Stem Cell Reports

Supplemental Information

A Simple and Robust Method for Establishing Homogeneous Mouse Epiblast Stem Cell Lines by Wnt Inhibition

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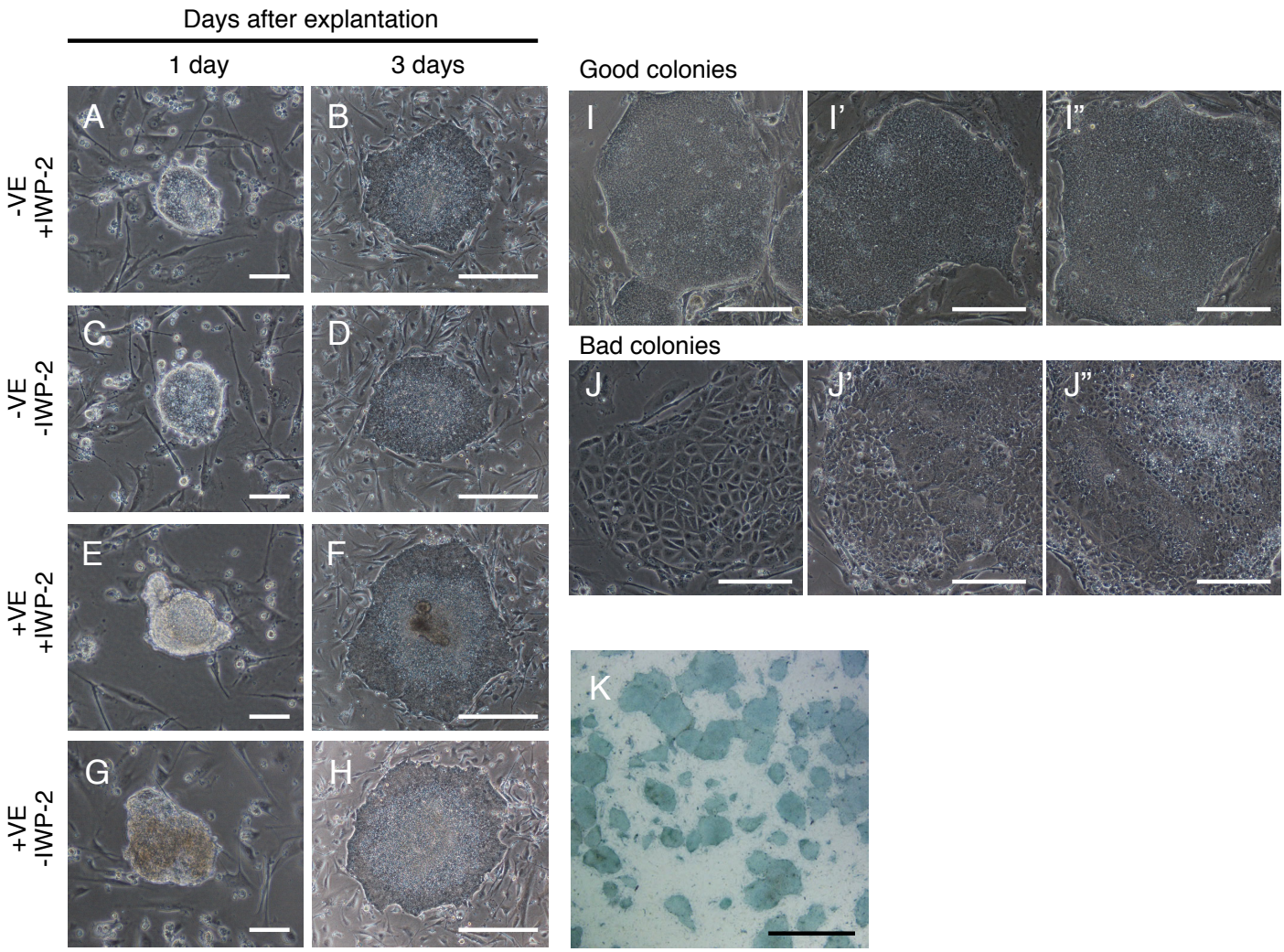
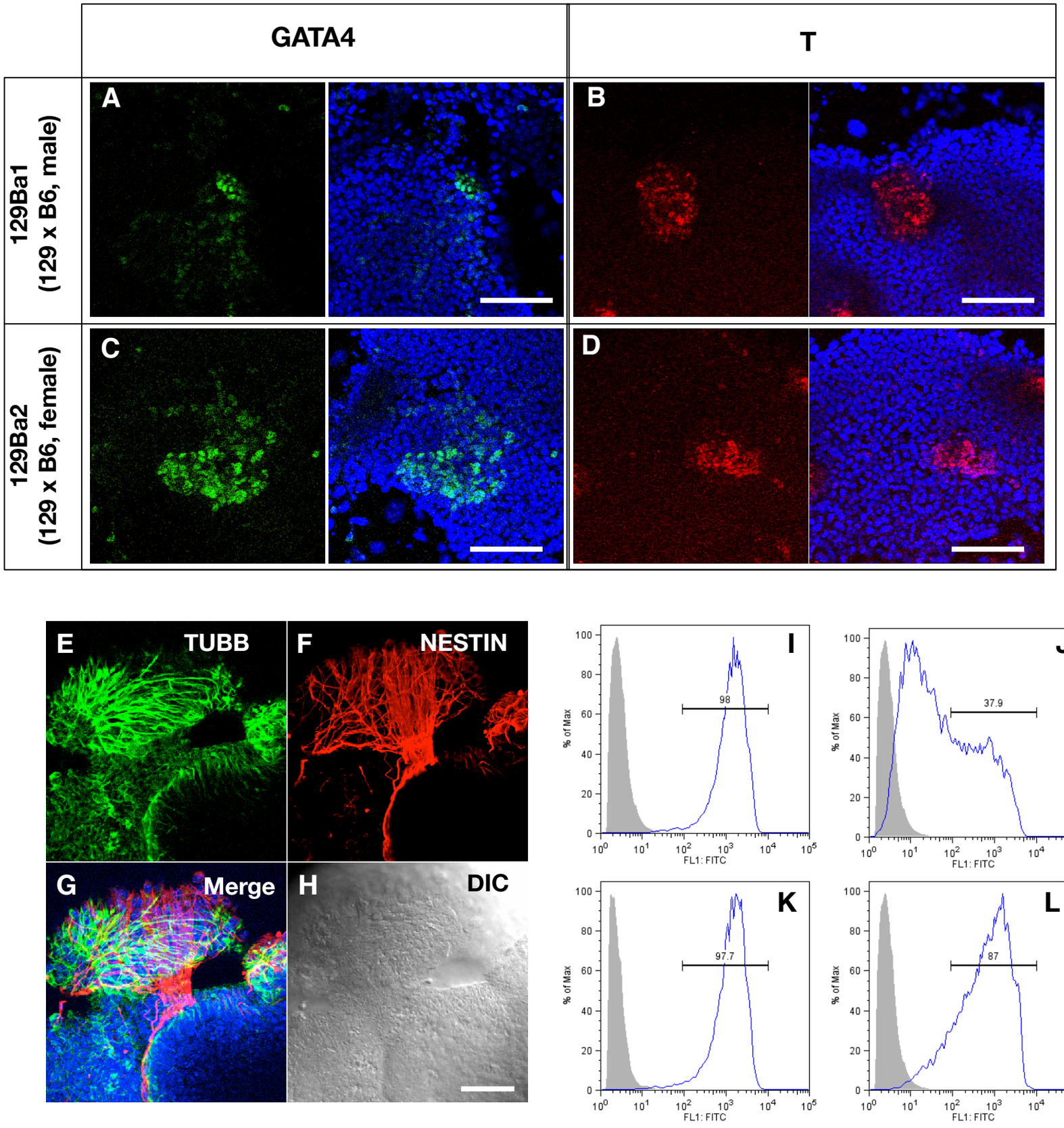
