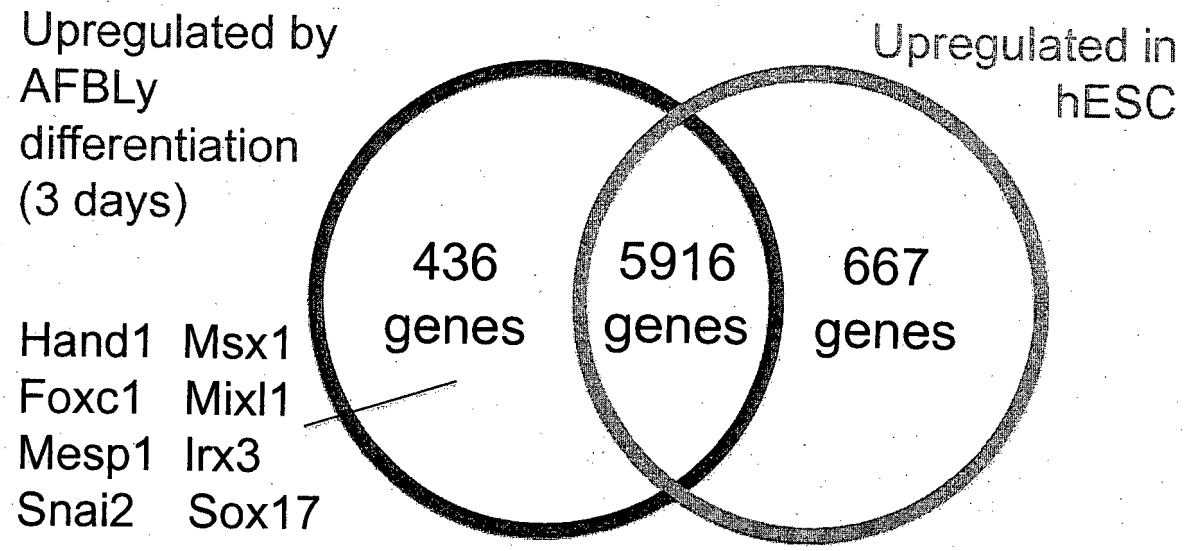
FIG. 1




GO functions:

Skeletal system development ($P$=3.3x10$^{-10}$)

Mesoderm development ($P$=2.4x10$^{-9}$)

Vasculature development ($P$=1.6x10$^{-8}$)

Heart development ($P$=6.6x10$^{-8}$)

