007; Zhao et al., 2007).

[00109] Based on functional lineage and clonogenic self-renewal bias among c- KIT^{+/−} and A2B5^{+/−} subsets of hESCs, using established sequential ChIP methods (Pan et al., 2007) the relative enrichment of H3K4me3 (1st ChIP) and H3K27me3 (2nd ChIP) was examined on the Oct4 and Nanog loci in c-KIT^{+/−} and A2B5^{+/−} subfractions of hESCs (Figs. 6d-e). We observed low levels of H3K27me3 enrichment indicative of gene activation at the Oct4 locus in c-KIT⁺, c-KIT[−], and A2B5[−] subfractions (Figs. 6d-e), a result in accordance with flow cytometry analysis (Figs. 1d-e) and Oct-4 transcript expression (Figs. 3a-b). Oct-4 and Nanog were activated in all fractions of hESCs (Figs. 6d-e), however, A2B5⁺ hESCs showed low levels of H3K4me3 enrichment at the Oct4 locus (Fig. 6e), indicative of histone methylation bivalency. To explore lineage-specific loci, the relative enrichment of H3K4me3 and H3K27me3 on the 5' regions of mesoderm lineage-associated genes was examined; Brachyury, MIXL1, Meox1, Eomes, and Tbx6, in c-KIT⁺ c-KIT[−] hESCs. Extensive enrichment of H3K4me3 on these loci in c-KIT⁺ hESCs was observed, while enrichment of H3K27me3 was displayed in c-KIT[−] hESCs (Figs. 6f). When these same subpopulations of hESCs were analyzed for chromatin state of neural associated gene loci, such as Pax6 and NF-68, chromatin marks indicative of repression (Figs. 6g) were observed. Activated marks on these loci in c-KIT⁺ hESCs correlate with the increased expression of Brachyury and MixL1 genes observed by transcript measurement and flow cytometry analysis, and functionally demonstrated by more permissive hematopoietic developmental potential (Figs. 3c,e and Fig. 4).

[00110] Based on these observations, the analysis was extended to neural-associated Pax6, NF-68, Mash1, Nestin, and Sox1 loci in A2B5⁺ and A2B5[−] hESCs. Bivalent domains were observed for all above loci in both UF cells (Fig. 6c). However, sequential ChIP analysis identified loss of H3K27me3 on these loci in A2B5⁺ hESCs when compared to the UF or A2B5[−]

subfractions (Fig. 6h). Similar to correlations of histone marks and gene transcription, these observations are consistent with gene expression levels of Pax6 and NF-68 of A2B5⁺ hESCs (Fig. 3d). On the contrary, analysis of mesodermal associated gene loci (Brachyury and MIXL1) in A2B5⁻ hESC subsets showed reduced activation (Fig 6i). Control experimentation using fibroblasts showed substantial levels of H3K27me3 marks, but negligible H3K4me3 (Fig. 13) and identical results were generated using these lineage-specific genes using reversed sequential ChIPs (eg. H3K27me3 1st ChIP followed by H3K4me3 2nd ChIP, data not shown). These results provide confirmation of the unique profile of activation and repression domains within these subfractions of hESCs.

[00111] Of all cells comprising hESC cultures, nearly 50% are devoid of both c-KIT and A2B5 expression (Fig. 1c). Based on the monovalent encoded chromatin marks revealed in subsets of c-KIT⁺ and A2B5⁺ hESCs, we sought to evaluate the chromatin state within the subset of c-KIT⁻A2B5⁻ hESCs most likely to harbor bivalent chromatin marks due to the absence of lineage associated protein expression (Fig 1c). Oct4 and Nanog gene loci showed extensive enrichment of H3K4Me3 marks (indicative of activation) in c-KIT⁻A2B5⁻ hESCs (Fig 7a). Unlike bivalent domains observed in unfractionated hESCs (Fig 6a-c) strong enrichment of H3K27Me3 marks (indicative of repression) were revealed on mesodermal associated loci (Fig 7b) and neural associated loci (Fig 7c) loci. These results were consistent for all loci analyzed by sequential ChIP assays (IP H3K27 ; Re-IP-H3K4) for purified c-KIT⁻A2B5⁻ hESCs, confirming the inability to detect bivalent marks in all subfractions analyzed, in contrast to analysis performed on unfractionated hESC cultures.

[00112] Taken together, these studies suggest that biased lineage developmental potential of hESC subfractions is encoded by histone modifications of H3K27me3 and H3K4me3 methylation found at lineage-associated and pluripotency genes. In addition, fractionation of previously unknown subpopulations of hESCs resolves apparent bivalent histone methylation marks of unfractionated hESC cultures into monovalent

signatures that can be correlated to functional lineage potential via robust differentiation assays.

Discussion

5 **[00113]** The present study describes a previously unappreciated level of concealed developmental potential among hESCs that has direct implications to the differentiation and cell fate decisions of hESCs. Using c-KIT or A2B5 expression as examples, the inventors delineate that seemingly equivalent hESCs possess a relationship between phenotypic heterogeneity and
10 functional propensity to participate in self-renewal versus differentiation programs that are uniquely encoded by histone modifications of hESC subsets.

[00114] Pluripotency-associated cell surface markers such as SSEA3, GCTM-2, and CD9 allow for isolation of sub-populations of cells from hESC
15 cultures that display high levels of pluripotency gene transcripts (Enver et al., 2005; Hough et al., 2009; Stewart et al., 2006). These studies established that heterogeneity is typical in hESC cultures, but did not elucidate the identity and functional capabilities of these hESC compartments. The combined analysis and correlation of c-KIT⁺ or A2B5⁺ subfractions, expression profiling, and
20 lineage potential measured by the RA-EB assays illustrate that hESC cultures are a complex mosaic of cell types that cover the spectrum from self-renewing undifferentiated stem cells to incipient lineage-biased cells.

[00115] The accession of lineage markers is typically associated with the loss of pluripotency markers (Hough et al., 2009; Toyooka et al., 2008).
25 Surprisingly, the data presented herein shows that the mutually exclusive relationship often described between pluripotency and fate markers is not always maintained. Although A2B5⁺ hESCs showed marked down-regulation of pluripotency markers at both the transcript and protein levels, they expressed these proteins and were capable of clonogenic self-renewal
30 capacity. For c-KIT⁺ hESCs there was little change in Oct4 and Nanog levels,

and pluripotency marker SSEA3 was equally distributed in c-KIT⁺ vs c-KIT⁻ populations of hESCs. The functional validation of these results by the hESC-CIC assay demonstrated that the c-KIT⁺, c-KIT⁻ and A2B5⁺, A2B5⁻ subpopulations of hESCs are all similar in their ability to re-initiate hESC colonies and cultures, but vary in frequency of clonogenic self-renewal cells.

[00116] Several reports describe the epigenetic modification patterns present within ESCs that are thought to control pluripotency and cell fate specification (Azuara et al., 2006; Bernstein et al., 2006; Boyer et al., 2006; Pan et al., 2007; Zhao et al., 2007). Concomitant H3K4me3 (activating) and H3K27me3 (repressive) methylation patterns mark untranscribed lineage-specific gene loci, termed “bivalent domains”. The results described herein are unique to the view that describes hESCs as being concomitantly pluripotent and primed for fate decisions by competing constellations of histone modifications that position loci in a bivalent state (Fig. 7d). Alternatively, the phenomenon of histone modification bivalency may be the direct result of collectively assaying a diverse range of cell types resident within hESC cultures, suggesting that bivalency reflects a population of hESCs with unequivalent developmental potentials and not necessary the state of individual hPSCs. The data provided herein demonstrates that bivalent domains are detectable in unfractionated hESC cultures. However, when the hESC cultures are fractionated into mesoderm (c-KIT⁺) or neural (A2B5⁺) progenitors, the co-occupancy of H3K27me3 and H3K4me3 marks at mesoderm neural-related gene loci is reduced to a monovalent activation or repression methylation signature. Moreover, the epigenetic signature at the mesoderm or neural lineage genes that were assayed appears to be a direct predictor of the fate attainable when the specific prospective populations (c-KIT^{+/+} or A2B5^{+/+}) are differentiated. Recent studies have established that bivalent methylation patterns at gene loci are not the norm in the frog embryo, and that genes with associated bivalent domains are typically transcribed and not repressed as posited in ESCs (Akkers et al., 2009). Based on these results, when heterogeneity among hESCs is deconstructed by fractionation,

the overall state of human pluripotent may best be described as a plastic and dynamic gradient of clonogenic and lineage specification potentials (Fig. 7e).

[00117] The proposed model to describe hPSC state is further supported by a recent publication from Scholer et al (Cherry and Daley, ; Han et al.) wherein mouse EpiSCs showed a metastable subpopulation of cells that represent various epiblast stages *in vivo*. Since human ESCs share basic similarities with mouse EpiSCs, it is believed that observed heterogeneity is inherent to the cells of the epiblast stage. Human ESCs might possess the intrinsic memory of the location and proximity to be primed to differentiate *in vitro*, similar to that of the transient epiblast cells. Nonetheless recent studies demonstrated bFGF-responsive hESC cultures could be converted to mouse ES-like LIF-dependent colonies by inhibition of multiple signaling pathways including GSK and ERK1/2 (Hanna et al, PNAS 2010), providing further evidence of the intricate signaling that might be responsible for observed priming of lineage potentials. Nevertheless, the present Example reveals an unpredicted biodiversity within the undifferentiated state demonstrating monovalent epigenetic marks at lineage-associated gene loci that provide an encoded predictive index of eventual cell fate progression, thereby forming a unique perspective for understanding the mechanisms of lineage specification from pluripotent stem cells in the human.

EXAMPLE 2: Reversible Modulation of Self-Renewal and Differentiation Potential by the Embryonic Stem Cell Culture Environment

Summary

[00118] Clinical use of human stem cells requires efficient cellular expansion, that must be followed by the ability to generate specialized progeny. Although self-renewal and differentiation are deemed inherent hallmarks of human pluripotent stem cells (hPSCs), a growing body of observations suggests that culture conditions used to maintain hPSCs govern self-renewal, and differentiation. The present Example reveals that defined

culture conditions using mTeSR1 media favor self-renewal amounting to enhanced cellular expansion at the expense of hematopoietic lineage differentiation. However, this culture induced self-renewing state is reversible, as subsequent passage using mouse embryonic fibroblast conditioned medium (MEF-CM) allows mTeSR1 expanded hPSCs to re-establish self-renewal levels and differentiation potential for hematopoietic specification. Moreover, this lineage differentiation potential can be predicted via surrogate markers expressed on hPSCs to measure propensity for differentiation towards hematopoietic and neural cellular types. As described herein, hPSCs exist in a range of functional states spanning the balance of self-renewal to differentiation potential that can be modulated by culture conditions and predicted in a quantitative manner.

Background

[00119] Stem cells react to biochemical and biophysical signals that when present in a particular combination allow for the maintenance of self-renewal capacity while retaining differentiation potential. This can be exemplified by the work of Gilbert et al. (2010) demonstrating that mouse muscle stem cells cultured on a bioengineered substrate and exposed to a particular biochemical combination display improved self-renewal capacity while contributing to tissue regeneration in vivo. Precise control of stem cell self-renewal and differentiation is paramount to clinical translation of regenerative medicine products that depend on the exponential proliferation of pluripotent cells followed by differentiation competency into specific cell types of interest (Thomson et al. 1998). Yet modulation of these two stem cell states upon command remains grossly unachieved.

[00120] The definition of ideal stem cell culture conditions allowing self-renewal and differentiation without the introduction of undefined materials or materials of non-human animal origin has been the subject of multiple reports (Ludwig et al. 2006; Hannoun et al. 2010; Lu et al. 2006; Yao et al. 2006; Li et al. 2005; Rajala et al. 2007; Rajala et al. 2010; Akopian et al. 2010), nevertheless the basis for the majority of those compositions has been

essentially empirical in nature with media formulations often aiming at mimicking embryonic systems or being extrapolated from tissue culture conditions.

[00121] In vitro, multiple levels of intrinsic and external controls
5 regulating stem cell function have also been reported (Watt and Hogan, 2000), and must be considered during the creation of media formulations. Among those, external regulators are of particular importance since they can act directly on the cells or indirectly through the activation of intrinsic regulators such as transcription factors and cellular clocks. Furthermore,
10 secreted factors have been demonstrated to undergo active modulation by the chemical environment existent within media formulations (Bendall et al. 2007).

[00122] Despite some progress in the development of improved culture conditions such as the elimination of feeder layers of animal origin (Xu et al. 2001), adopted stem cell culture systems remain suboptimal especially in
15 terms of support to differentiation since most formulations are primarily evaluated based on their ability preserve pluripotency (Lim and Bodnar, 2002; Prowse AB et al. 2005). Among those are commercially available semi-defined and humanized media preparations, all of which must be thoroughly investigated for maintenance of differentiation potential prior to their utilization
20 in product development.

[00123] The present Example provides insights into the modulation of proliferation and differentiation properties of human pluripotent stem cells in culture using broadly adopted semi-defined media formulations that demonstrate the need for in-depth evaluation of culture conditions for the
25 maximization of cellular output.

Methods

[00124] Human Embryonic Stem Cell Culture

[00125] In order to compare the effect of hESC culture medium on the
30 cellular expansion and differentiation capacities of pluripotent stem cells, three

different hESC lines (H1, H9 and CA2) maintained under feeder-free condition (Xu et al., 2001; Chadwick et al. 2003; Wang et al. 2004) were passaged in either MEF-CM supplemented with 8ng/ml of bFGF or mTeSR1 with 1X mTeSR1 supplement (StemCell Technologies) for 9 consecutive passages.

5 Cells were passaged using 200U/ml of Collagenase IV and mechanical scoring prior to plating onto Matrigel-coated dishes as recommended by the manufacturer. Culture effect reversibility studies were performed by culturing hESCs for 5 passages in either MEF-CM or mTeSR1 followed by mTeSR1 and MEF-CM media respectively for additional 4 passages.

10 **[00126]** Hematopoietic or Neural Differentiation of hESCs

[00127] Confluent hESC cultures at day 7 were harvested after treatment with Collagenase IV to form suspension embryoid bodies (EBs) as previously described (Chadwick et al. 2003; Wang et al. 2004). Hematopoietic differentiation was carried out by culturing EBs for 20 days in 20% FBS
15 containing DMEM/F12 medium supplemented with cytokines such as SCF, Flt-3L, IL-3, -6, G-CSF and BMP4. EBs were then analyzed for Colony-Forming Unit capacity and the generation of CD45+ cells by flow cytometry. EBs differentiated without hematopoietic cytokines were also analyzed as a negative control.

20 **[00128]** For neural differentiation, EBs were generated by suspension culture in neuro-proliferating media (DMEM/F12 supplemented with 1% N2, 1% B27, 20 ng/ml EGF, and 20 ng/ml FGF-2) and cultured for 7 days with media changes being performed at two-day intervals. To further expand and differentiate into neural stem cells, EBs were collected and dissociated into
25 single cells using Accutase (Sigma). Dissociated single cells were plated into low attachment 6-well plates (20K/well at passage #5 or 200K/well at passage #5+4) with neuro-proliferating media and allowed to generate neurospheres for 7 days with media change every three days. At the end of each expansion and differentiation, neurospheres were collected from each well, dissociated
30 into single cells, and counted. Neural cells were identified based on the expression of nestin by flow cytometry.

[00129] Hematopoietic Colony Formation Assay

[00130] CFU assays were performed with day 20 EBs formed with hESC cultures per treatment 5 passages. Differentiated EBs were dissociated into single cells by the serial treatment of Collagenase B and cell dissociation
5 buffer and then 15K cells were plated into methylcellulose H4230, supplemented with BMP4 and cytokines including SCF, Flt-3L, IL-3, IL-6 and G-CSF. Hematopoietic cell clusters displaying more than 50 cells were counted as colonies after incubation for 14 days at 37°C in 5% CO₂.

[00131] FACS Analysis

10 **[00132]** In order to identify SSEA3 and A2B5 in undifferentiated hESC cultures, cells were dissociated with Cell Dissociation Buffer (Gibco) for 5-10 min. Dissociated single cells incubated with SSEA3 (rat anti-mouse IgM, Hybridoma Bank) or A2B5 (mouse anti-human IgM, Chemicon) antibodies for 40 min and then localized with fluorescent conjugated secondary antibodies
15 (Alexa 647-conjugated goat anti-rat IgM or Alexa 647-conjugated goat anti-mouse IgM, BD). Stained Oct4 (BD) was localized using Alexa 647-conjugated goat anti-mouse IgG (BD). Additionally c-Kit was stained with APC-conjugated antibody (BD). Hematopoietic or neural cells derived from day 20 EBs were detected using CD45 (hematopoietic, BD), nestin and
20 (neural) antibodies. Following each staining, live cells were distinguished by 7-AAD (BD). Expression of SSEA3, Nestin, A2B5, c-kit and CD45 was analyzed on FACS Calibur (BDIS), Cell Quest Software (BDIS) and FlowJo version 8.5.3 (Treestar).

[00133] Image-based analysis of hESC morphology

25 **[00134]** Human ES cells, seeded at 1×10^4 cells per well (96-well imaging plate) were cultured for 5 days in either MEF-CM or mTeSR1 before fixation and permeabilization with Cytofix/Cytoperm kit (BD). Following blocking and washing procedure, hESCs were immunolabelled with a primary anti-Oct4
30 (BD) antibody and with a secondary fluorescent-conjugated antibody (Alexa Flour 488-conjugated). Nuclear staining was performed using Hoechst 33258.

After the staining, all the images were acquired with a 5x (0.4NA) and 10x (0.7NA) NA objective on a Cellomics Arrayscan (Thermofisher). Images were processed and analyzed using Perkin Elmer's Acapella 2.5. or ImageJ (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997-2005.) Representative images were processed in ImageJ, after stitching together 9 adjacent fields of view with Perkin Elmer Acapella 2.5.

[00135] Kinetic cell death analysis

[00136] Cells were seeded at 5×10^4 cells per well using 24-well plates treated with either MEF-CM or mTeSR1 and cultured for 5 days. Cells were incubated in a Nikon BiostationCT allowing automated image acquisition of the cultures. AnnexinV-R-phycoerythrin (Invitrogen) was added to the media (1:1000) 24 hours post seeding and the media dye exchanged each day. Phase contrast and fluorescent images of manually selected colonies were acquired every 4 hours at 2x for quantification and 10x for visualization.

[00137] Images were processed and analyzed as described above.

[00138] Statistical Analysis

[00139] Student's *t*-test was applied for the statistical analysis.

20 **Results**

[00140] Stem cell culture media modulate key human embryonic stem cell characteristics.

[00141] The present Example demonstrates that culture of hESCs in mTeSR1 media altered the morphological properties of colonies and cells previously treated with MEF-CM (Figure 14A). More specifically, hESCs maintained in the presence of MEF-CM possessed typical human embryonic stem cell (hESC) morphology including multiple compact colonies surrounded by hESC-derived fibroblasts (hdFs), whereas mTeSR1 produced irregular-shaped multi-layer colonies with a higher proportion of hdFs (Figure 14A).

These differences were confirmed using high content analysis of microscopic images that showed a dramatic increase in the number of colonies of mTeSR1 cultures (Figure 14B). Image-based quantification of Oct4 and Hoechst labeled cultures (Figure 14C) indicated that mTeSR1 colonies had smaller surface area (Figure 14D); contained more cells than the ones in MEF-CM (Figure 14E); displayed cells with a smaller nuclear area and cell area (Figures 14F and 14G). Using Annexin-V staining, an early apoptosis marker, an increase in the number of labeled cells when they were cultured in MEF-CM media was observed suggesting a decrease in cell death or an increase in cell survival of cells treated with mTeSR1 (Figure 14H and I). This experiment was based on the kinetic image acquisition and analysis of Annexin-V labeled cells followed by image stitching (Figure 14H shows representative images) and quantitative analysis (Figure 14I).

[00142] The above mentioned mTeSR1 induced alterations in cellular and colony characteristics lead the inventors to question the ability of culture media to induce changes in stem cell self-renewal. In order to assess self-renewal, hESCs were maintained for 5 weeks under two media conditions, namely, mTeSR1 and MEF-CM and monitored weekly for their proliferation, viability and expression of pluripotency markers by flow cytometry.