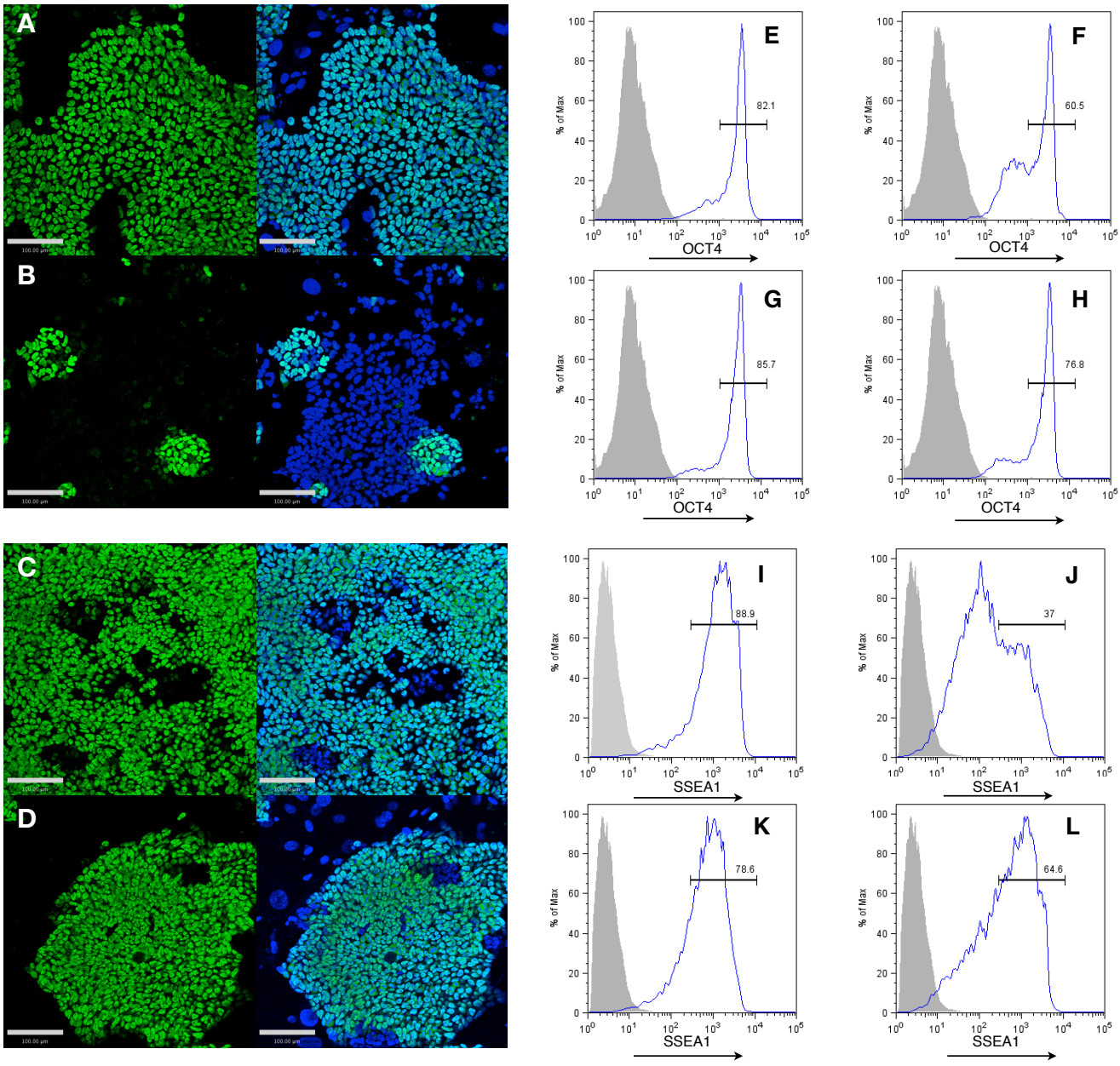
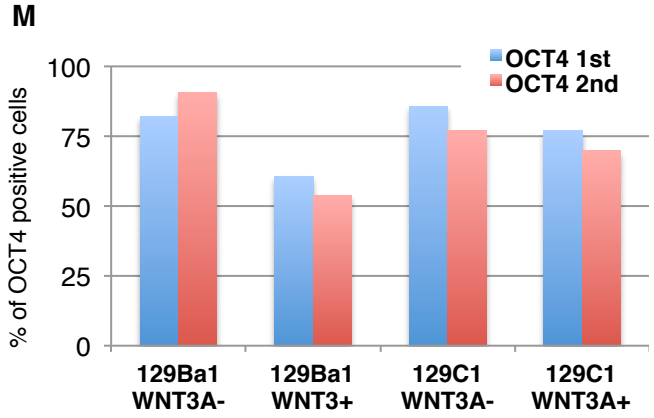
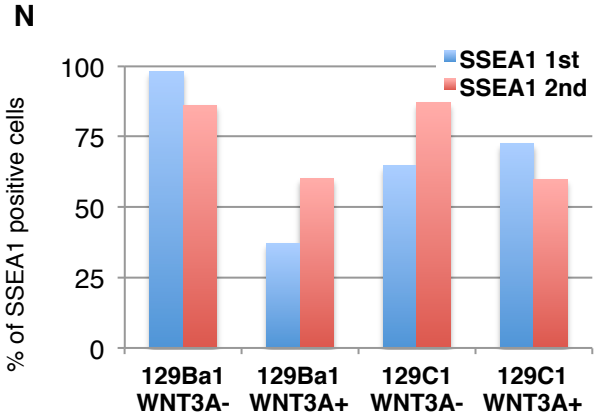
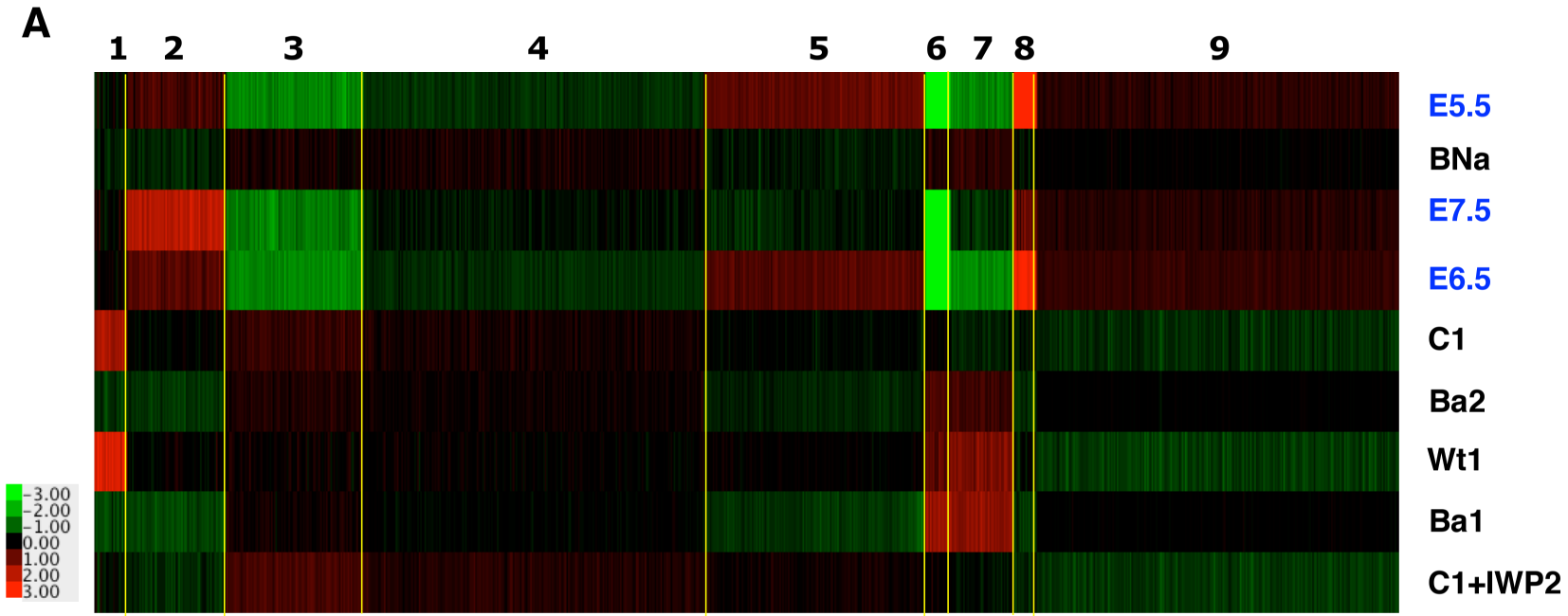