FIG. 2

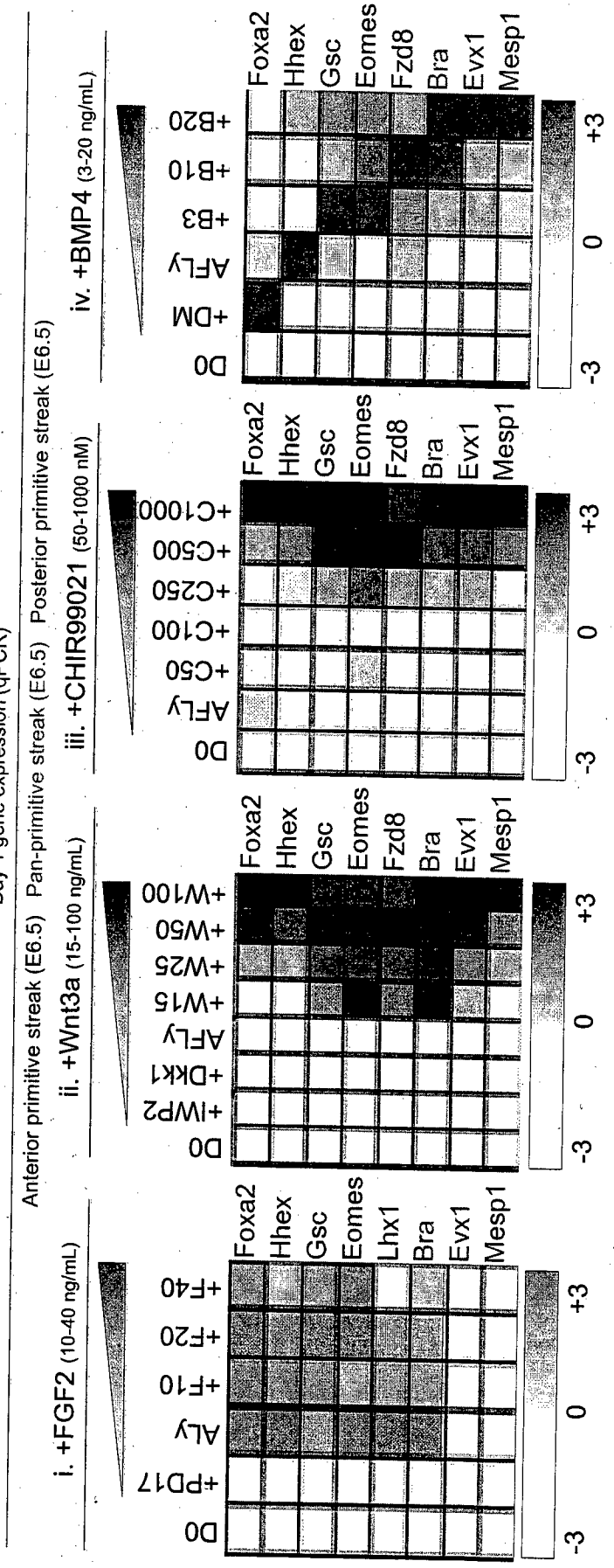

Day 1 gene expression (qPCR)
Anterior primitive streak (E6.5) Pan-primitive streak (E6.5) Posterior primitive streak (E6.5)
i. +FGF2 (10-40 ng/mL)
ii. +Wnt3a (15-100 ng/mL)
iii. +CHIR99021 (50-1000 nM)
iv. +BMP4 (3-20 ng/mL)
D0
+PD17
ALy
+F10
+F20
+F40
+IWP2
+Dkk1
AFLy
+W15
+W25
+W50
+W100
+C50
+C100
+C250
+C500
+C1000
+DM
+B3
+B10
+B20
Foxa2
Hhex
Gsc
Eomes
Lhx1
Bra
Evx1
Mesp1
Fzd8
-3
0
+3