[00143] Interestingly, an increase in the total cell number was observed in cultures maintained using mTeSR1 media in comparison to MEF-CM and was sustained throughout the 5-week period (Figure 14J). mTeSR1 hESC cultures also showed a consistent and significant higher percentage of live cells throughout the culture period (Figure 14K) but no significant differences in the percentage of cells expressing the pluripotency marker SSEA3 (Figure 14L); overall resulting in a greater accumulated total and SSEA3+ cell numbers (Figure 14J inset). These initial data suggested that mTeSR1 media favors self-renewal of hESCs in culture potentiating expansion of pluripotent stem cells.

[00144] Together, these findings clearly show that culture conditions influence the stem cell culture dynamics inducing alterations of cell growth,

morphology and pluripotency which underlie the higher capacity of mTeSR1 to accelerate self-renewal of treated hESCs.

[00145] Hematopoietic and neural differentiation are altered in mTeSR1-expanded cells.

5 **[00146]** Differentiation experiments using embryoid bodies (EBs) evidenced that cultures treated with mTeSR1 media for a minimum of 3 weeks partially lost their ability to differentiate towards the hematopoietic lineage. Despite the morphological similarities of EBs generated using the two culture conditions (Figure 15A), CD45+ blood cells and hematopoietic colony-
10 forming unit levels were reduced by approximately 2-fold (Figure 15B and 2C).

[00147] Neural lineage differentiation was assessed through the generation of neurospheres. Human ESCs expanded in mTeSR1 showed a much greater number of neurospheres than hESCs expanded in MEF-CM
15 (Figure 15E). No difference in the number of cells per neurosphere was identified after the media treatments (Figure 15F). Overall expansion of hESCs in mTeSR1 generated a much greater number of cells with a statically significant higher percentage of nestin positive cells yielding a greater total number of nestin positive cells when contrasted to cells expanded in MEF-CM
20 (Figures 15G, 15H and 15I), finally suggesting a gain in neural differentiation potential under this treatment. Neural differentiation commitment of cells within neurospheres expressing nestin was further confirmed by immunocytochemistry of MEF-CM and mTeSR1 cultured cells with antibodies against oct-4, TuJ 1 and GFAP. All of which showed positive cellular staining
25 (Figure 15J).

[00148] These data, taken together, indicate that expansion of hESC in mTeSR1 favors neural differentiation at the expense of hematopoietic differentiation potential while the reverse is true for embryonic stem cells subjected to culture in MEF-CM.

30 **[00149]** Culture medium-induced gain in cellular expansion is reversible

[00150] In order to investigate the reversibility of culture induced alterations in hESC growth, culture media was swapped after 5 passages with cells remaining in the new culture media for additional 4 passages (Figure 16A). Cellular proliferation, pluripotency and morphology were monitored weekly during the 4-week period following media swap. The exchange of culture media induced a gain in cell growth by cells initially cultured in MEF-CM and then exposed to mTeSR1 (Figure 16B) while mTeSR1 cultured cells exposed to MEF-CM showed a reduction in the total number of cells in culture (Figure 16C) both of which contributed to the progressive increase in the accumulated total cell number of cells swapped into mTeSR1 and a decrease of accumulated total cell number in cells treated with MEF-CM after exposure to mTeSR1. No changes in the percentage of pluripotent cells, as evidenced by SSEA3+ staining, were observed during the 4-week culture after media swap in relation to the respective controls (Figures 16D and 16E). Altogether, the observed changes in cellular expansion while pluripotency levels were maintained constant accounted for statically significant higher cumulative SSEA3+ total cell numbers in cultures exposed to TeSR media after the swap at week 5 (Figures 16F and 16G). Furthermore, a morphological recovery (Figure 16H) of cellular and colony phenotypes were gradually regained after the media exchange event in week 4 under both culture conditions (MEF-CM and mTeSR1).

[00151] Hematopoietic differentiation potentials of hESC is a reversible characteristic modulated by the culture environment of pluripotent stem cells.

[00152] Reversibility of culture media induced alterations in the differentiation potentials of hESC were investigated through the quantification of blood progenitor colony formation and CD45+ blood cell emergency after media exchange at week 5 of culture.

[00153] Colony forming unit assays of cells expanded in T-T, T-M, M-T and M-M treatments were performed for four consecutive weeks after the media exchange. After single cell seeding in methylcellulose, cells expanded under the different treatment and control conditions displayed the capacity to

generate erythroid, granulocytic and macrophage colonies without significant morphological differences (Figure 17A). Accumulated total CFU numbers decreased when hESCs were transferred from MEF-CM to mTeSR1 (Figure 17B); inversely, CFU counts increased after the exposure of mTeSR1 expanded hESCs to MEF-CM (Figure 17C). The reversibility of alterations in the hematopoietic differentiation capacity of pluripotent cells exposed to different media conditions was further confirmed by a reduction in the proportion and accumulated CD45+ blood cells when MEF-CM treated cells were moved into mTeSR1 (Figures 17D and 17G). Flow cytometric analysis (Figure 17F) also indicated that emergence of CD45+ blood cells was improved when mTeSR1 cultured hESCs were treated with MEF-CM (Figure 17E). Thus leading to a higher cumulative count of CD45+ cells over the 4-week period (Figure 17H). Overall, a positive correlation between progenitor cell generation evidenced by CFU assays and CD45+ cell emergence was observed under all culture conditions.

[00154] In summary, these data show for the first time that particular hESC features such as self-renewal and differentiation can be reversibly modulated *in vitro* by culture conditions.

[00155] Expression of early neural and hematopoietic markers in hESCs correlate with late differentiation potentials.

[00156] Using immunocytochemistry and flow cytometry we identified a correlation between the levels of c-kit receptor and A2B5 expression and the hematopoietic and neural differentiation capacities of cells cultured in MEF-CM and mTeSR1 (Figure 18). Only minimal levels (less than 15%) of c-kit expressing cells were identified in mTeSR1 cultures, while cells exposed to MEF-CM showed a c-kit positive fraction of nearly 40% (Figure 18A). Reversibly, the expression of the neural precursor marker A2B5 was significantly higher in mTeSR1 cultures when compared to the ones maintained with MEF-CM (Figure 18A). The correlation of these data and the skewed differentiation potential of MEF-CM and mTeSR1 cultures towards the

hematopoietic and neural lineages respectively, validate the use of c-kit and A2B5 as early surrogate markers of hESCs differentiation potential.

[00157] In order to expand on the application of these predictive markers of differentiation to culture media evaluation, the inventors proceeded with measurements of their expression upon media exchange at week 5. Cells cultured in MEF-CM and then mTeSR1 showed a significant decrease in the relative expression of c-kit and a greater level of expression of A2B5 in relation to MEF-CM treated cells (Figure 18B). Inversely, MEF-CM media hESC culture following mTeSR1 treatments induced a gain in c-kit and a decrease in A2B5 expression in relation to mTeSR1 controls (Figure 18C). All of which further support the use of A2B5 and c-Kit as predictors of differentiation potential.

Discussion

[00158] The present Example evidences the yet unappreciated impact of culture conditions on key stem cell characteristics such as cellular expansion and differentiation. Furthermore, it is demonstrated for the first time that pluripotent stem cells are capable to undergo reversible changes of early cell fate decision in response to culture conditions. The ability of specific culture medium to command pluripotent stem cells primed for hematopoietic differentiation to commit to the neural lineage illustrate the heterogeneous and dynamic nature of the pluripotent stem cell state while delineating studies that must be performed for accurate characterization of culture systems (Figure 17).

[00159] More specifically, the data provided herein observations indicate that mTeSR1 medium is supportive of improved neural differentiation at the expense of hematopoietic differentiation while inducing an increase in proliferation of hESCs. In addition, these features could be modulated in a reversible manner within a fairly short timeframe. Early prediction of stem cell differentiation potential becomes paramount in the evaluation of culture systems. The identification of early surrogate markers of neural and

hematopoietic differentiation described herein enables the early prediction of stem cell differentiation potential.

[00160] The composition and complexity of media traditionally required for human embryonic stem cell maintenance has long been identified as a significant weakness of the system, limiting production scale-up due to reproducibility and cost issues in addition to potentially contaminating transplant grade cells with proteins of animal origin. The ideal commercial culture media for embryonic stem cell production would have a defined composition minimizing among other features current batch-to-batch variations. These variations have been implicated in cell quality and output fluctuations observed in stem cell cultures maintained using complex media such as MEF-CM. Although in theory this is a fairly trivial challenge in practice such media has not been identified or adopted.

[00161] At the heart of this issue is the inability of researchers to identify media conditions that are favorable to both, cellular expansion through self-renewal and differentiation towards lineages of interest. Historically, hESC pluripotency has been defined based on the ability of stem cells to display differentiation capacity towards several cell lineages *in vivo* as demonstrated by teratoma generation in immunosuppressed mice. Although indicative of self-renewal and differentiation potential, this assay is non-quantitative in nature and unable to provide insights into the balance of these two key features. This can be clearly evidenced by the work of Werbowetski-Ogilvie et al. (2009) who demonstrated that certain genetic alterations of hESCs can lead to an increase in self-renewal capacity at the expense of differentiation potential. At a first glance the increase in proliferation of hESCs could be deemed an improvement to the stem cell culture system especially considering that teratoma assays are unable to define the extent of alterations in differentiation capacity. However, in practice these cells should not be utilized for cellular therapy due to their biased self-renewal aptitude and neoplastic features.

[00162] Similarly to that study, the present Example demonstrates that a semi-defined and commercially available stem cell media composition, namely mTeSR1, biases self-renewal of hESC in culture while being unable to support the hemogenic differentiation capacity of hESCs for longer than one
5 passage.

[00163] Nonetheless, the skewed balance of self-renewal and differentiation towards the hematopoietic lineage was not related to genetic alterations in this case as it was proven reversible upon swapping of culture conditions. This is the first demonstration of reversibility of stem cell lineage
10 priming induced by the cell culture environment, a knowledge that will be key to the development of culture conditions supportive of specific lineage differentiation. Lineage priming has been traditionally viewed as the first step towards lineage commitment with cells progressively extinguishing the expression of pluripotency-associated genes and turning on transcripts
15 characteristic of specific lineages. In contrast, lineage priming should be viewed as a reversible property of hESCs directly influenced by the culture environment.

[00164] This particular observation is a statement to the high level of plasticity of hESCs while adding to the notion of heterogeneity of cellular
20 ground states within pluripotent cultures. The heterogeneity of pluripotent cultures in terms of morphology and function has been illustrated by work showing the differential cellular behavior of subpopulations segregated based on the expression of certain surface markers (Bendall et al. 2007). This concept has now been broadened by investigations indicating the existence of
25 multiple epigenetic and gene expression states among cells expressing core markers of pluripotency (Adewumi et al. 2007).

[00165] From a gene expression and epigenetic point of view meta-analysis of multiple data sets obtained after microarray studies of hESCs (Assou et al. 2007) identified networks of genes over-expressed and down-
30 regulated in ES cells robustly associated with the pluripotency and differentiation states respectively. This subset of pluripotency-associated

genes was further refined to reflect multiple cell lines (Adewumi et al. 2007); yet a high degree of variability is still identified among pluripotent populations. This can be partially explained by the presence of bivalent chromatin modifications marking genes encoding lineage-specific transcription factors (Bernstein et al. 2006) placing the chromatin structure (Meshorer et al. 2006) in a state of high plasticity which could potentially explain the ability of stem cells to promptly react to cues originating from the culture environment altering fate commitment or at least its early stages.

[00166] Several publications have focused on the role that culture environments play on stem cell function and behavior; nevertheless, efficient modulation of stem cell proliferation and differentiation *in vitro*, and in particular under culture conditions compatible with cellular replacement therapy, have yet to be achieved. One key consideration with regards to this issue is the fact that this balance is not known to exist in nature. Instead stem cells *in vivo* are believed to be present in either a self-renewing or a differentiating state both of which are triggered by diverse physiological conditions. Additionally, prolonged and continuous self-renewal as observed in hES cultures is also an artificial state not replicated *in vivo*. Embryos only transitorily enter this state during the first week of development; the same type of transitory response is observed in adult stem cells after injury. Upon completion of repair stem cells become once again quiescent; nonetheless, selection of ES cell media has been heavily centered on expansion of pluripotent stem cells. Thus evidencing the need for independent assessment of proliferation and differentiation capacity and an upfront understanding that the benefit of one of these features will most likely always be associated to the detriment of the other. Similarly, the identification of culture conditions favoring the maintenance of stem cells in a truly pluripotent state without lineage biases seems far-fetched. Instead it is more likely that certain stem cell culture media will only maximize a particular lineage as demonstrated here using mTeSR1 and MEF-CM. In this scenario it becomes essential to predict differentiation trends early avoiding unnecessary experimentation. This can be achieved through surrogate marker expression monitoring; in

particular, c-Kit and A2B5 allow the early prediction of hematopoietic and neural differentiation capacities respectively.

[00167] As shown herein, the heterogeneity of pluripotent stem cells extends to functional characteristics that can be reversibly modulated by the culture environment. Whether this modulation is mediated by the niche or by directly impacting pluripotent stem cell function remains unclear; nevertheless it illustrates the importance of the choice of culture conditions for regenerative medicine applications.

[00168] While the present disclosure has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the disclosure is not limited to the disclosed examples.

- 56 -

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Claims:

1. An isolated embryonic stem cell (ESC) that expresses i) one or more pluripotency markers and ii) A2B5 or both A2B5 and c-KIT.
2. The isolated ESC of claim 1, wherein the ESC expresses one or more pluripotency markers selected from OCT4, SSEA3, SSEA4, nanog, TRA-1-60, TRA-1-81, Sox2, Rex1, GCTM-2 and CD-9.
3. The isolated ESC of claim 2, wherein the ESC expresses OCT4 and nanog.
4. The isolated ESC of claim 2, wherein the ESC expresses OCT4, nanog, SSEA3 and TRA-1-60.
5. The isolated ESC of any one of claims 1 to 4, wherein the ESC is predisposed to differentiate into neural lineages relative to ESCs that do not express A2B5.
6. The isolated ESC of claim 5, wherein the ESC is predisposed to differentiate into neurons or glia cells.
7. The isolated ESC of claim 6, wherein the glia cells are astrocytes or oligodendrocytes.
8. The isolated ESC of any one of claims 1 to 7, wherein the ESC is a mammalian ESC.
9. The isolated ESC of claim 8, wherein the ESC is a human ESC.
10. A method of producing a population of human embryonic stem cells (ESCs) predisposed to differentiate into neural lineages, the method comprising:
separating ESCs that express A2B5 from ESCs that do not express A2B5;
and
culturing the ESCs that express A2B5 to produce the population of ESCs predisposed to differentiate into neural lineages.
11. The method of claim 10, wherein the ESCs express one or more pluripotency markers selected from OCT4, SSEA3, SSEA4, nanog, TRA-1-60, TRA-1-81, Sox2, Rex1, GCTM-2 and CD-9.

12. The method of claim 11, wherein the ESCs express OCT4 and nanog.
13. The method of any one of claims 10 to 12, wherein the step of separating ESCs that express A2B5 from ESCs that do not express A2B5 comprises positive or negative selection.
14. The method of any one of claims 10 to 13, wherein the step of separating ESCs that express A2B5 from ESCs that do not express A2B5 comprises Fluorescent Activated Cell Sorting (FACS).
15. The method of any one of claims 10 to 14, wherein the step of separating ESCs that express A2B5 from ESCs that do not express A2B5 comprises contacting the cells with an antibody selective for A2B5.
16. The method of any one of claims 10 to 15, further comprising differentiating the isolated ESCs to produce neural cells, optionally neurons or glial cells.
17. The method of any one of claims 10 to 16, wherein the ESC is a mammalian ESC.
18. The method of claim 17, wherein the ESC is a human ESC.
19. A method for predicting the differentiation potential of an embryonic stem cell (ESC) comprising testing the ESC for expression of A2B5, wherein cells that express A2B5 are identified as predisposed to differentiate into neural lineages.
20. The method of claim 19, wherein the ESC expresses one or more pluripotency markers selected from OCT4, SSEA3, SSEA4, nanog, TRA-1-60, TRA-1-81, Sox2, Rex1, GCTM-2 and CD-9.
21. The method of claim 20, wherein the ESC expresses one or more pluripotency markers selected from OCT4, nanog, SSEA3 and TRA-1-60.
22. The method of claim 19, wherein the ESC expresses OCT4 and nanog.
23. The method of claim any one of claims 19 to 22, further comprising testing the cells for one or more mesodermal markers, optionally Brachyury or MIXL1.

24. The method of claim any one of claims 19 to 22, wherein cells that express A2B5 are predisposed to differentiate into neurons or glia cells relative to cells that do not express A2B5.
25. The method of claim 24, wherein the glia cells are astrocytes or oligodendrocytes.
26. The method of claim 24 or 25, further comprising testing the cells for one or more neural lineage markers, optionally Pax6 or NF-68.
27. The method of any one of claims 19 to 26, wherein testing the ESC for expression of A2B5 comprises contacting the ESC with antibodies for A2B5.
28. The method of any one of claims 19 to 27, further comprising testing ESCs cells that express A2B5 for relative enrichment of H3K4me3 histone methylation for one or more neural markers selected from Pax6, NF-68, Mash1, Nestin and Sox1.
29. The method of any one of claims 19 to 28, wherein the ESC is a mammalian ESC.
30. The method of claim 29, wherein the ESC is a human ESC.
31. A method of screening embryonic stem cells (ESCs) for capacity for expansion and self-renewal comprising testing the ESCs for expression of A2B5, wherein ESCs that do not express A2B5 have an increased capacity for expansion and self-renewal relative to stem cells that express A2B5.
32. The method of claim 31, wherein the ESCs express one or more pluripotency markers selected from OCT4, SSEA3, SSEA4, nanog, TRA-1-60, TRA-1-81, Sox2, Rex1, GCTM-2 and CD-9.
33. The method of claim 32, wherein the ESCs express OCT4 and nanog.
34. The method of any one of claims 31 to 33, wherein testing the ESCs for the expression of A2B5 comprises contacting the cells with an antibody selective for A2B5.

35. The method of any one of claims 31 to 34, further comprising separating ESCs that do not express A2B5 from ESCs that express A2B5 to produce a population of ESCs with increased capacity for expansion and self-renewal.

36. The method of claim 35, wherein separating ESCs that do not express A2B5 from ESCs that express A2B5 comprises positive or negative selection, optionally Fluorescence Activated Cell Sorting (FACS).

37. The method of any one of claims 31 to 36, wherein the ESCs are mammalian ESCs.

38. The method of claim 37, wherein the ESCs are human ESCs.

39. A method for identifying an effect of culture media on the differentiation potential of embryonic stem cells (ESCs) comprising:

culturing the ESCs in the culture media, wherein the ESCs express one or more pluripotency markers; and

testing the ESCs for expression of markers that indicate the differentiation potential of the stem cells,

wherein the ESCs are tested for the expression of A2B5 and expression of A2B5 indicates the culture media is supportive of neural differentiation of ESCs.

40. The method of claim 39, wherein the one or more pluripotency markers are selected from OCT4, SSEA3, SSEA4, nanog, TRA-1-60, TRA-1-81, Sox2, Rex1, GCTM-2 and CD-9.

41. The method of any one of claims 39 or 40, wherein the ESC is a mammalian ESC.

42. The method of claim 41, wherein the ESC is a human ESC.

43. An isolated embryonic stem cell (ESC) or a population of embryonic stem cells produced by the method of any one of 10 to 18.