Days after explantation
1 day
3 days
-VE
+IWP-2
-VE
-IWP-2
+VE
+IWP-2
+VE
-IWP-2
A
B
C
D
E
F
G
H
Good colonies
I
I'
I"
Bad colonies
J
J'
J"
K

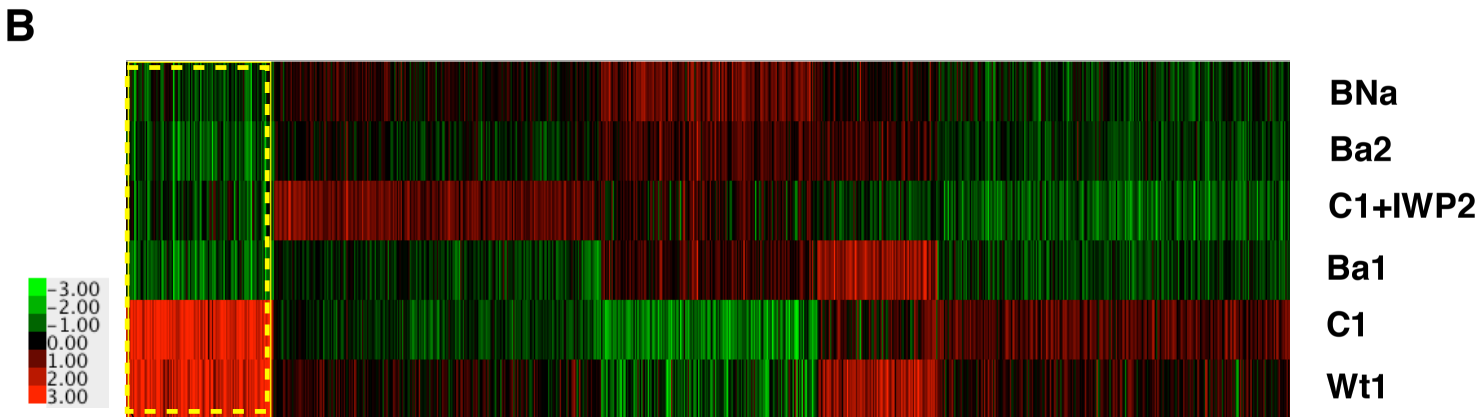

GATA4
T
129Ba1
(129 x B6, male)
129Ba2
(129 x B6, female)
A
B
C
D
E
TUBB
F
NESTIN
G
Merge
H
DIC
I
J
K
L
% of Max
FL1: FITC
98
37.9
97.7
87