FIG. 3

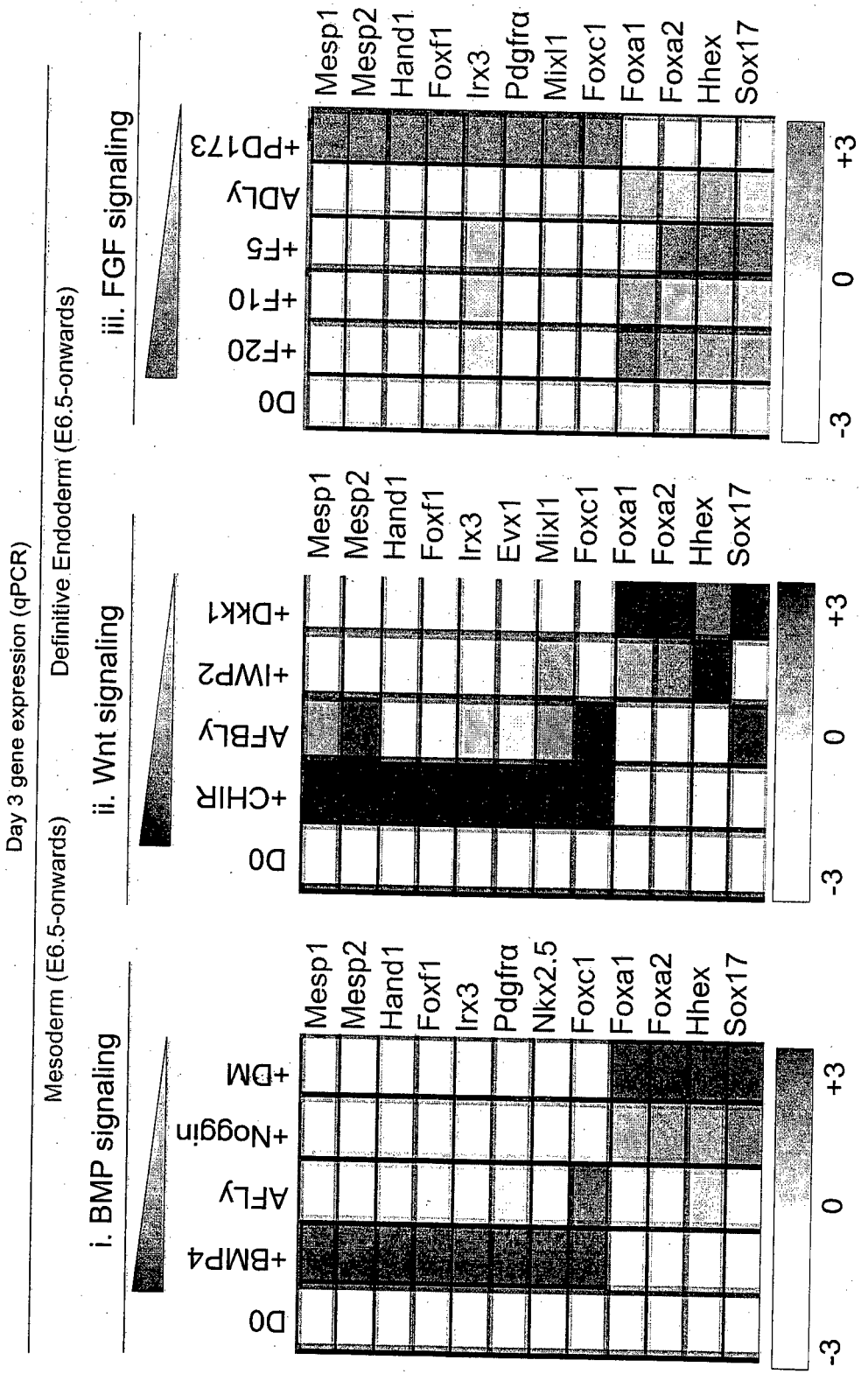
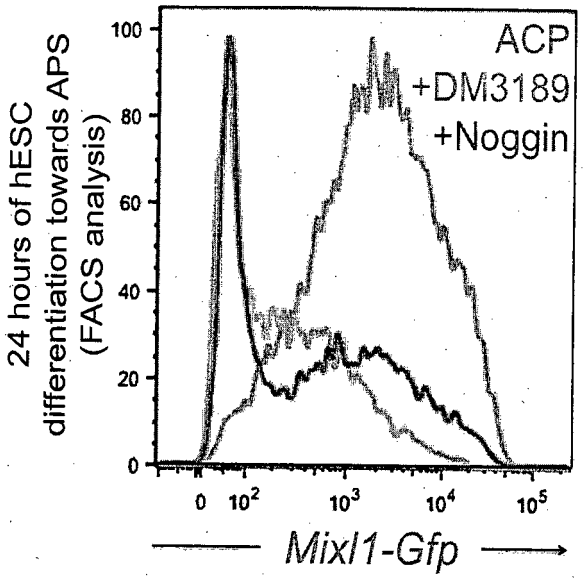

Day 3 gene expression (qPCR)
Mesoderm (E6.5-onwards)
Definitive Endoderm (E6.5-onwards)
i. BMP signaling
D0
+BMP4
AFLy
+Noggin
+DM
Mesp1
Mesp2
Hand1
Foxf1
Irx3
Pdgfrα
Nkx2.5
Foxc1
Foxa1
Foxa2
Hhex
Sox17
-3
0
+3
ii. Wnt signaling
D0
+CHIR
AFBLy
+IWP2
+Dkk1
Mesp1
Mesp2
Hand1
Foxf1
Irx3
Evx1
Mixl1
Foxc1
Foxa1
Foxa2
Hhex
Sox17
-3
0
+3
iii. FGF signaling
D0
+F20
+F10
+F5
ADLy
+PD173
Mesp1
Mesp2
Hand1
Foxf1
Irx3
Pdgfrα
Mixl1
Foxc1
Foxa1
Foxa2
Hhex
Sox17
-3
0
+3