44. An isolated embryonic stem cell (ESC) that expresses one or more pluripotency markers and does not express A2B5.

45. The isolated ESC of claim 44, wherein the ESC expresses one or more pluripotency markers selected from OCT4, SSEA3, SSEA4, nanog, TRA-1-60, TRA-1-81, Sox2, Rex1, GCTM-2 and CD-9.
46. The isolated ESC of claim 45, wherein the ESC expresses OCT4 and nanog.
47. The isolated ESC of any one of claims 44 to 46 that wherein the ESC has increased capacity for expansion and self-renewal relative to an ESC that expresses A2B5.
48. The isolated ESC of any one of claims 44 to 47, wherein the ESC is a mammalian ESC, optionally a human ESC.
49. An isolated embryonic stem cell (ESC) that expresses i) one or more pluripotency markers and ii) c-KIT or A2B5 or both c-KIT and A2B5.
50. The isolated ESC of claim 49, wherein the ESC expresses one or more pluripotency markers selected from OCT4, SSEA3, SSEA4, nanog, TRA-1-60, TRA-1-81, Sox2, Rex1, GCTM-2 and CD-9.
51. The isolated ESC of claim 50, wherein the ESC expresses OCT4 and nanog.
52. The isolated ESC of claim 50, wherein the ESC expresses OCT4, nanog, SSEA3 and TRA-1-60.
53. The isolated ESC of any one of claims 49 to 52, wherein the ESC expresses c-KIT.
54. The isolated ESC of claim 53, wherein the ESC is predisposed to differentiate into mesodermal lineages relative to hESCs that do not express c-KIT.
55. The isolated ESC of claim 54, wherein the ESC is predisposed to differentiate into hematopoietic lineages.
56. The isolated ESC of claim 55, wherein the ESC is predisposed to differentiate into hemogenic precursors, primitive hematopoietic progenitors or mature hematopoietic cells.

57. A method of producing a population of embryonic stem cells (ESCs) predisposed to differentiate into mesodermal lineages, the method comprising:
separating ESCs that express c-KIT from ESCs that do not express c-KIT;
and
culturing the ESCs that express c-KIT to produce the population of ESCs predisposed to differentiate into mesodermal lineages.
58. The method of claim 57, wherein the ESCs express one or more pluripotency markers selected from OCT4, SSEA3, SSEA4, nanog, TRA-1-60, TRA-1-81, Sox2, Rex1, GCTM-2 and CD-9.
59. The method of claim 58, wherein the ESCs express OCT4 and nanog.
60. The method of any one of claims 57 to 59, wherein the step of separating ESCs that express c-KIT from ESCs that do not express c-KIT comprises testing the population of ESCs for the expression of c-KIT.
61. The method of any one of claims 57 to 60, wherein the step of separating ESCs that express c-KIT from ESCs that do not express c-KIT comprises positive or negative selection.
62. The method of any one of claims 57 to 61, wherein the step of separating ESCs that express c-KIT from ESCs that do not express c-KIT comprises Fluorescent Activated Cell Sorting (FACS).
63. The method of any one of claims 57 to 62, wherein the step of separating ESCs that express c-KIT from ESCs that do not express c-KIT comprises contacting the cells with an antibody selective for c-KIT.
64. The method of claim 63, further comprising differentiating the isolated ESCs to produce mesodermal cells, optionally hematopoietic cells.
65. The method of any one of claims 57 to 22, wherein the ESC is a mammalian ESC.
66. The method of claim 65, wherein the ESC is a human ESC.

67. A method for predicting the differentiation potential of an embryonic stem cell (ESC) comprising testing the ESC for expression of c-KIT or A2B5, wherein cells that express c-KIT are identified as predisposed to differentiate into mesodermal lineages and cells that express A2B5 are identified as predisposed to differentiate into neural lineages.

68. The method of claim 67, wherein the ESC expresses one or more pluripotency markers selected from OCT4, SSEA3, SSEA4, nanog, TRA-1-60, TRA-1-81, Sox2, Rex1, GCTM-2 and CD-9.

69. The method of claim 68, wherein the ESC expresses one or more pluripotency markers selected from OCT4, nanog, SSEA3 and TRA-1-60.

70. The method of claim 67, wherein the ESC expresses OCT4 and nanog.

71. The method of any one of claims 67 to 70, wherein cells that express c-KIT are predisposed to differentiate into hematopoietic lineages relative to cells that do not express c-KIT.

72. The method of claim 71, wherein cells that express c-KIT are predisposed to differentiate into hemogenic precursors, primitive hematopoietic progenitors or mature hematopoietic cells.

73. The method of claim any one of claims 67 to 72, further comprising testing the cells for one or more mesodermal markers, optionally Brachyury or MIXL1.

74. The method of claim any one of claims 67 to 72, wherein cells that express A2B5 are predisposed to differentiate into neurons or glia cells relative to cells that do not express A2B5.

75. The method of claim 74, wherein the glia cells are astrocytes or oligodendrocytes.

76. The method of claim 74 or 75, further comprising testing the cells for one or more neural lineage markers, optionally Pax6 or NF-68.

77. The method of any one of claims 67 to 76, wherein testing the ESC for expression of c-KIT or A2B5 comprises contacting the ESC with antibodies for c-KIT or A2B5.

78. The method of any one of claims 67 to 77, further comprising testing ESCs cells that express c-KIT for relative enrichment of H3K4me3 histone methylation for one or more mesodermal markers selected from Brachyury, MIXL1, Meox1, Eomes and TBx6.

79. The method of any one of claims 67 to 77, further comprising testing ESCs cells that express A2B5 for relative enrichment of H3K4me3 histone methylation for one or more neural markers selected from Pax6, NF-68, Mash1, Nestin and Sox1.

80. The method of any one of claims 67 to 79, wherein the ESC is a mammalian ESC.

81. The method of claim 80, wherein the ESC is a human ESC.

82. A method for identifying an effect of culture media on the differentiation potential of embryonic stem cells (ESCs) comprising:

culturing the ESCs in the culture media, wherein the ESCs express one or more pluripotency markers; and

testing the ESCs for expression of markers that indicate the differentiation potential of the stem cells, wherein the ESCs are tested for the expression of c-KIT and expression of c-KIT indicates the culture media is supportive of mesodermal differentiation of ESCs.

83. The method of claim 82, wherein the one or more pluripotency markers are selected from OCT4, SSEA3, SSEA4, nanog, TRA-1-60, TRA-1-81, Sox2, Rex1, GCTM-2 and CD-9.

84. The method of claim 82 or 83, wherein the ESC is a mammalian ESC.

85. The method of claim 84, wherein the ESC is a human ESC.

86. An isolated embryonic stem cell (ESC) or a population of embryonic stem cells produced by the method of any one of 57 to 66.

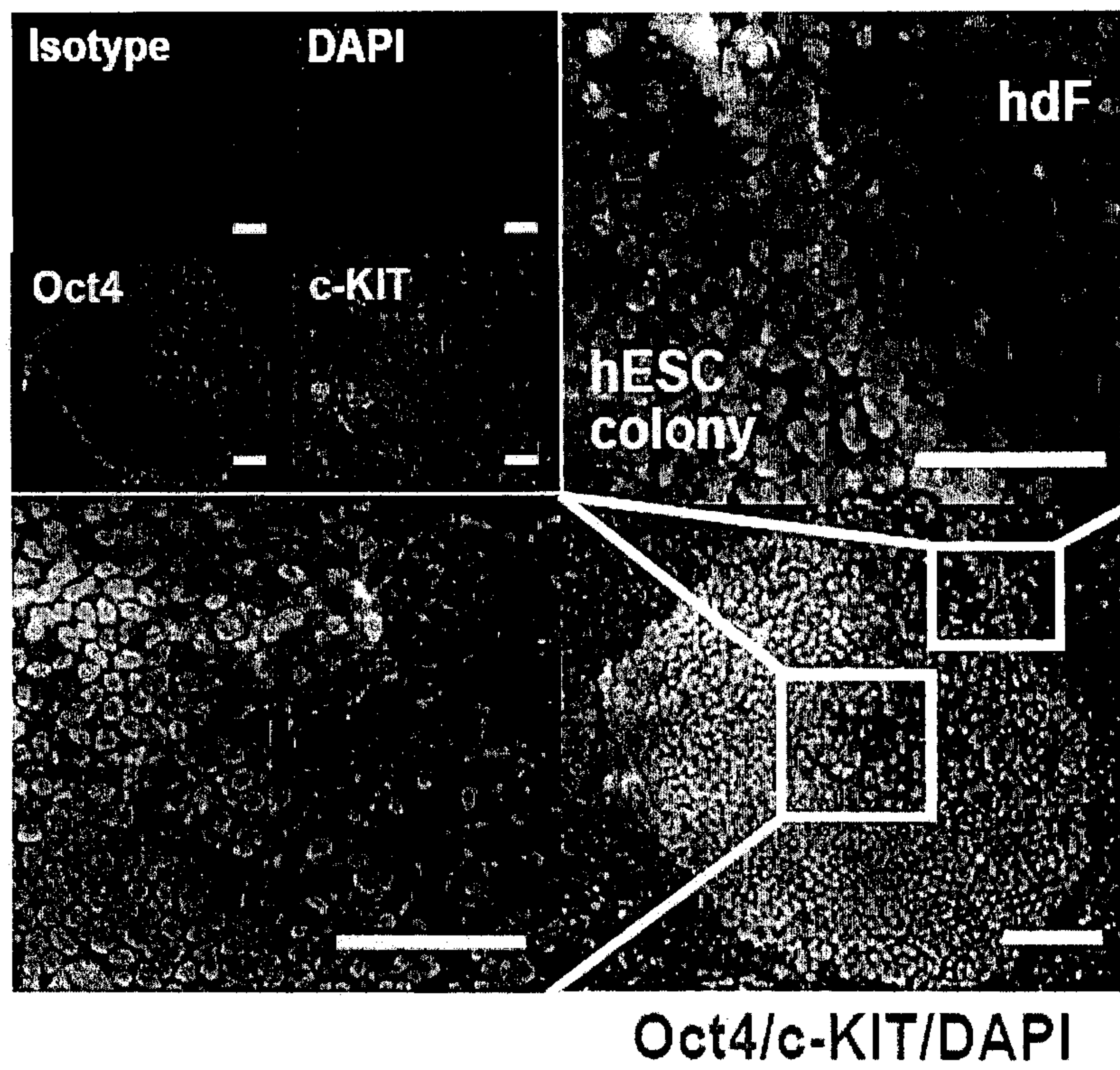
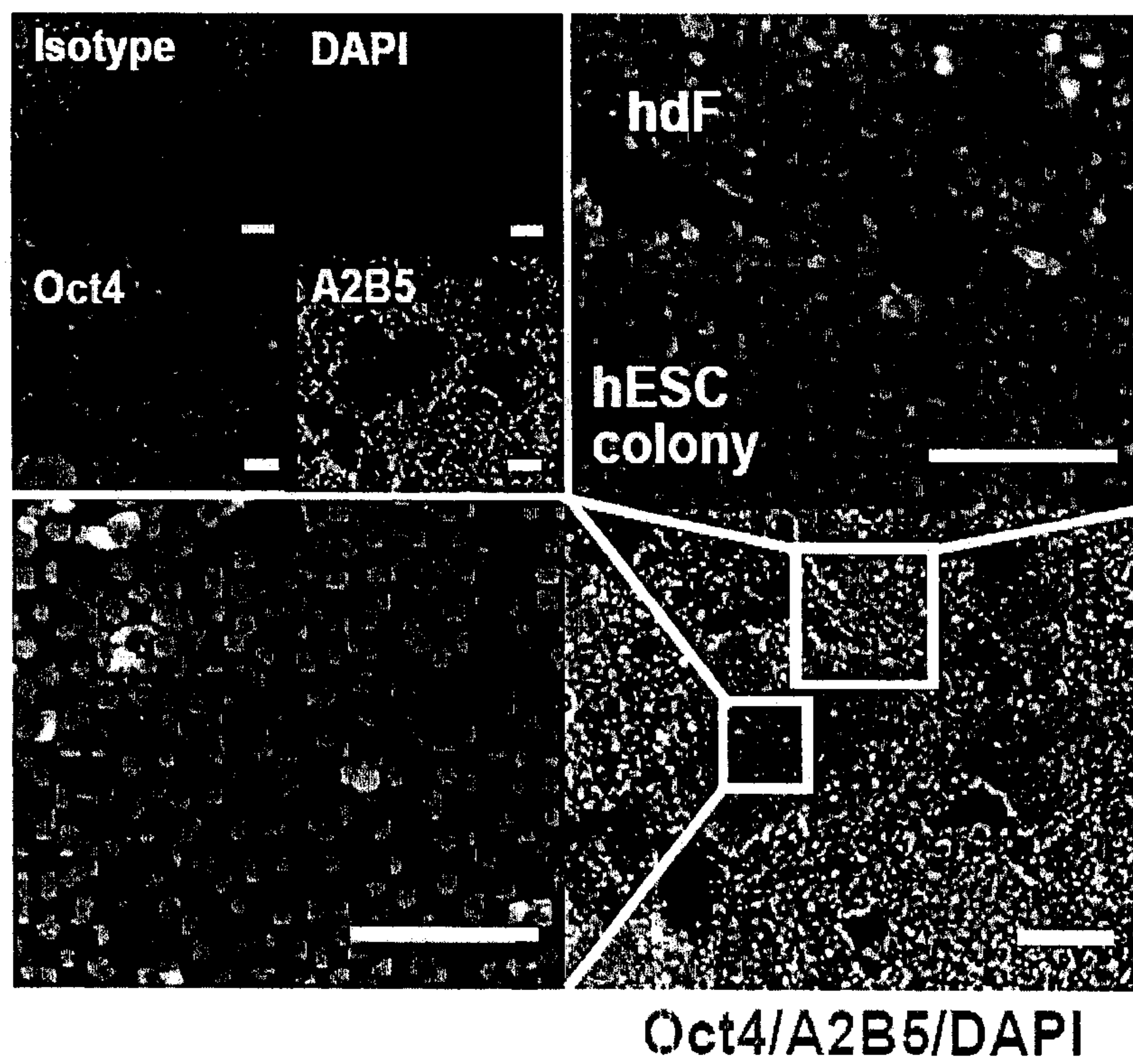
a**b**

Fig. 1

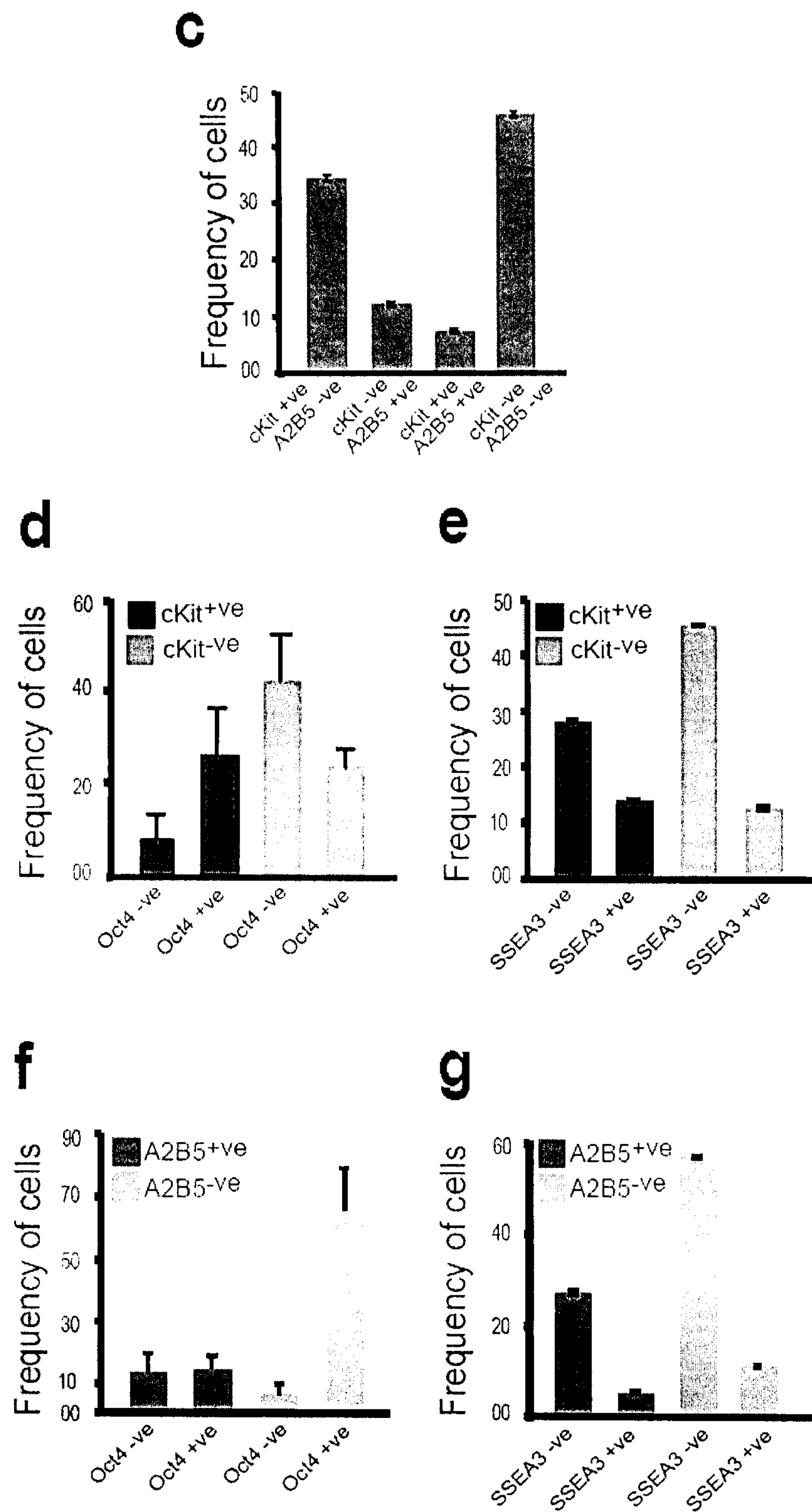


Fig. 1 (cont.)