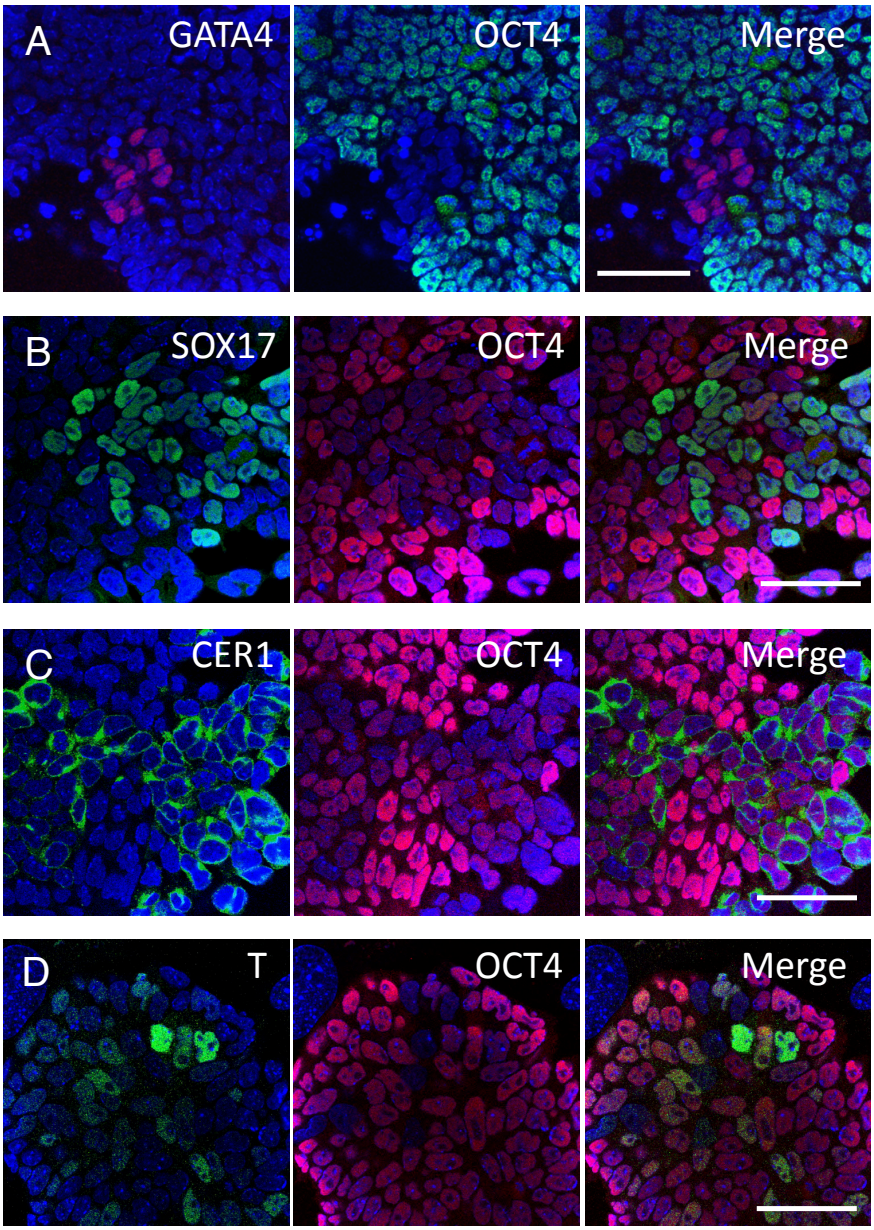

A
B
C
D
E
F
G
H
I
J
K
L
% of Max
OCT4
SSEA1
82.1
60.5
85.7
76.8
88.9
37
78.6
64.6