FIG. 4

i. BMP is essential for APS induction




ii. Signaling input truth table

iii. Current signaling paradigm

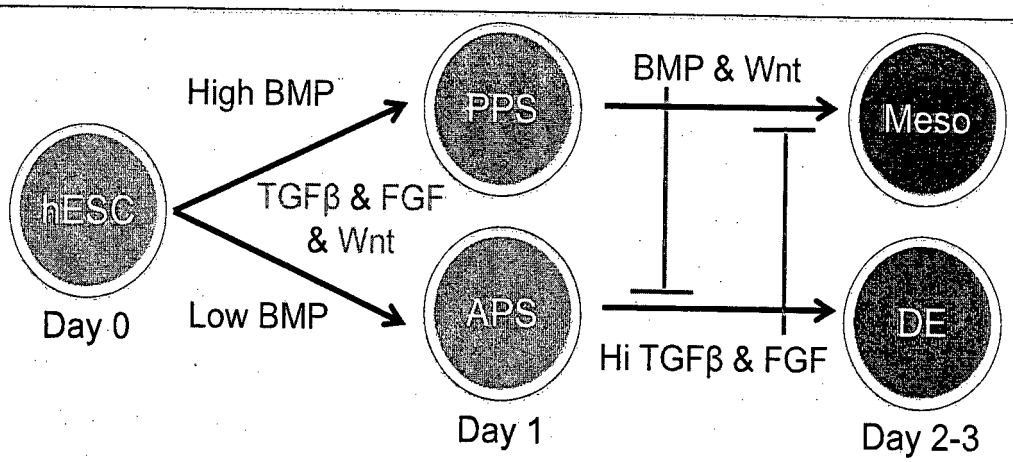


FIG. 5

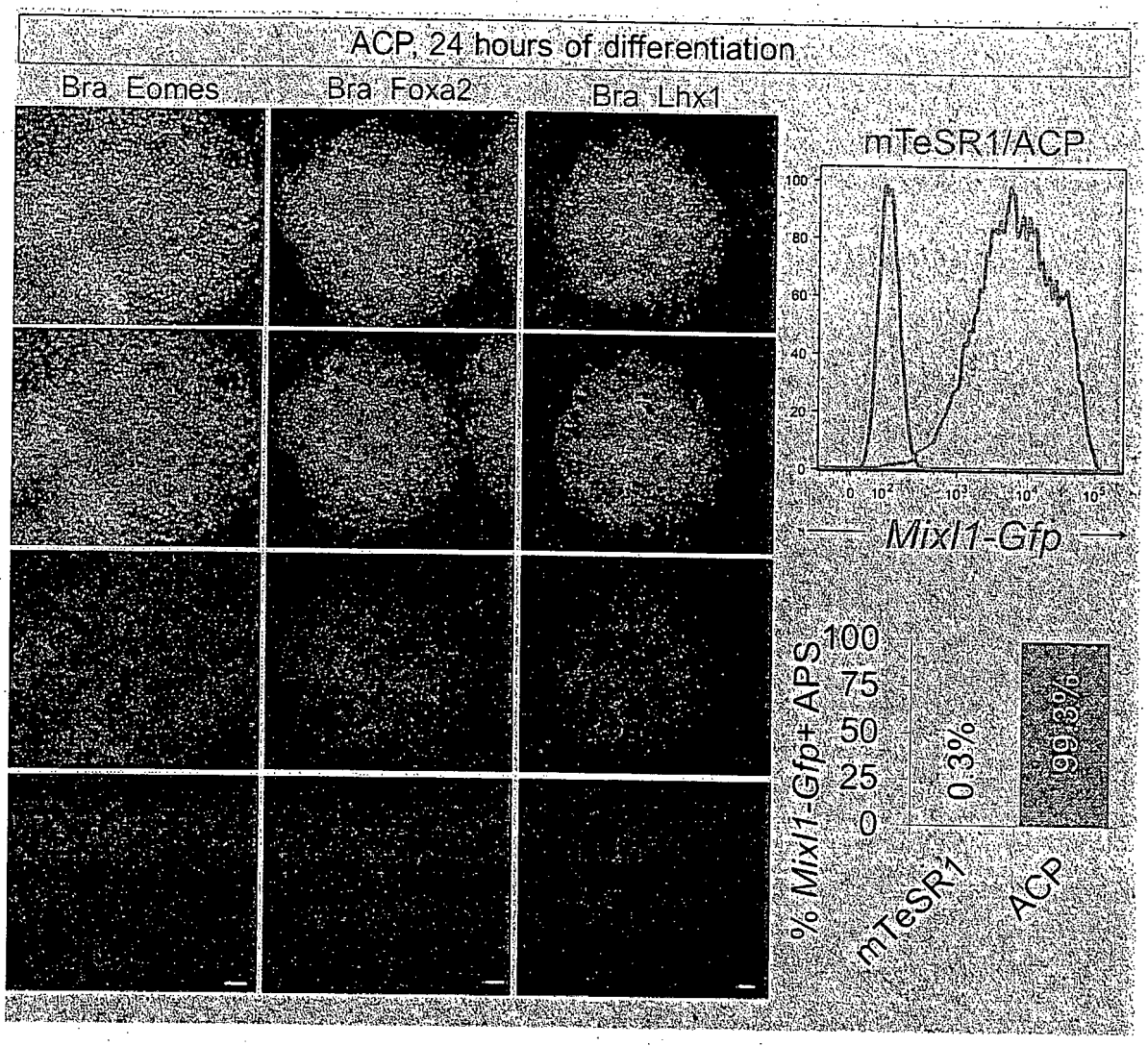

ACP, 24 hours of differentiation
Bra Eomes
Bra Foxa2
Bra Lhx1
mTeSR1/ACP
100
80
60
40
20
0
0
10²
10³
10⁴
10⁵
Mixl1-Gfp
% Mixl1-Gfp+ APS
100
75
50
25
0
0.3%
99.3%
mTeSR1
ACP