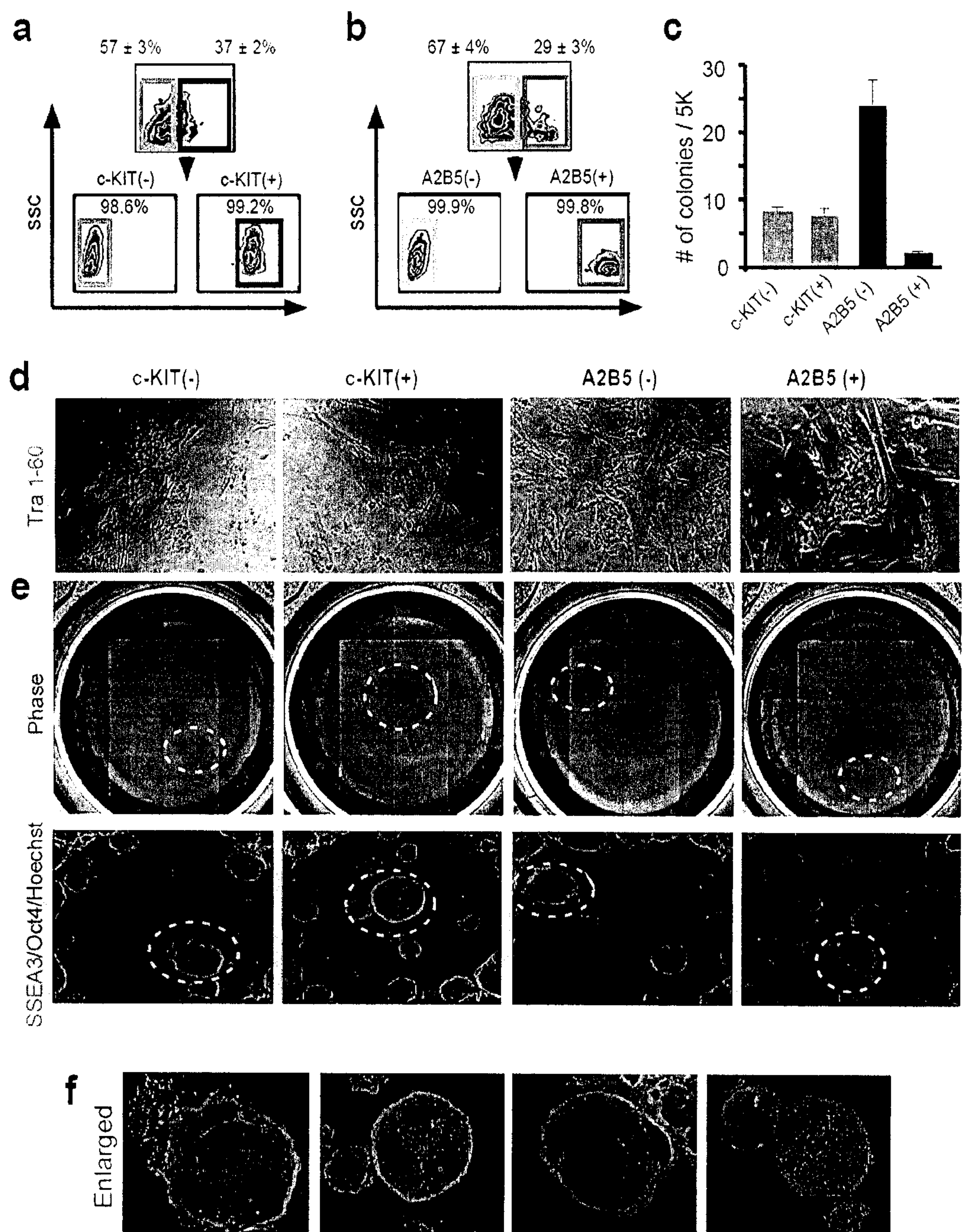


Fig. 2

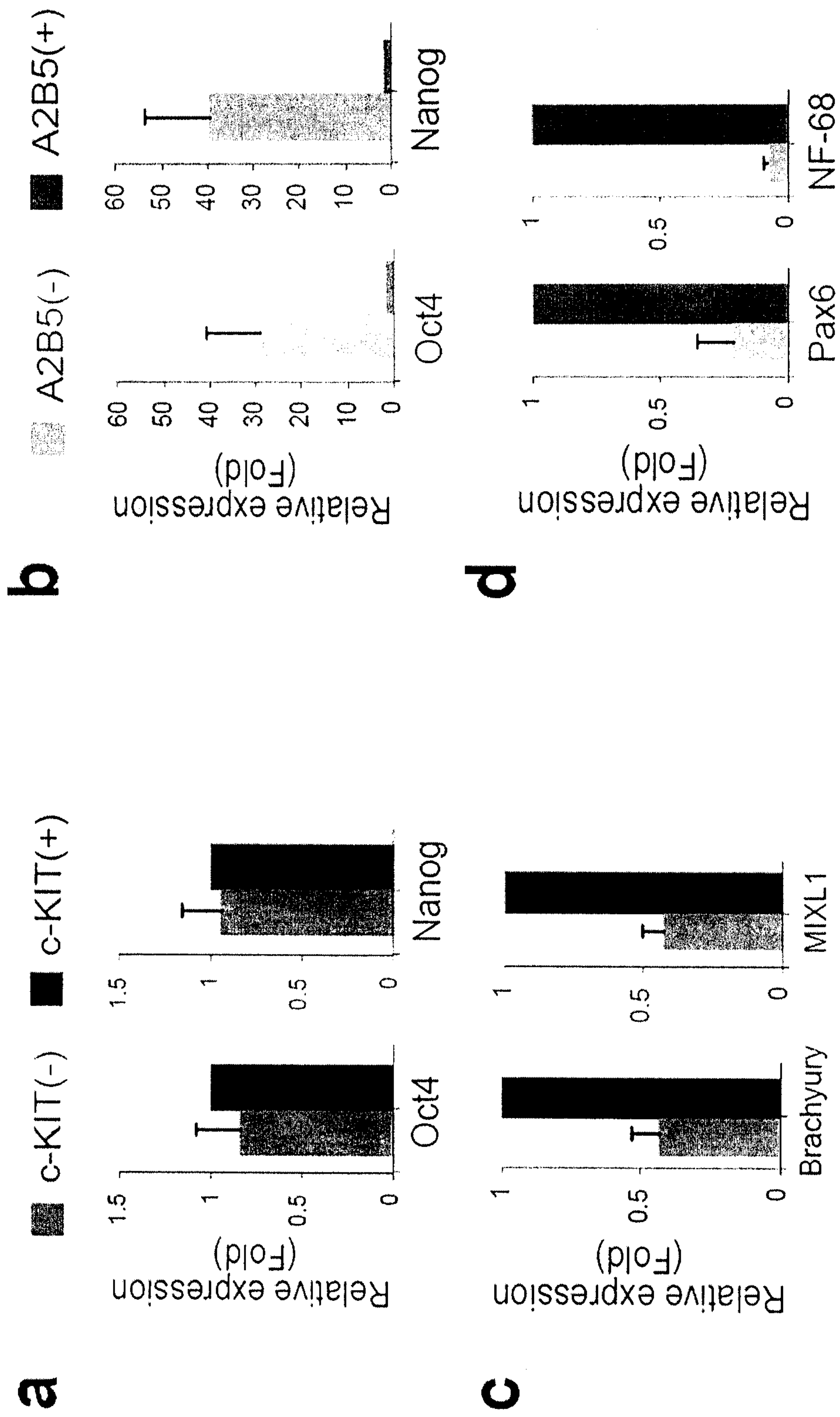


Fig.3

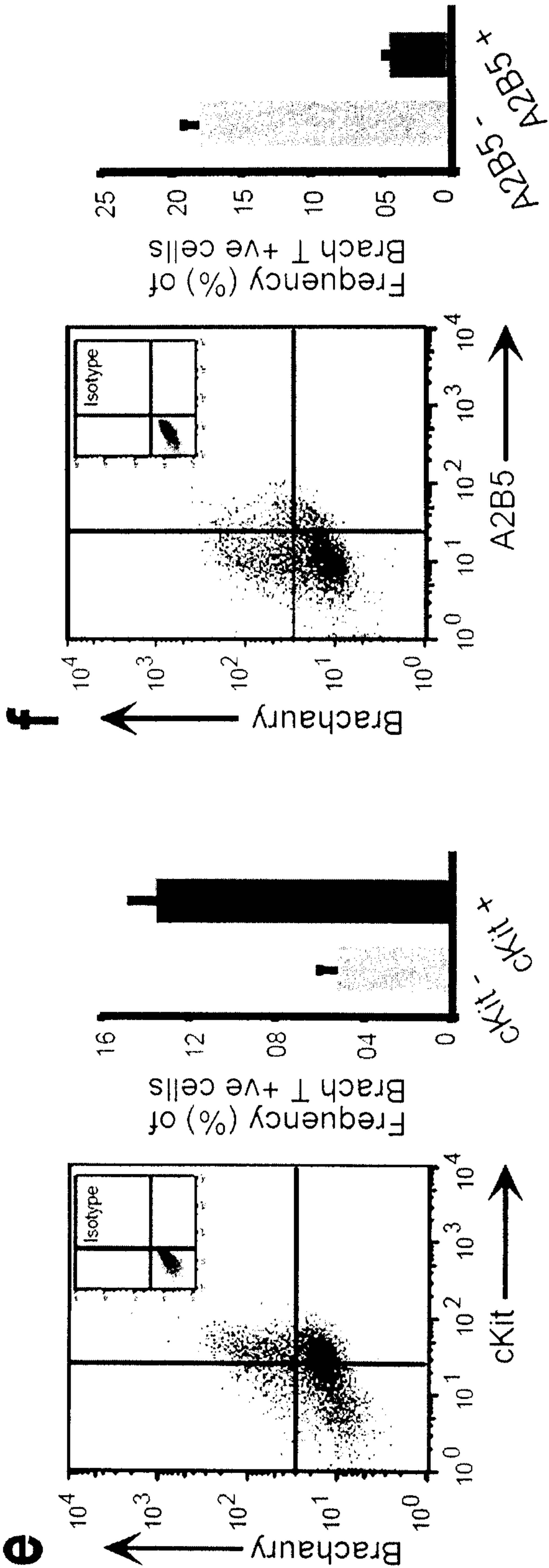


Fig. 3 (cont.)

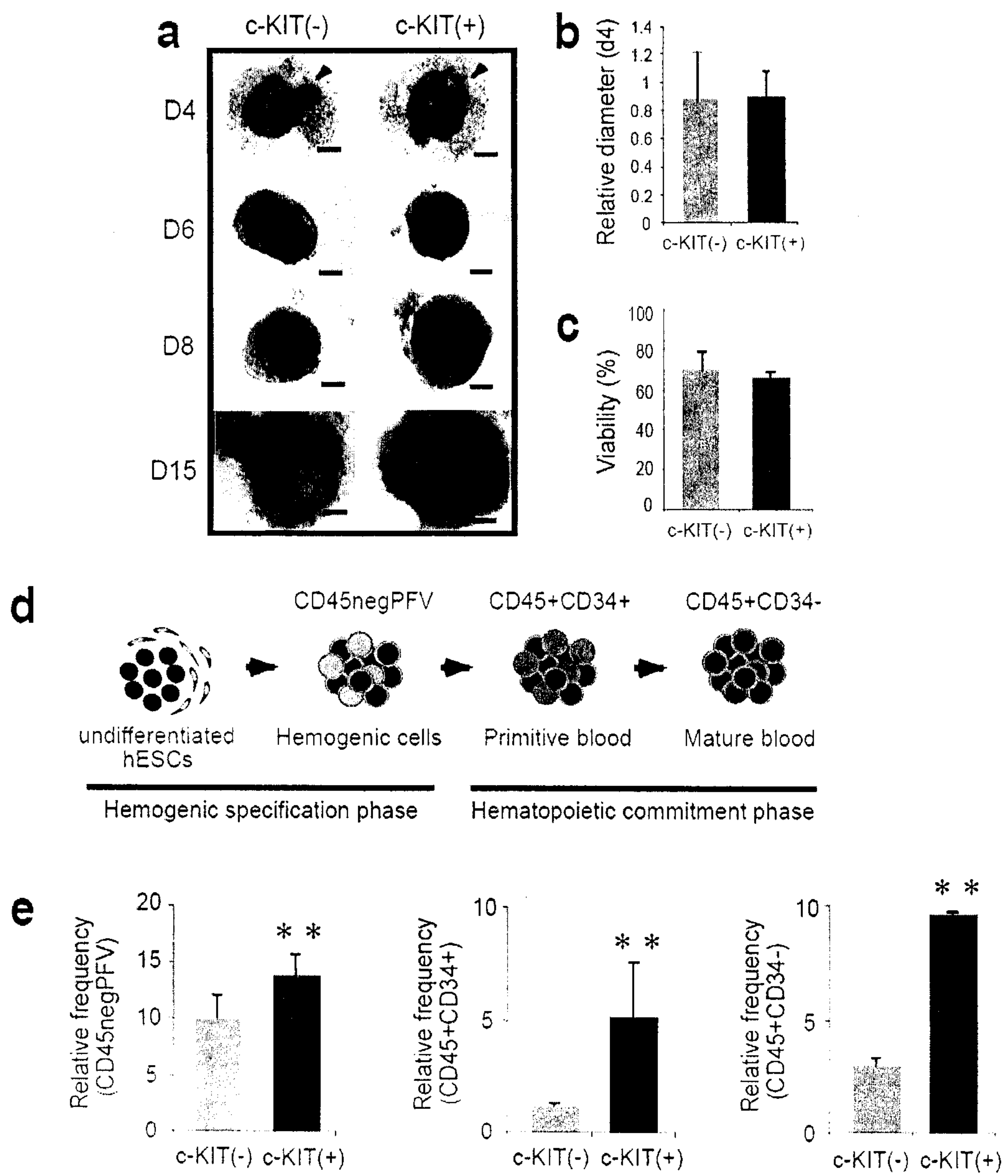


Fig. 4

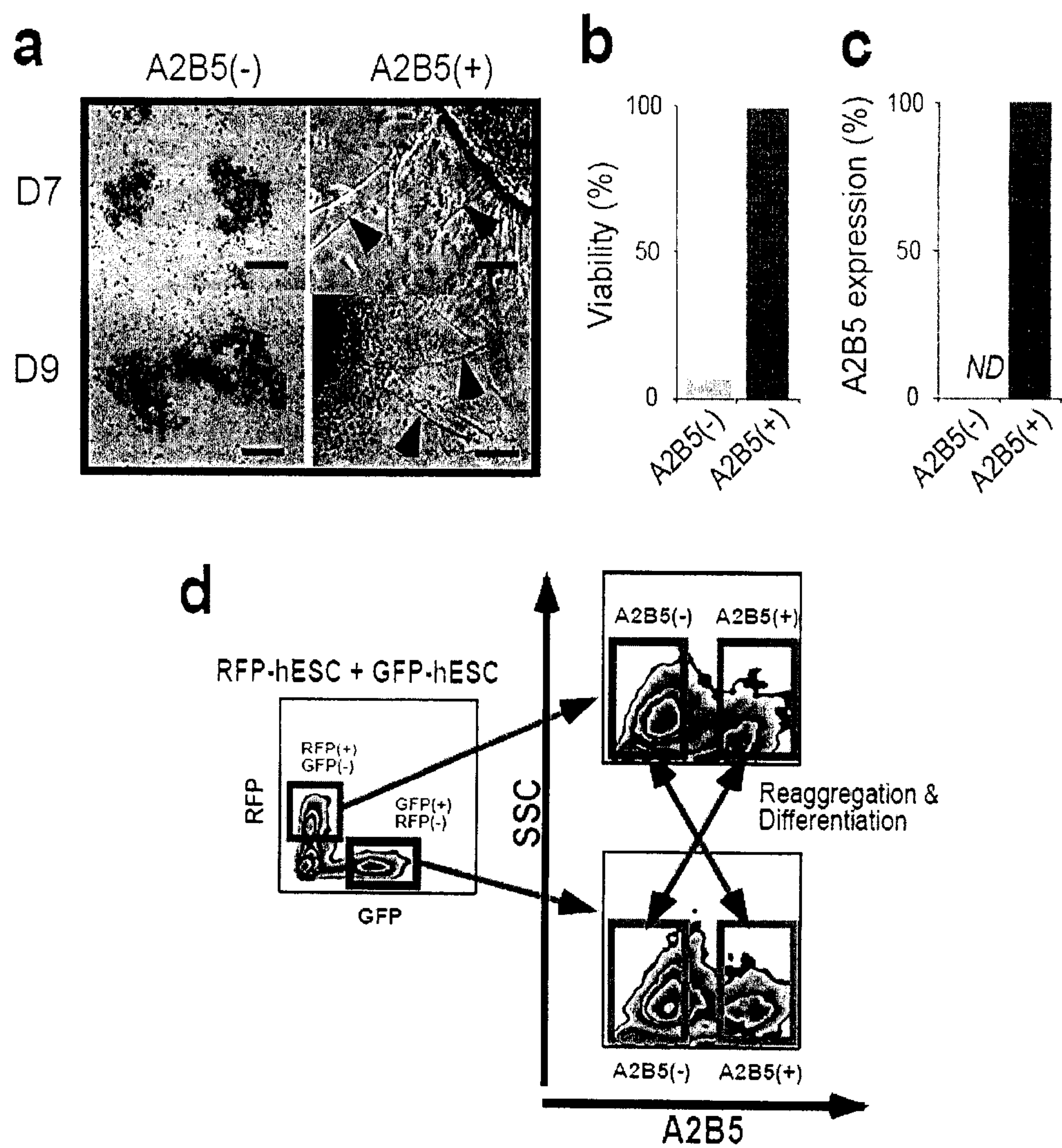


Fig. 5

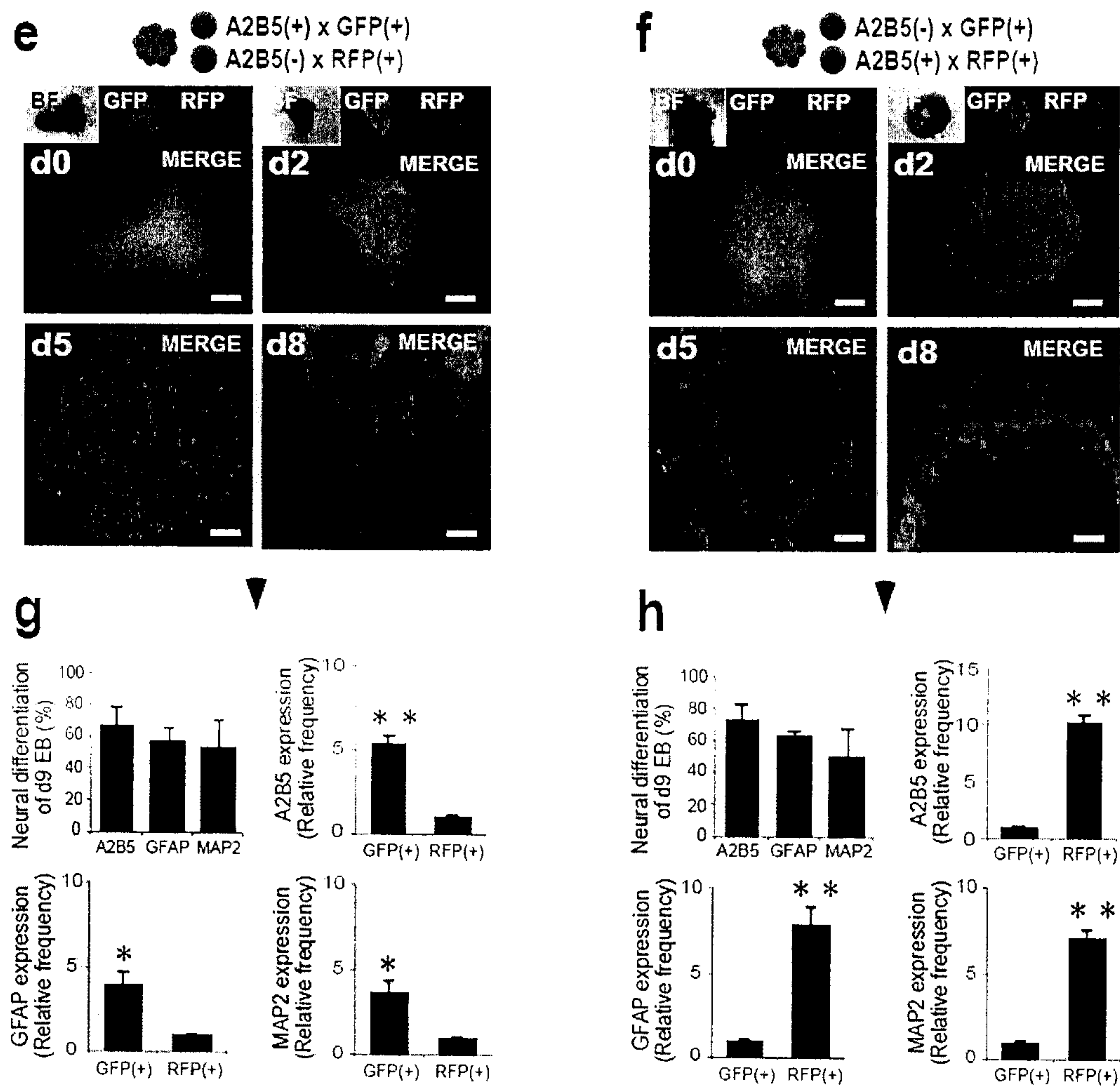


Fig. 5 (cont.)