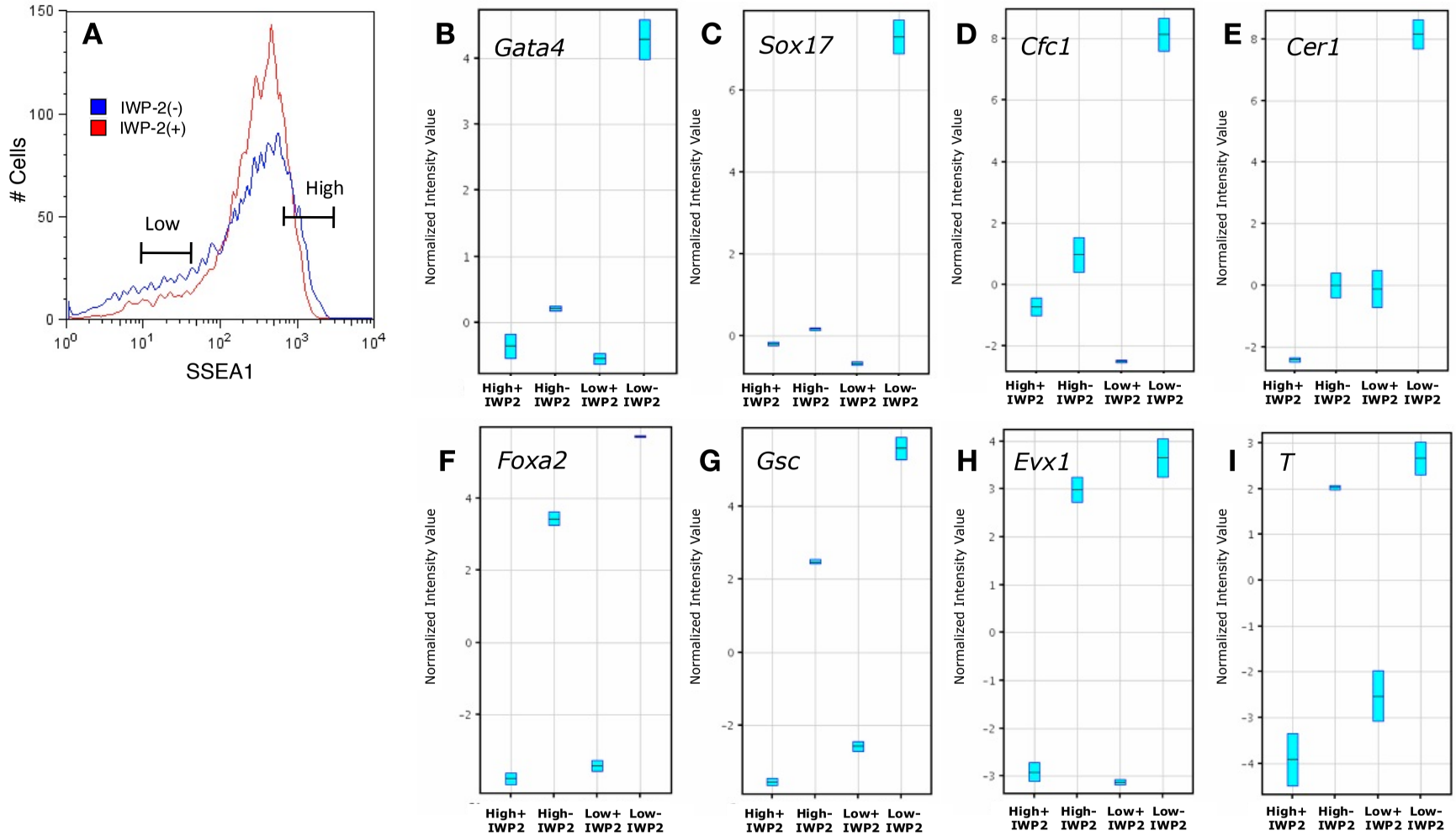

M
% of OCT4 positive cells
OCT4 1st
OCT4 2nd
129Ba1 WNT3A-
129Ba1 WNT3+
129C1 WNT3A-
129C1 WNT3A+