FIG. 6

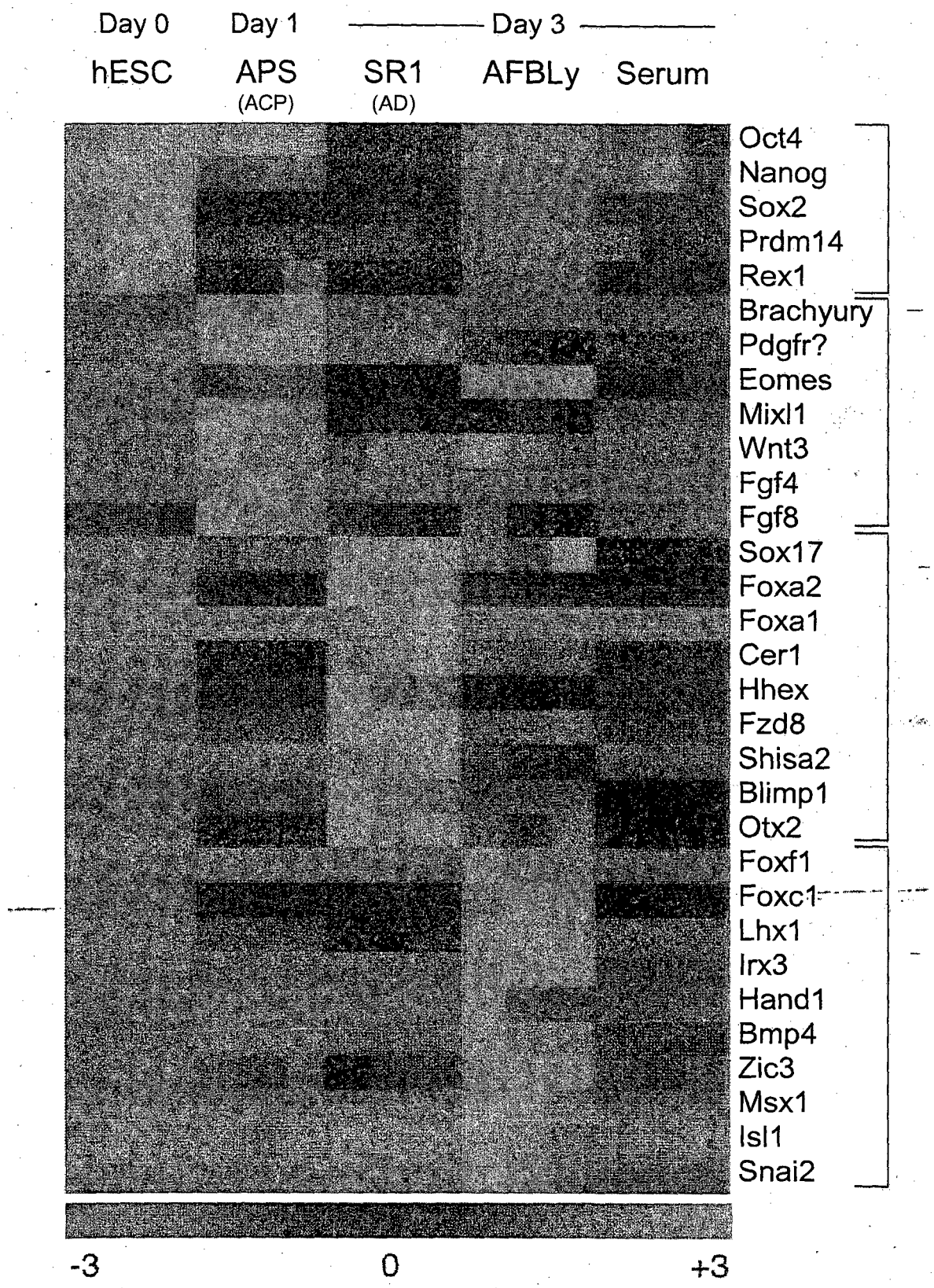