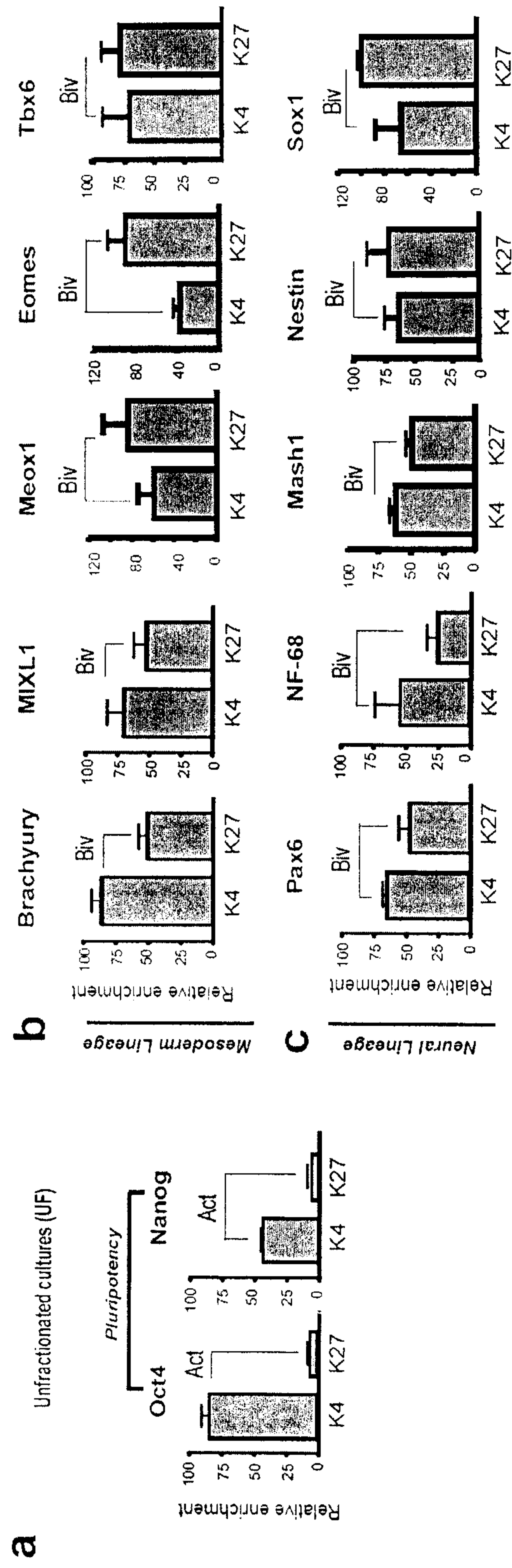


Fig. 6



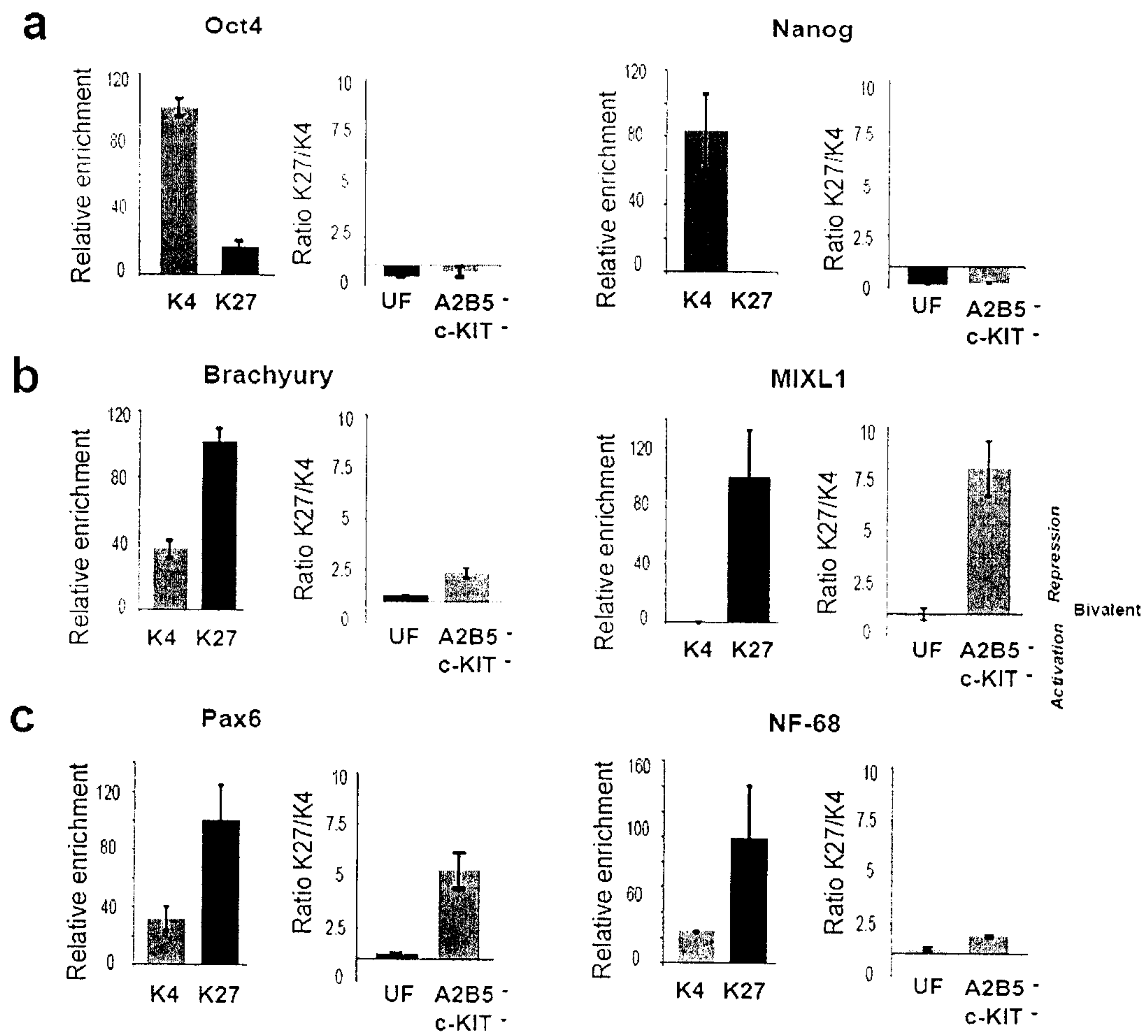


Fig. 7

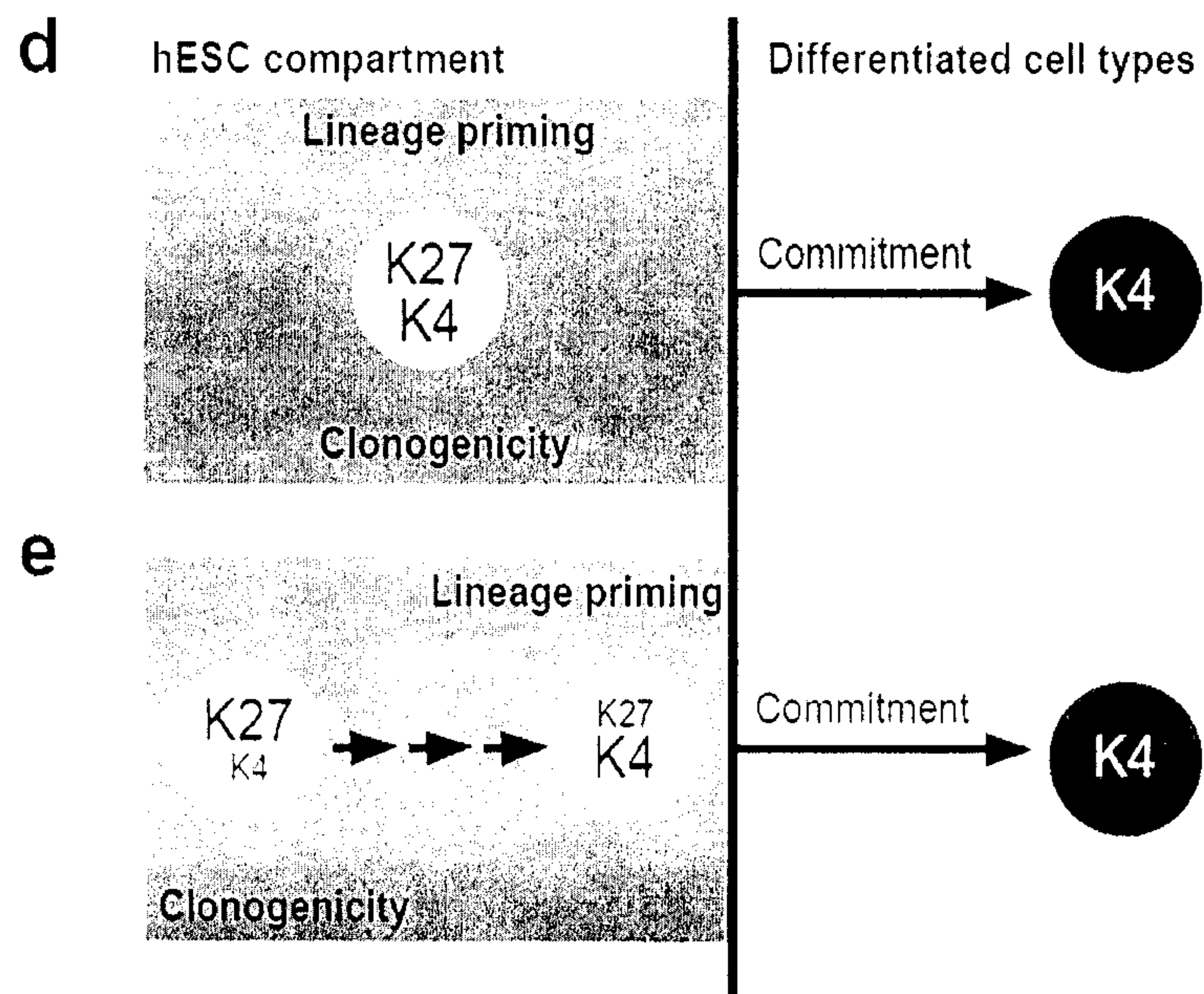


Fig. 7 (cont.)

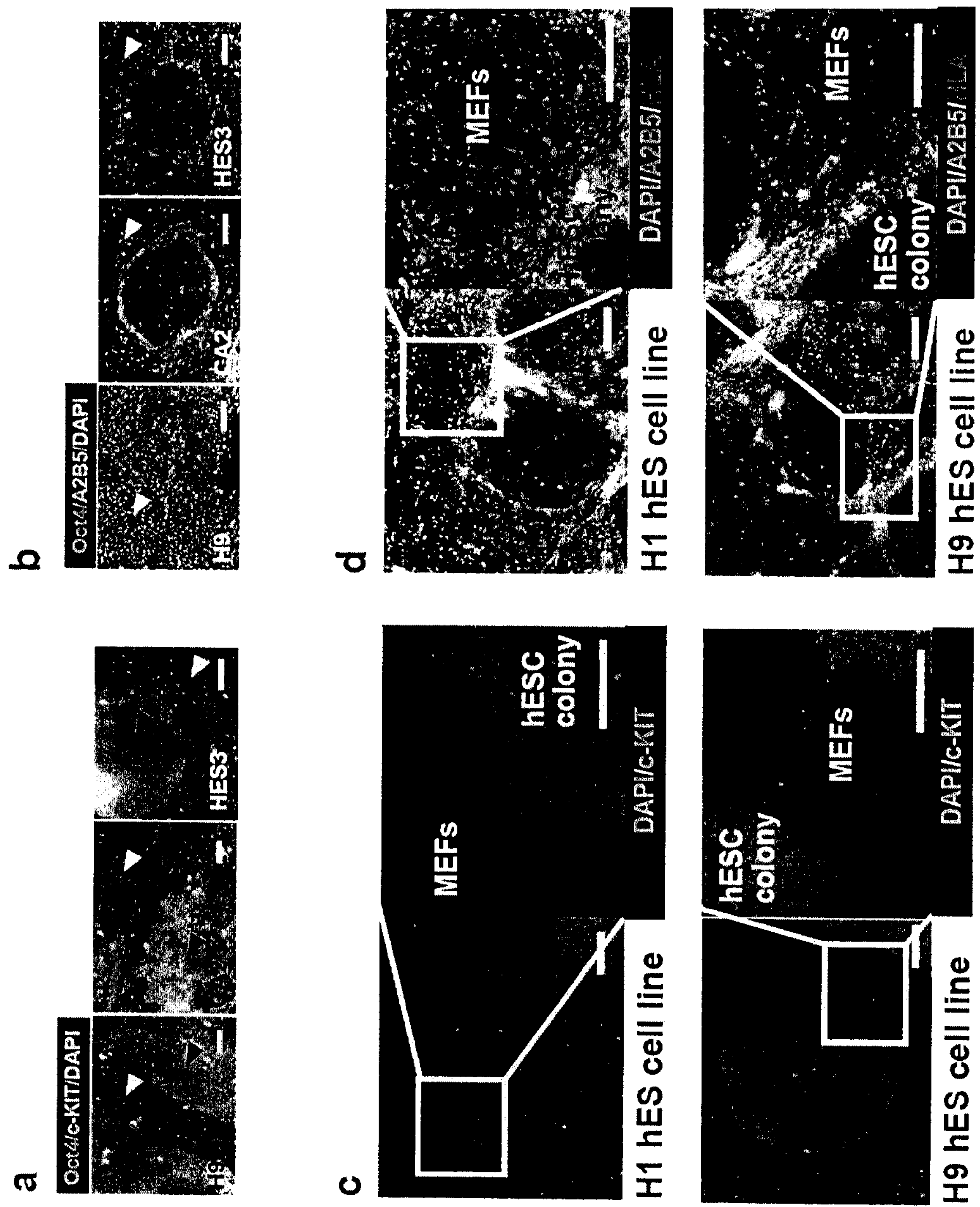


Fig. 8

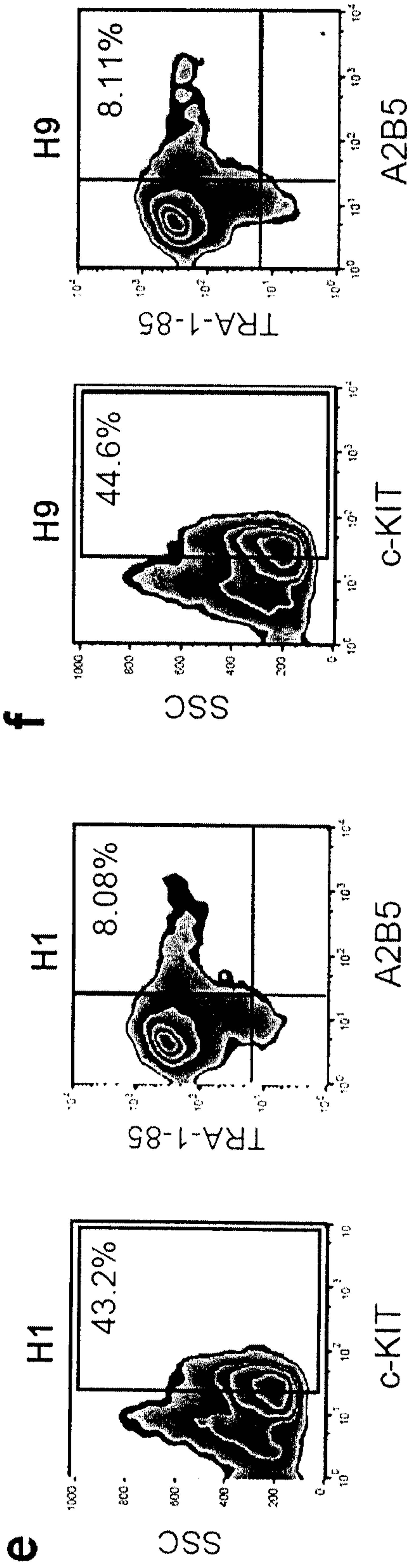


Fig. 8 (cont.)