N
% of SSEA1 positive cells
SSEA1 1st
SSEA1 2nd
129Ba1 WNT3A-
129Ba1 WNT3A+
129C1 WNT3A-
129C1 WNT3A+


A
1
2
3
4
5
6
7
8
9
E5.5
BNa
E7.5
E6.5
C1
Ba2
Wt1
Ba1
C1+IWP2
-3.00
-2.00
-1.00
0.00
1.00
2.00
3.00


B
BNa
Ba2
C1+IWP2
Ba1
C1
Wt1
-3.00
-2.00
-1.00
0.00
1.00
2.00
3.00


A
GATA4
OCT4
Merge
B
SOX17
OCT4
Merge
C
CER1
OCT4
Merge
D
T
OCT4
Merge


A
IWP-2(-)
IWP-2(+)
Low
High
Cells
SSEA1
B
Gata4
C
Sox17
D
Cfc1
E
Cer1
F
Foxa2
G
Gsc
H
Evx1
I
T
Normalized Intensity Value
High+ IWP2
High- IWP2
Low+ IWP2
Low- IWP2

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Epiblast Outgrowth during EpiSC Establishment , and Morphology of Undifferentiated EpiSC Colonies and Differentiated Colonies Observed during the Derivation Process, Related to Figure 1. Epiblasts of –VE/+IWP-2 (A and B), –VE/–IWP-2 (C and D), +VE/+IWP-2 (E and F), and +VE/–IWP-2 (G and H) attached to the bottom of the dishes one day after explantation (A, C, E, and G) and formed EpiSC-like colonies (B, D, F, and H). Scale bars, 0.1 mm (A, C, E, and G) and 0.5 mm (B, D, F, and H). Typical examples of "good" undifferentiated colonies found during establishment of EpiSC lines are shown (I, I', I"). Colonies exhibiting heterogeneous, differentiated morphologies were judged as "bad" differentiated cells (J, J', J"). Alkaline phosphatase staining of EpiSC colonies (K). Scale bars, 200 μm (I, I', I", J, J', J"), 2 mm (K).

Figure S2. Expression of Differentiation Marker Genes and Changes in SSEA1 Expression Profiles after Removal of IWP-2 from EpiSC Culture, Related to Figure 3. Immunofluorescence images of EpiSC colonies after removal of IWP-2 stained with anti-GATA4 (A, C) and with anti-T antibodies (B, D). (A, B) 129Ba1 EpiSC line, (C, D) 129Ba2 line. Immunofluorescence images for TUBB3 (E) and NESTIN (F); a merged image (G) and bright field image (H) are shown. Scale bar, 50 μm. EpiSCs were subjected to flow cytometry analysis as described in EXPERIMENTAL PROCEDURES to detect changes in SSEA1 expression profiles. (I) Profile of SSEA1 expression in 129Ba1 EpiSCs maintained in medium containing IWP-2 (blue line). Isotype control (gray). (J) 129Ba1 cultured for 4 days in medium containing no IWP-2 (blue). Isotype control (gray). Flow cytometry analysis was

conducted 4 day after medium change. (K) Profile of SSEA1 expression in 129C1 EpiSCs maintained in medium containing IWP-2 (blue). Isotype control (gray). (L) 129C1 cultured for 4 days in medium containing no IWP-2 (blue). Isotype control (gray). Flow cytometry analysis was performed four days after medium change. The analyses were performed at least twice for each condition, and representative data are presented.

Figure S3. Effect of WNT3A on Pluripotent Marker Expression, Related to Figure 3. (A) OCT4 expression (left, green) in 129Ba1 cultured in the presence of IWP-2. Nuclei were stained with TO-PRO3 (blue). Merged image (right). (B) OCT4 expression in 129Ba1 cultured in medium supplemented with IWP-2 and WNT3A (200 ng/ml). Merged image (right). Images were taken 2 days after addition of WNT3A. (C) 129C1 cells were cultured without WNT3A, and stained with OCT4 antibody (green) or TO-PRO3 (blue). Merged image (right). (D) 129C1 cells were cultured with WNT3A, and stained with anti-OCT4 (green) and TO-PRO3 (blue). Merged image (right). Images were taken 2 days after addition of WNT3A. Scale bar, 100 μm. The expression analyses were performed at least twice for each condition, and representative data are presented. (E-H) Flow cytometry analysis of OCT4 expression in 129Ba1 (E, F) and in 129C1 (G, H). (E, G) Control: each cell line was cultured without WNT3A. 129Ba1 was cultured with IWP-2, while 129C1 was cultured without IWP-2. (F) 129Ba1 line was cultured with IWP-2 and WNT3A. WNT3A was added to the culture at 200 ng/ml. (H) 129C1 was cultured with WNT3A (without IWP-2). (I-L) Flow cytometry analysis of SSEA1 expression in 129Ba1 (I, J) and in 129C1 (K, L). (I, K) Control: each cell line was cultured without WNT3A. 129Ba1 was cultured with IWP-2, while 129C1 was