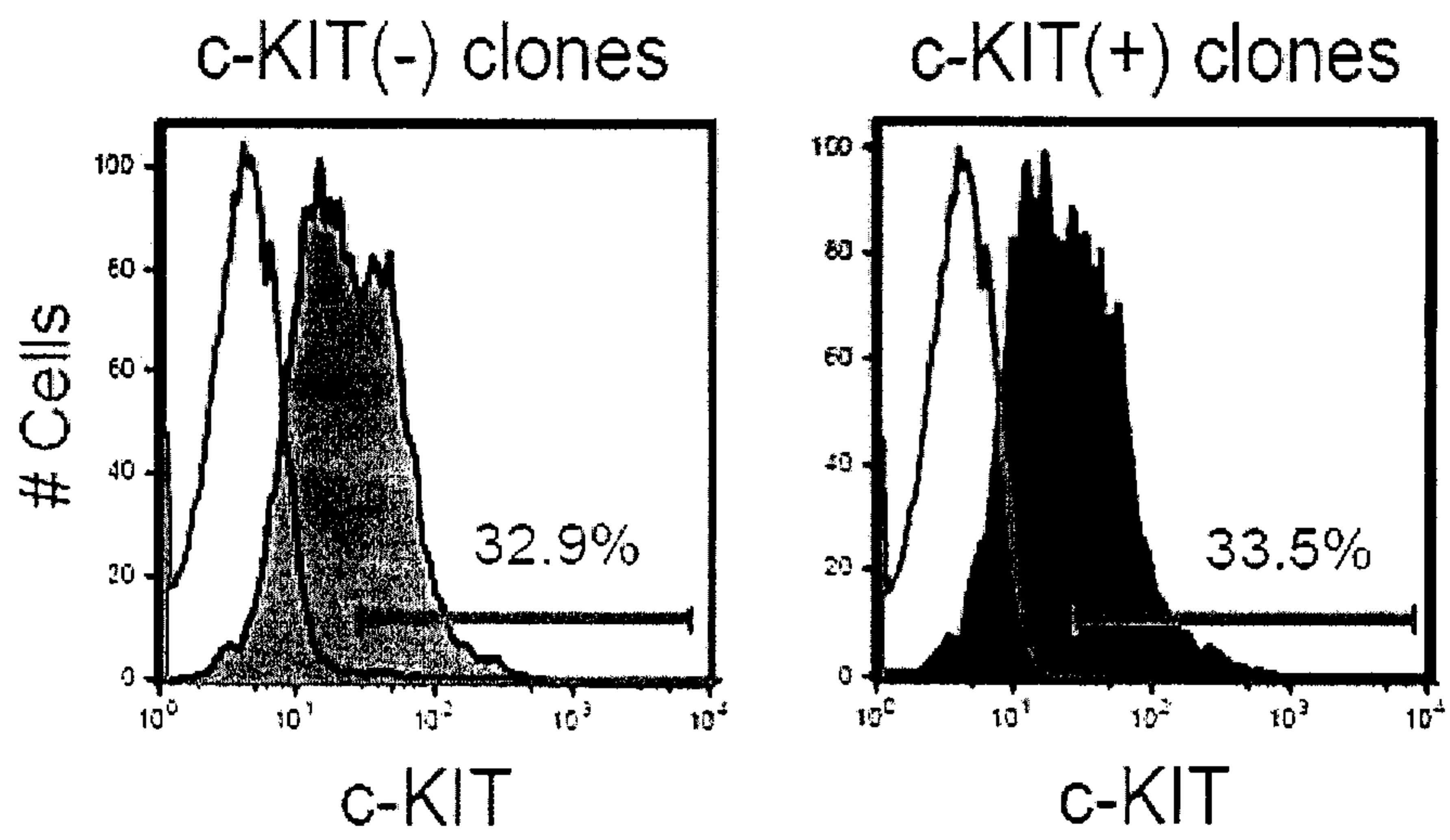
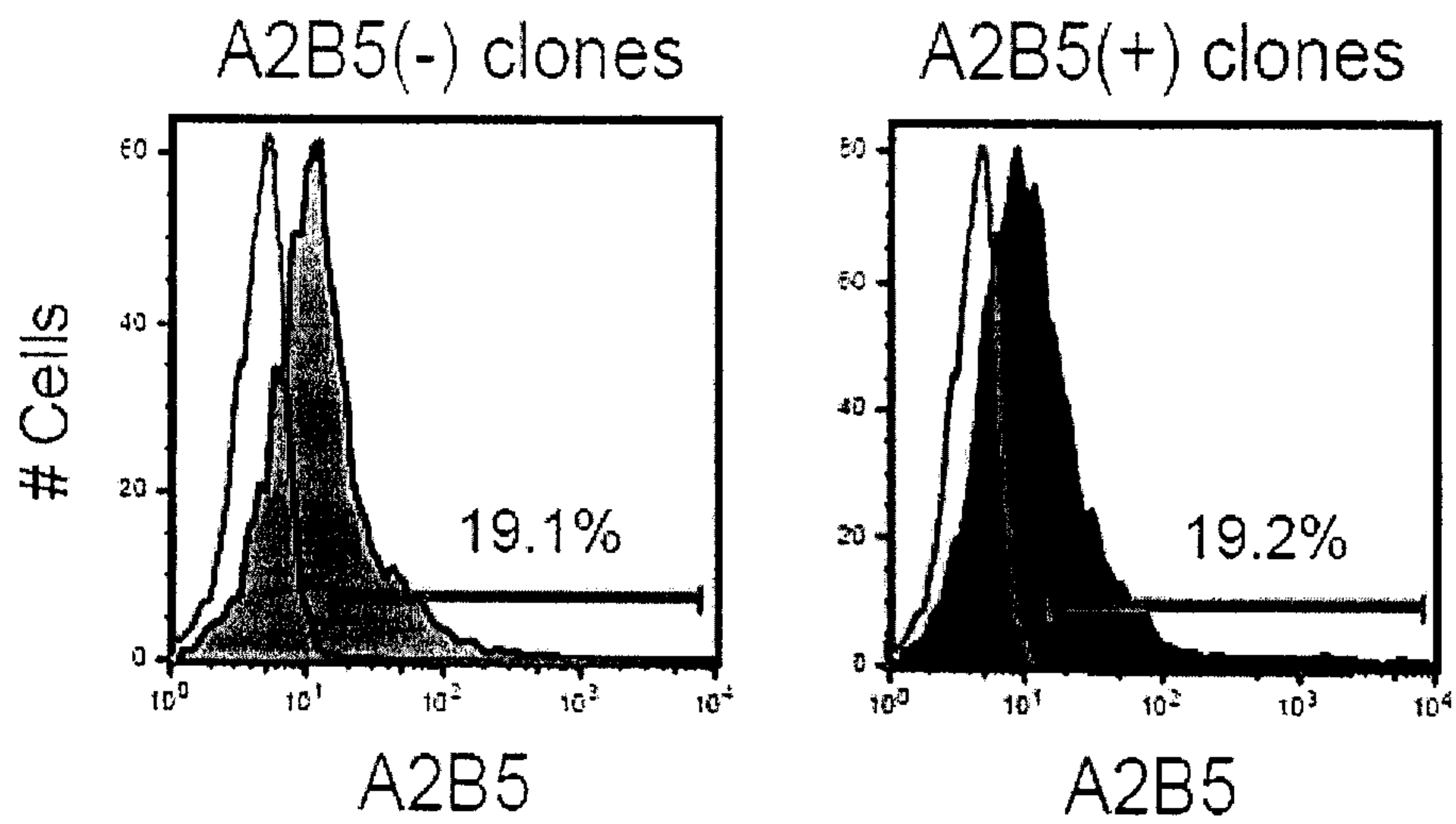
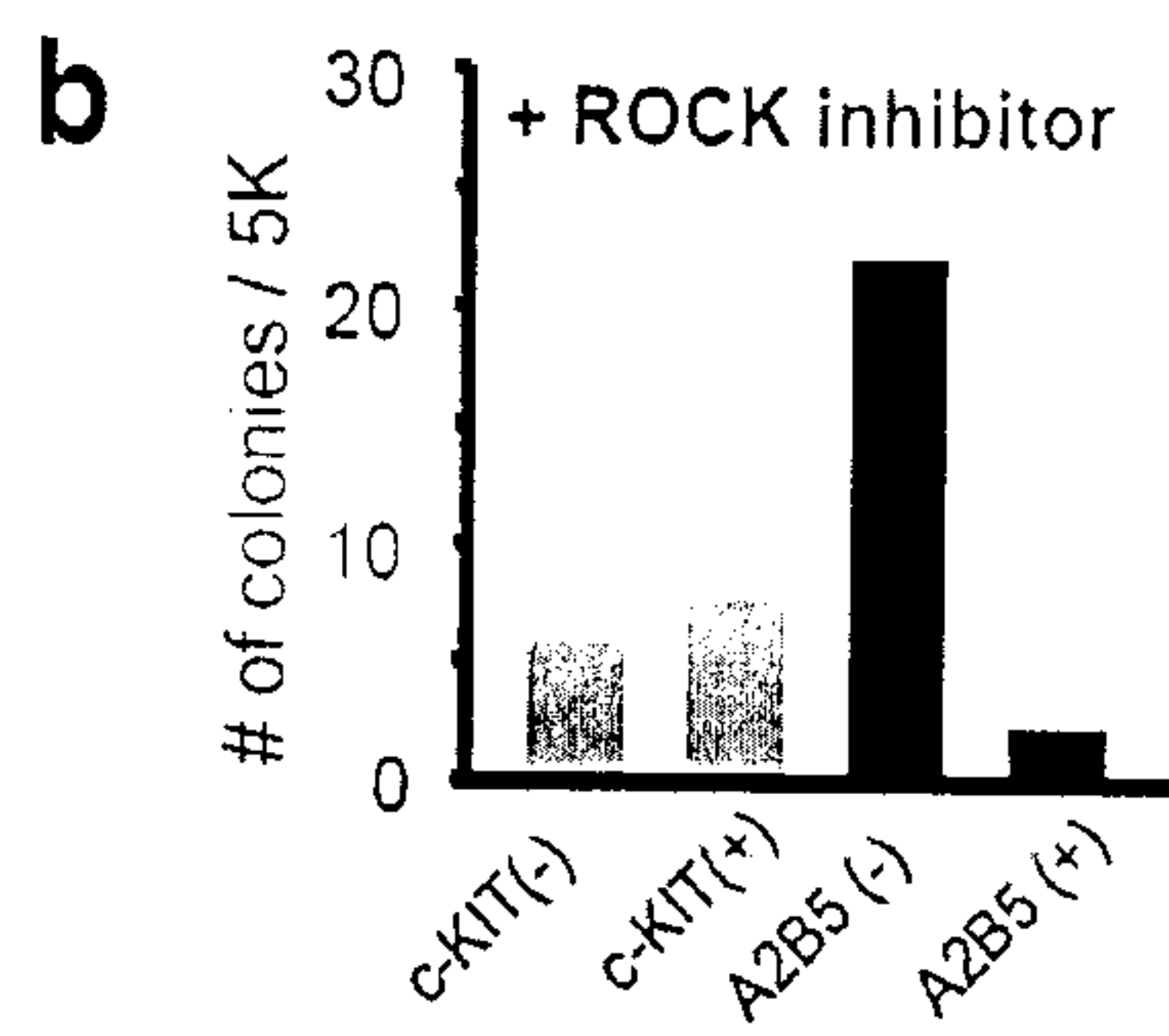
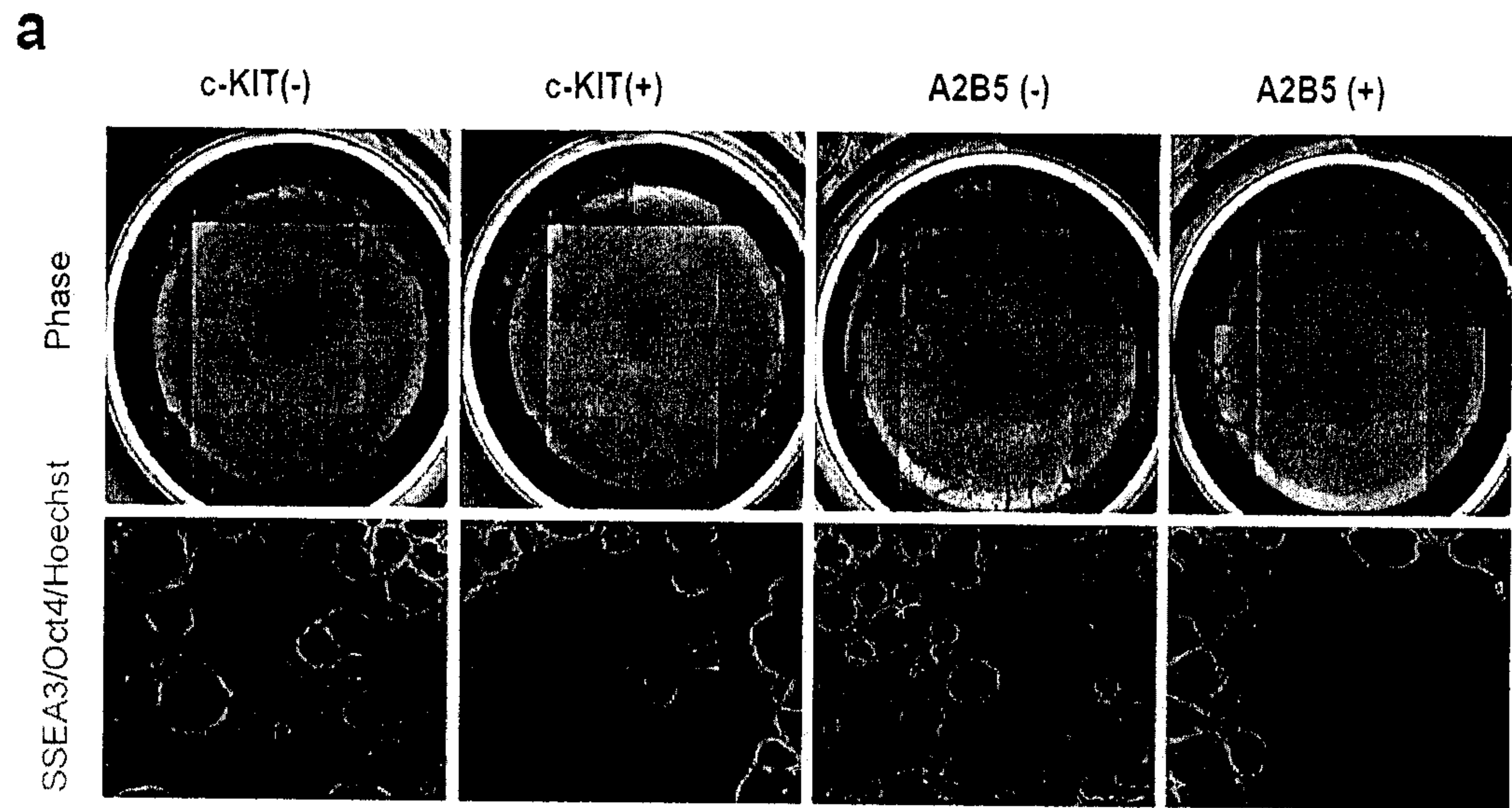
a**b**

Fig. 9

+ ROCK inhibitor**Fig. 10**

a	P1	P2	P3	P4
ENZYME	Coll IV	Coll IV	Coll IV	TrypLE
SORT				
Cells	Live cells	Live cells	Live cells	Live cells
Efficiency	73.5%	55.0%	86.3%	89.0%
Solution	3%FCS PBS	3%FCS PBS	3%FCS PBS	3%FCS EBM
EB FORMATION				
Cell No.	80K	80K	80K	80K
EB No.	14	8	7	14
Tool	CT	CT	CT	U-96W
Medium	EBM+hGFs	EBM+hGFs	EBM+hGFs	EBM+hGFs
Additive	NA	BSA	Matrigel	Matrigel
Spin	450 g/5 min	450 g/5 min	450 g/5 min	450 g/5 min
HEMATOPOIETIC DIFFERENTIATION				
Viability	30.9%	3.73%	58%	89.9% (92.6%)
CD45negPFV	0%	0%	0%	11.3% (8.12%)
CD45+CD34+	0.43%	0%	0%	3.76% (4.72%)
CD45+CD34-	0.29%	0%	0.08%	4.51% (5.39%)

Coll IV, Collagenase IV; TrypLE, Trypsin-like enzyme; PBS, Phosphate buffered saline; EBM, EB differentiation medium; hGFs, Hematopoietic growth factors; NA, No addition; FCS, Fetal calf serum; BSA, Bovine serum albumin; CT, Conical tube (1.5 ml effendorf centrifuge tube); U-96W, U-bottom low binding 96-well plate.
(). Unsorted cells (100K/CT/EBM+hGFs/Matrigel/450 g, 5 min)

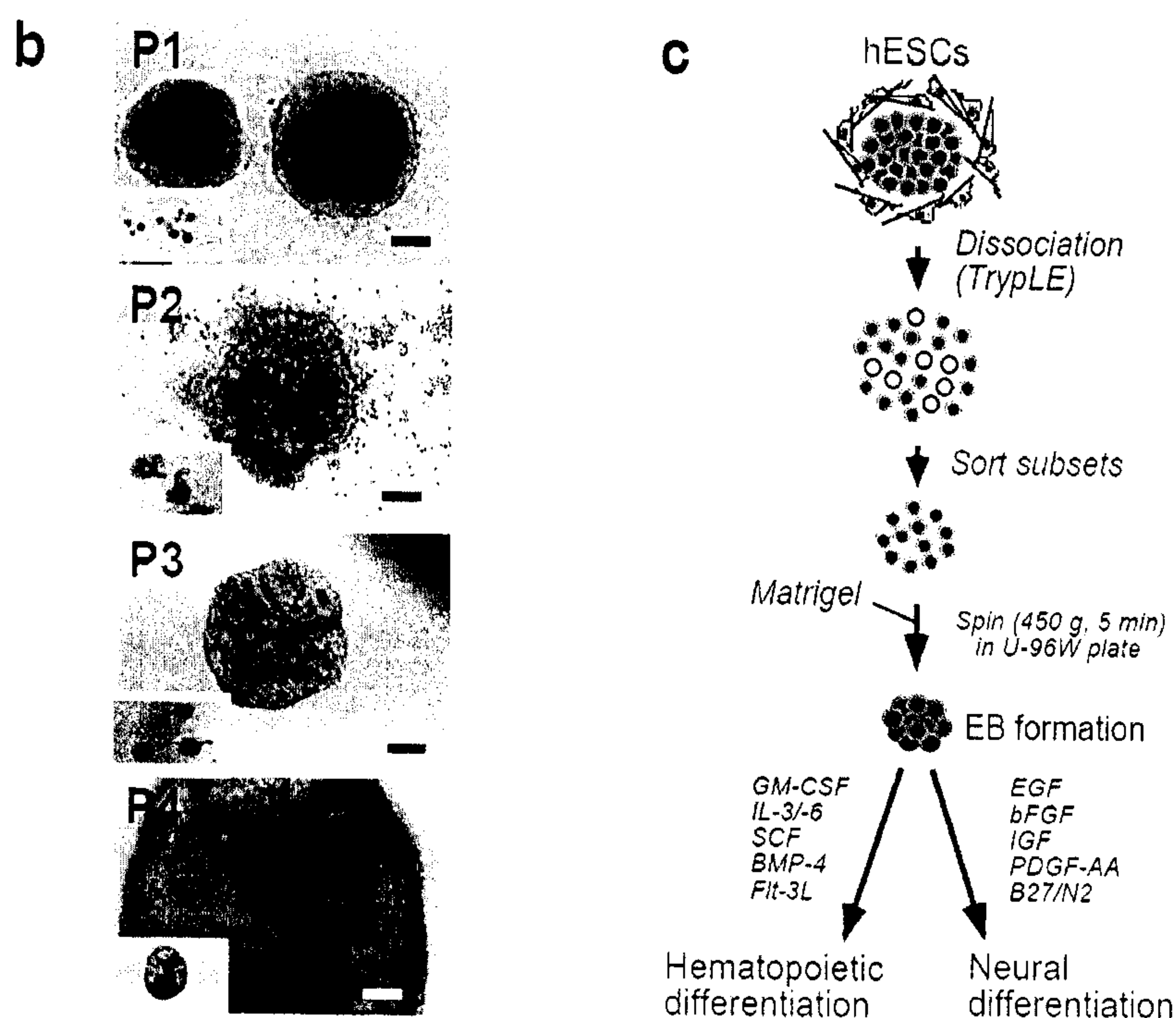


Fig. 11

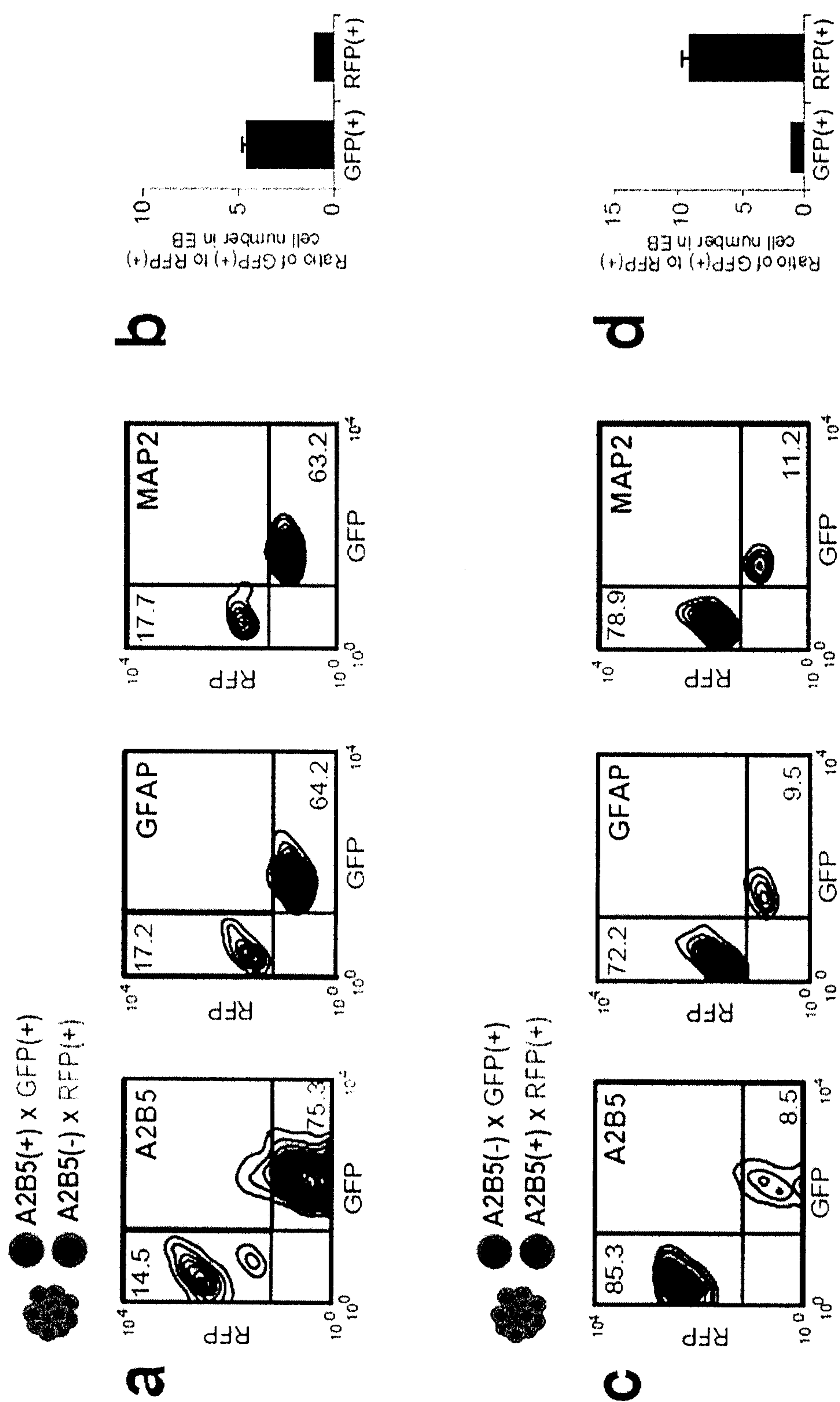


Fig. 12

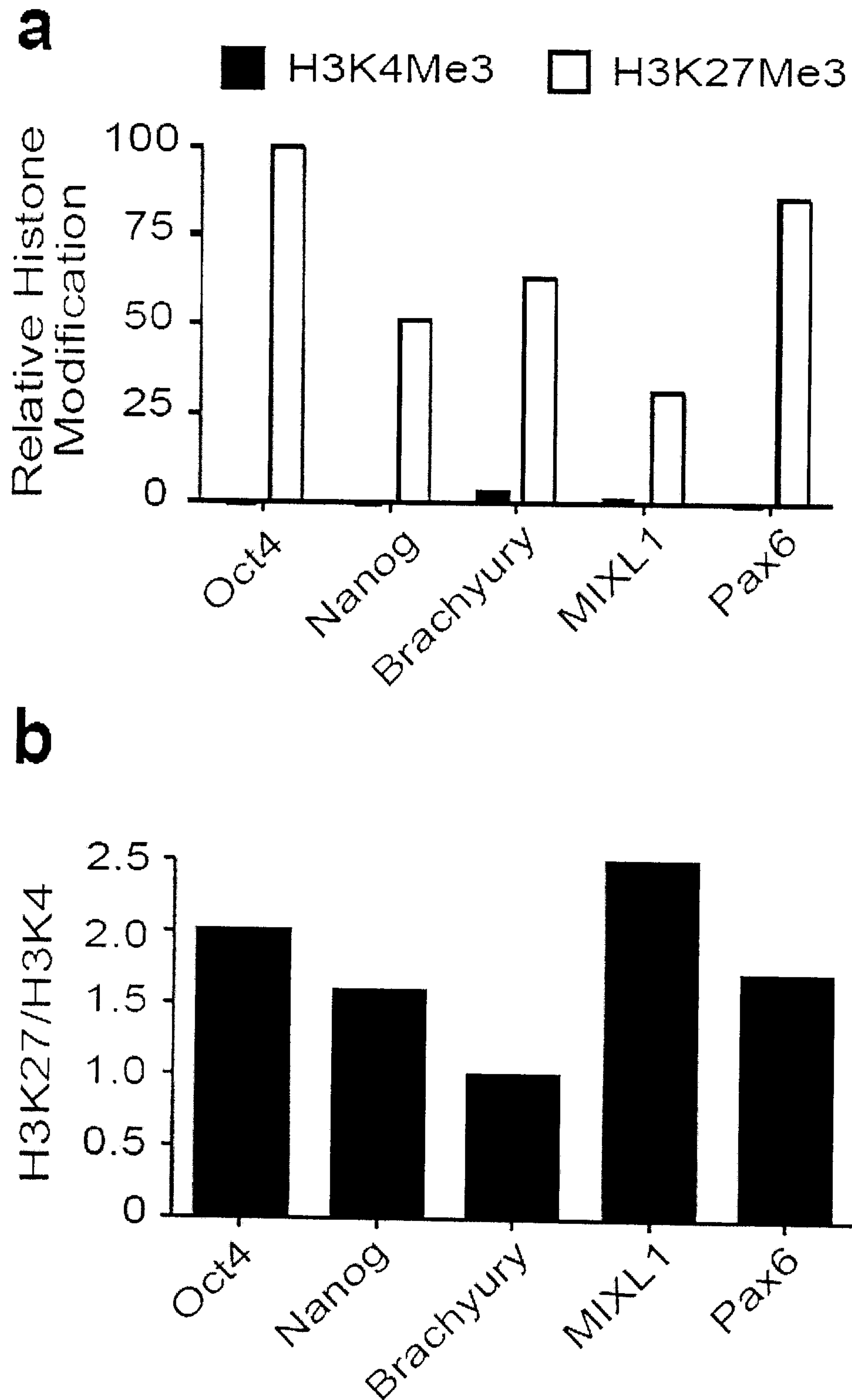


Fig. 13

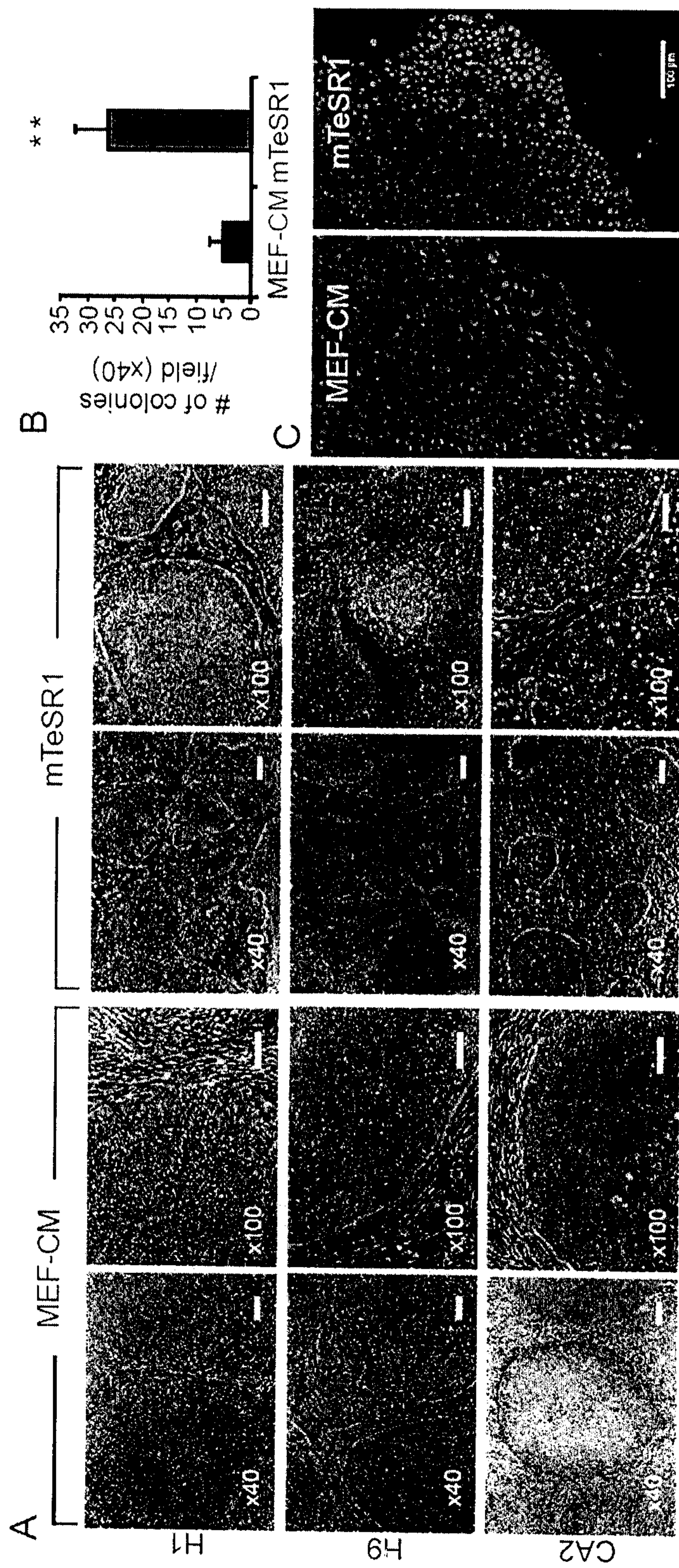


Fig. 14

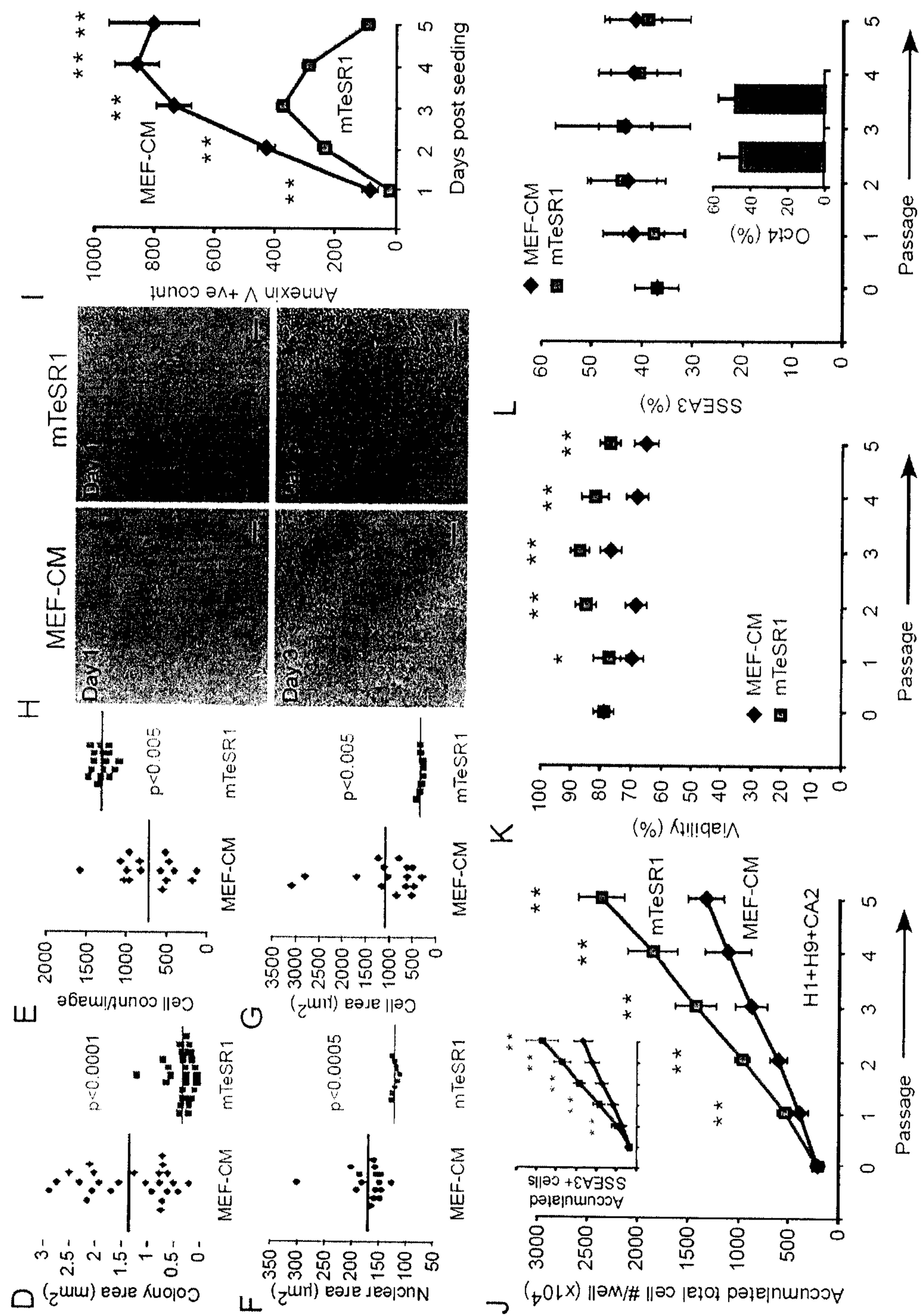


Fig. 14 (cont.)

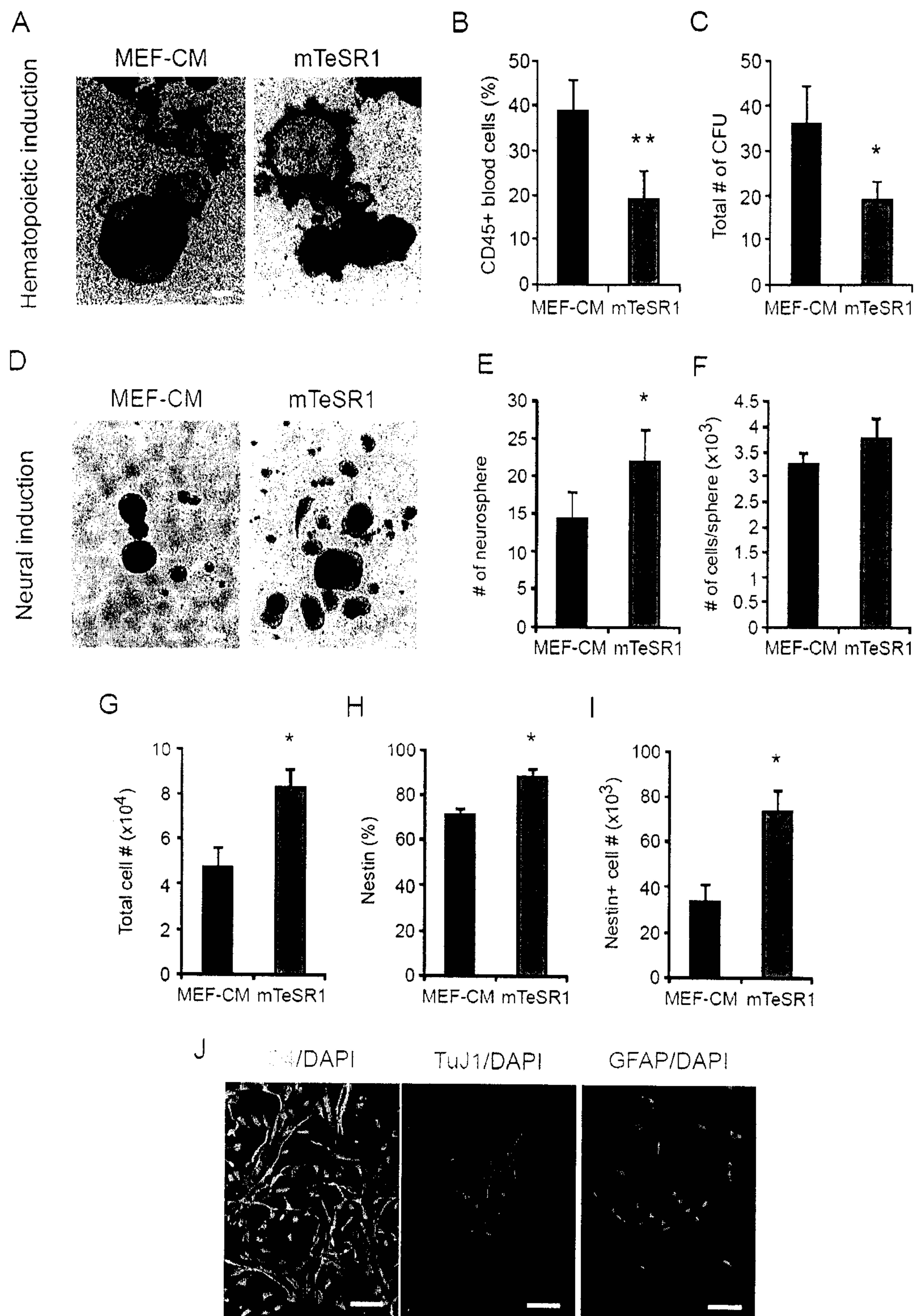


Fig. 15

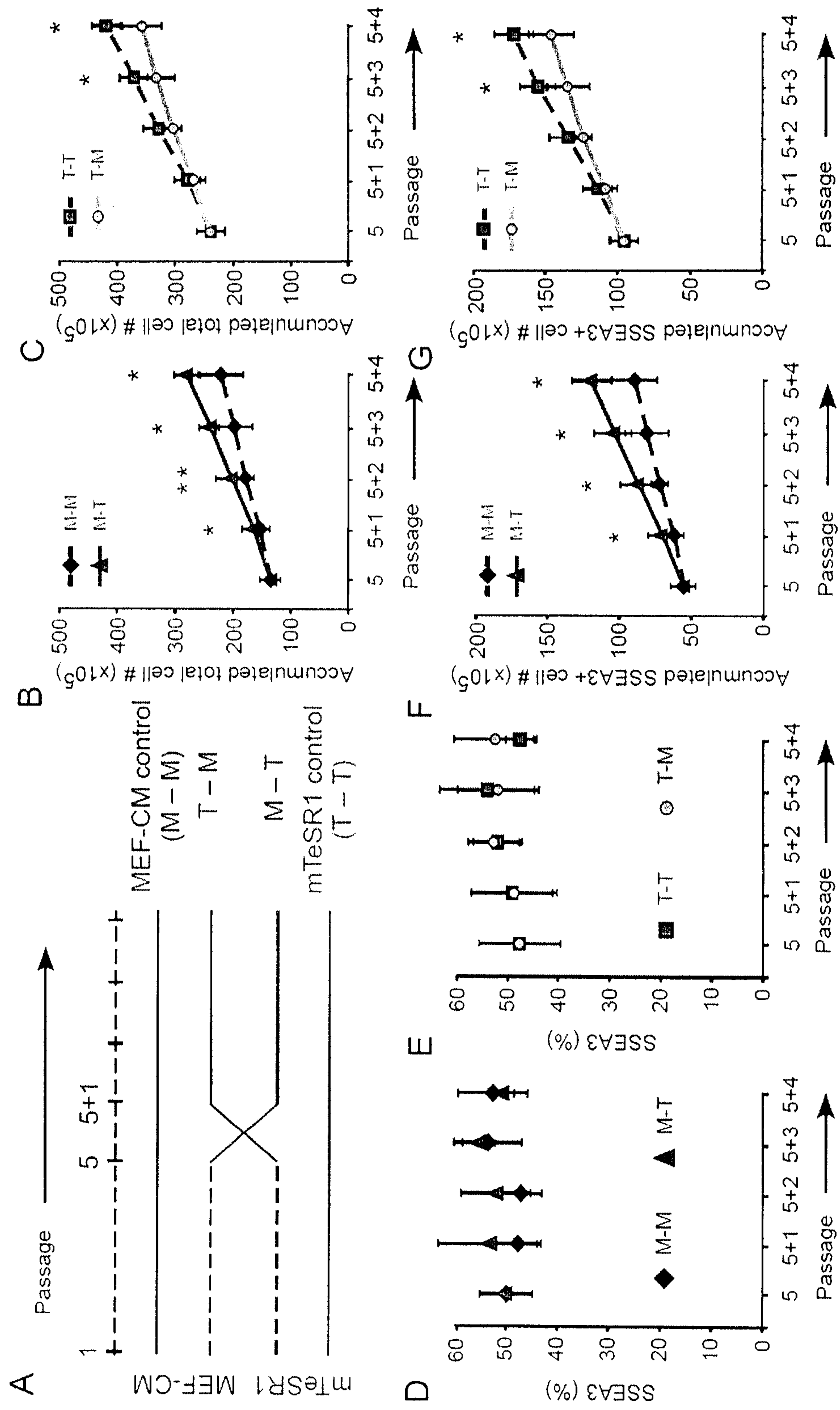


Fig. 16

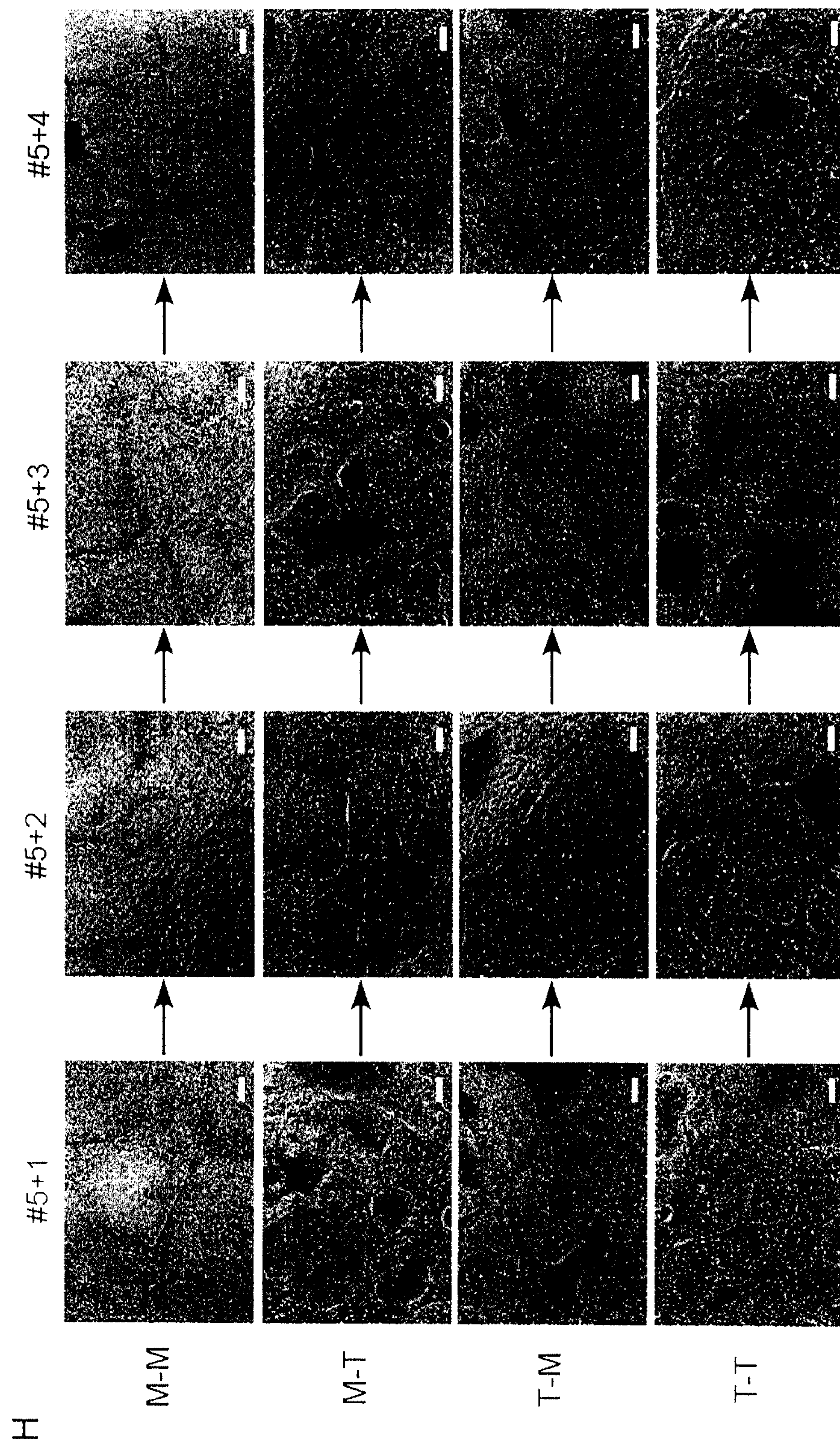


Fig. 16 (cont.)