cultured without IWP-2. (J) 129Ba1 was cultured with IWP-2 and WNT3A (200 ng/ml). (L) 129C1 was cultured with WNT3A (without IWP-2). Flow cytometry data from two independent experiments are shown as bar graphs; (M) OCT4, (N) SSEA1. Flow cytometry analyses were conducted 2 days after WNT3A addition. The analyses were performed at least twice for each condition, and representative data are presented in Fig.S3E-L.

Figure S4. K-Means Cluster Analyses of Gene Expression Profiles of EpiSCs and Epiblast/Embryonic Ectoderm, Related to Figure 4. (A) Expression profile data from all the samples were subjected to one-way ANOVA, and selected 17,819 features were classified into nine clusters by K-means cluster analysis. (B) Gene probes showing differences between 129C1 and 129C1+IWP-2 ($p<0.05$, fold change ≥ 2.0) were selected and subjected to K-means analysis. Probes displaying upregulation in 129C1 and Wt1 cell lines compared with other EpiSCs including 129C1+IWP-2 were detected (229 probes are surrounded by a yellow rectangle).

Figure S5. Heterogeneous Marker Expressions in EpiSCs, Related to Figure 5. Immunofluorescence images for GATA4 (red) and OCT4 (green) (A), SOX17 (green) and OCT4 (red) (B), CER1 (green) and OCT4 (red) (C), T (green) and OCT4 (red) (D) are shown. Scale bar, 50 μm.

Figure S6. Isolation of SSEA1^{high} and SSEA1^{low} Cell Fractions from 129C1 EpiSCs, and Expression Analysis of Selected Marker Genes in SSEA1^{high} and SSEA1^{low} Cell Fractions from 129C1 EpiSCs, Related to Figure 5. The 129C1 EpiSCs cultured with or without IWP-2 were subjected to BD FACS Aria III to isolate SSEA1^{high} fraction and

SSEA1^{low} cell fractions as shown in (A). (Red line) FACS pattern of 129C1 EpiSCs cultured in IWP-2 containing medium. (Blue line) FACS pattern of 129C1 EpiSCs cultured without IWP-2. Eight genes that exhibited significant differences between bulk 129C1 and 129C1(+)IWP-2 were selected, and their expression data were retrieved from microarray data obtained from 129C1 SSEA1^{high} and SSEA1^{low} samples . Normalized intensity value, an arbitrary value showing gene expression level (calculated by the Feature Extraction software 10.5.1.1 (Agilent Technologies)) of each gene in the SSEA1^{high}±IWP-2 and SSEA1^{low}±IWP-2 samples was used to draw a box-whisker plot. High+IWP2, data from 129C1 SSEA1^{high} (+) IWP-2 sample; High-IWP2, data from 129C1 SSEA1^{high} (-) IWP-2 sample; Low+IWP2, data from 129C1 SSEA1^{low} (+) IWP-2 sample; Low-IWP2, data from 129C1 SSEA1^{low} (-) IWP-2 sample. (B) *Gata4*, (C) *Sox17*, (D) *Cfc1*, (E) *Cer1*, (F) *Foxa2*, (G) *Gsc*, (H) *Evx1*, (I) *T* .

Table S1. EpiSC lines established from E6.5 epiblasts with or without IWP2, related to Table 1.

Genetic background	Visceral endoderm	IWP2	No. of embryos	No. of cell lines (%)
C57BL/6N x 129S2/Sv	-	-	4	0 (0.0)
C57BL/6N x C57BL/6N	-	-	16	2 (12.5)
129S2/Sv x 129S2/Sv	-	-	4	0 (0.0)
C57BL/6N x 129S2/Sv	-	+	4	4 (100.0)
129S2/Sv x 129S2/Sv	-	+	2	2 (100.0)