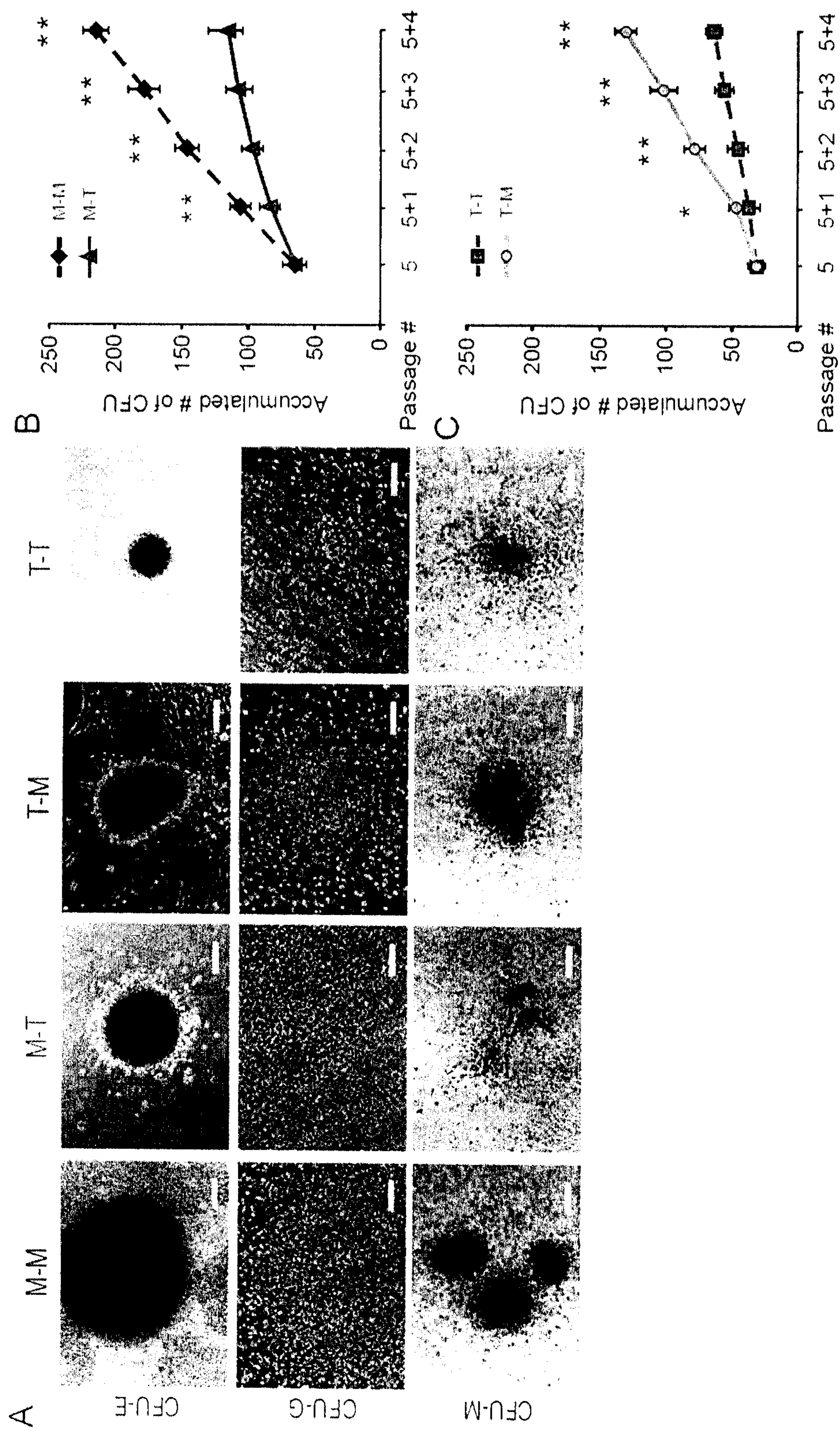


Fig. 17

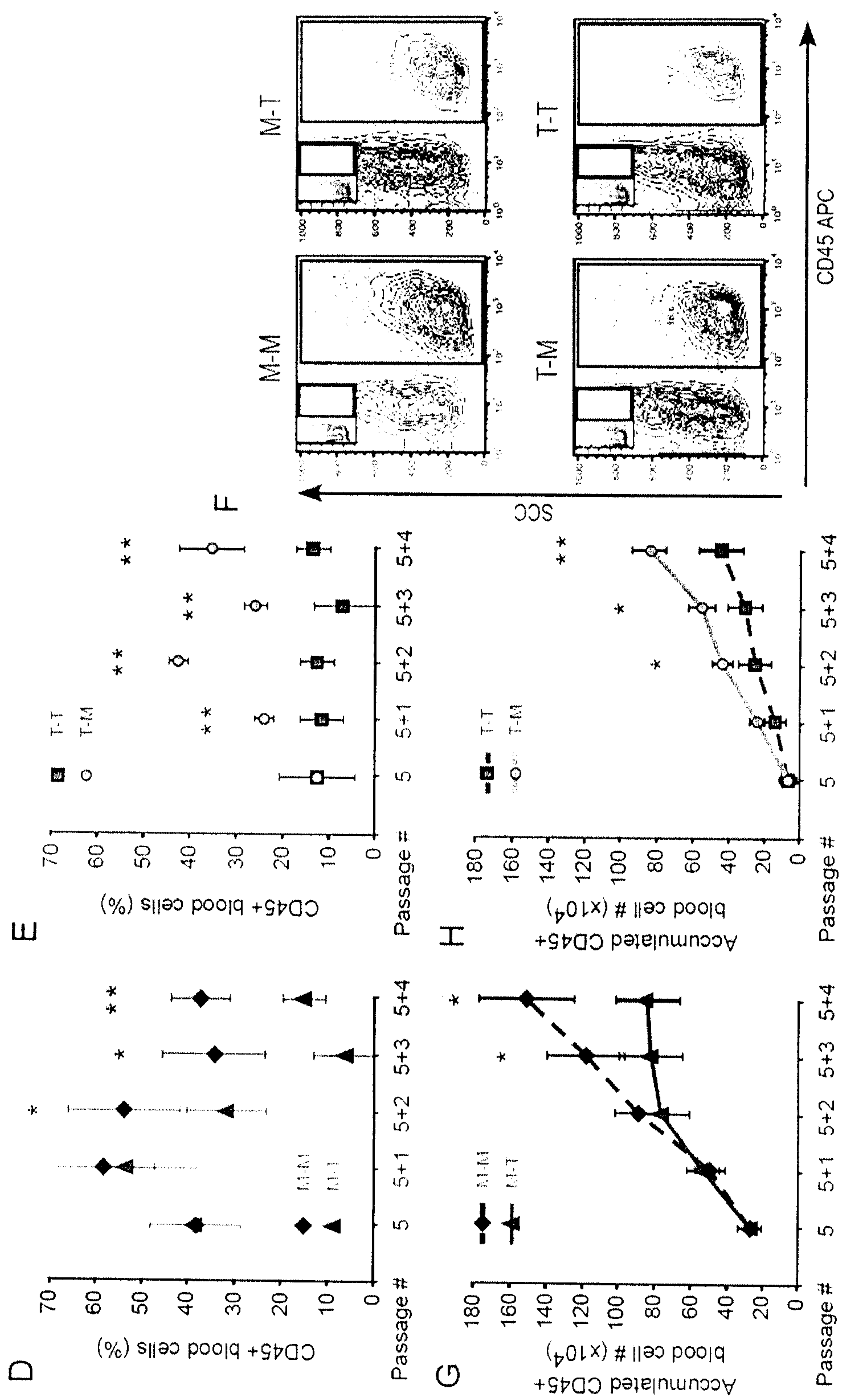
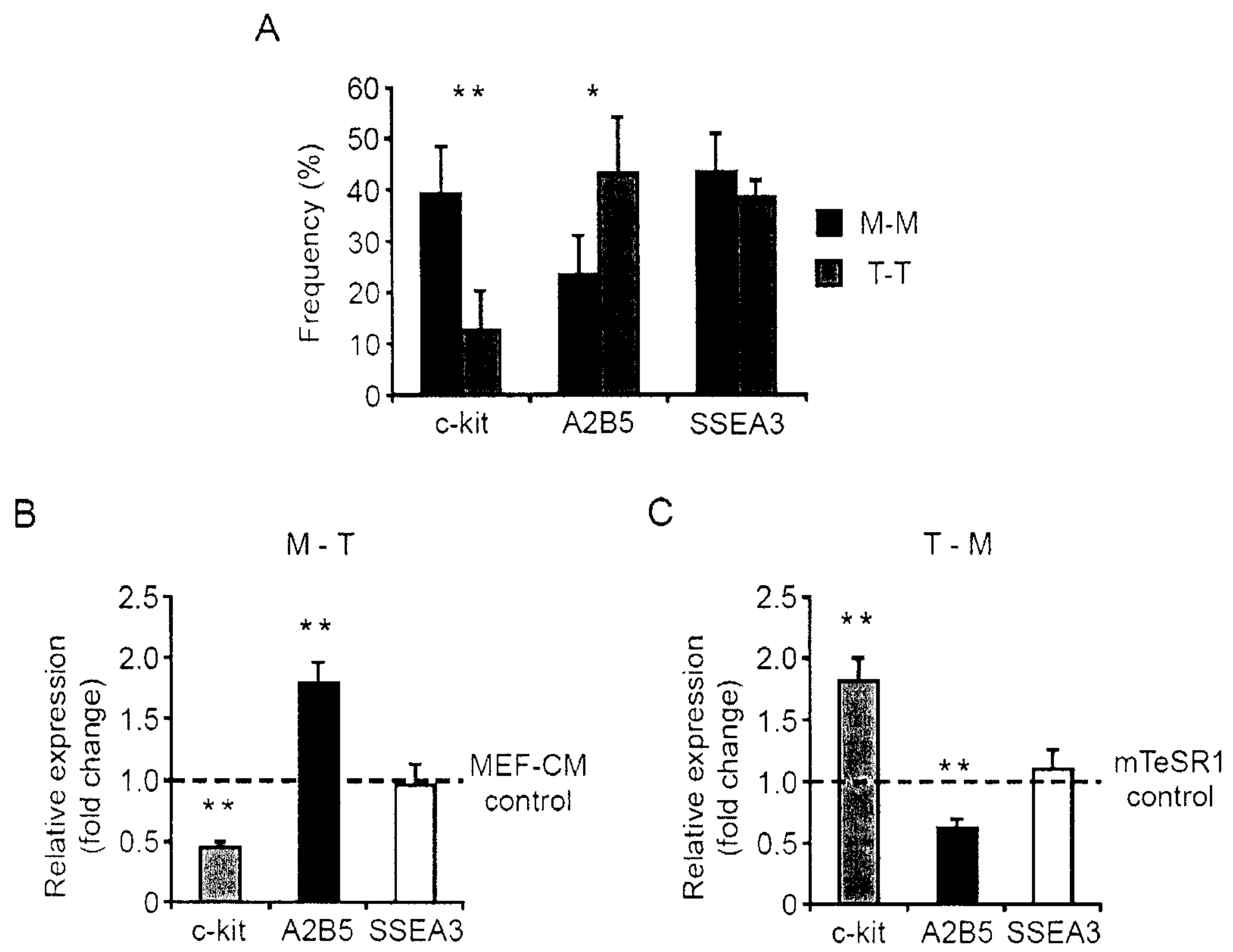
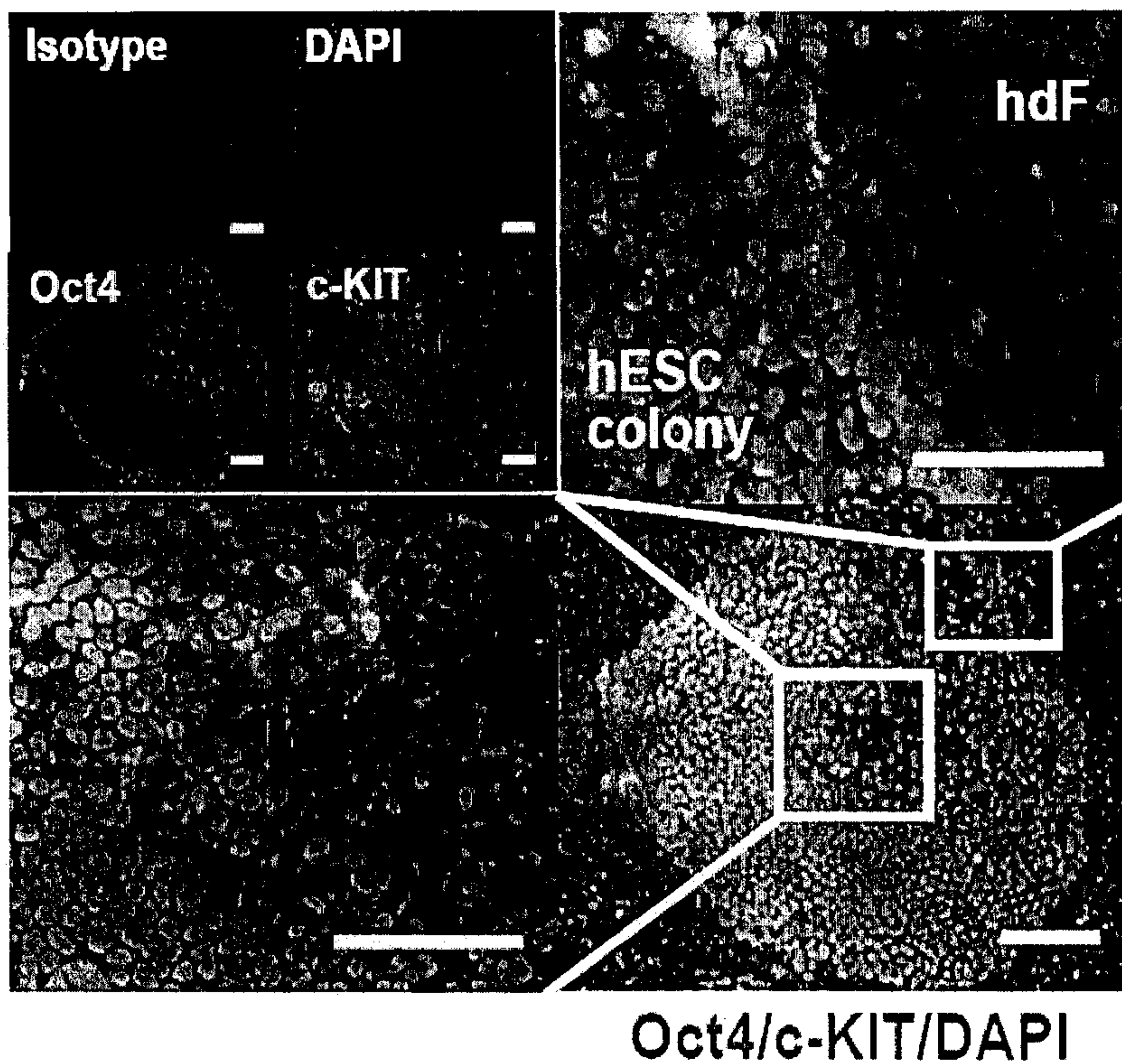


Fig. 17 (cont.)

**Fig. 18**

a



b

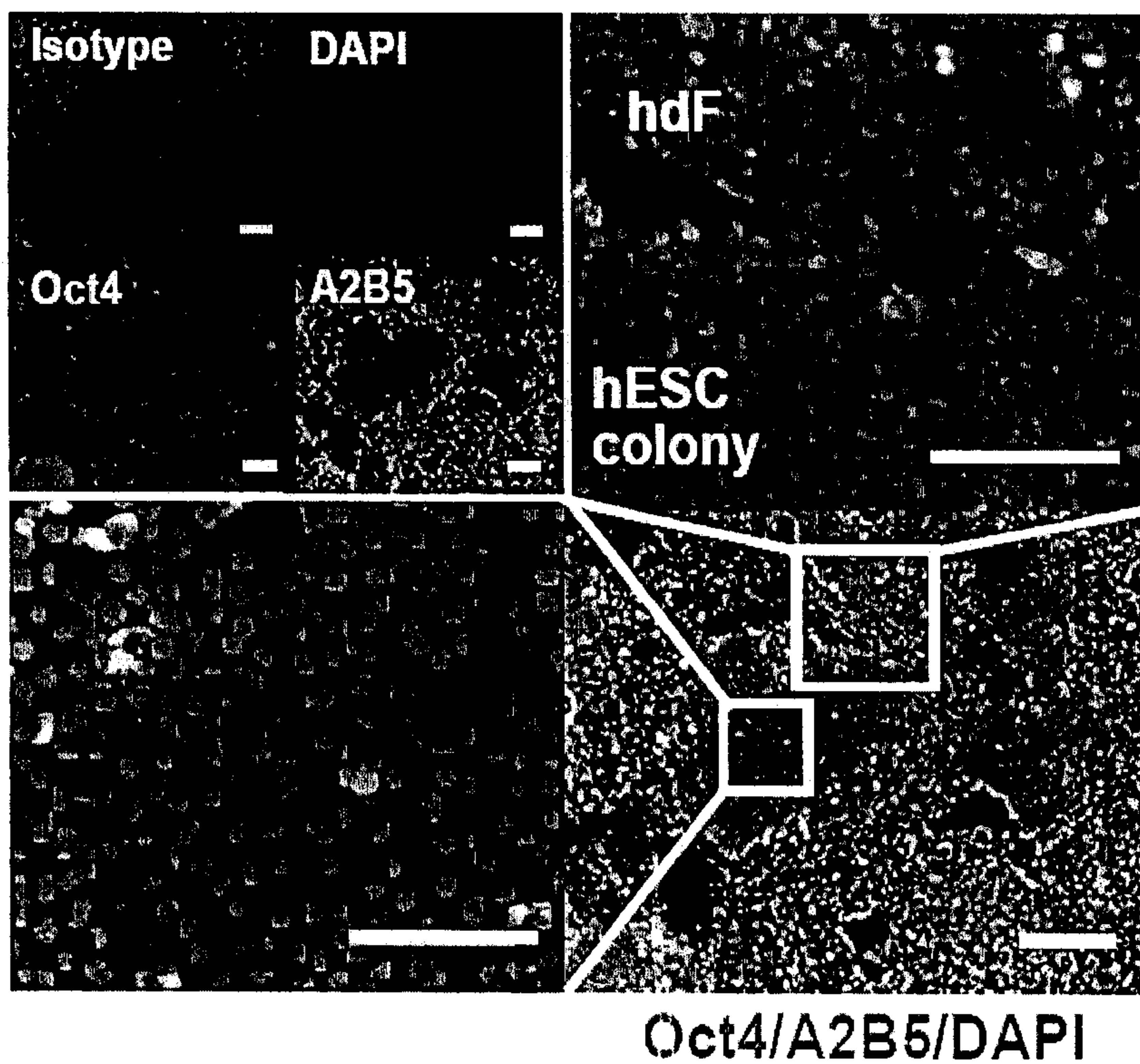


Fig. 1