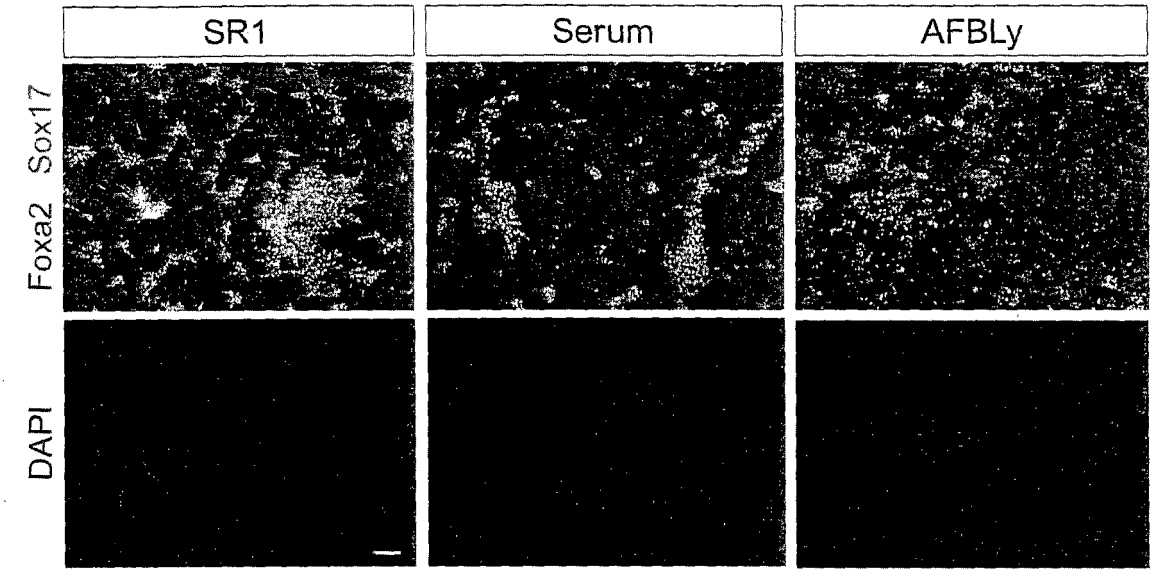
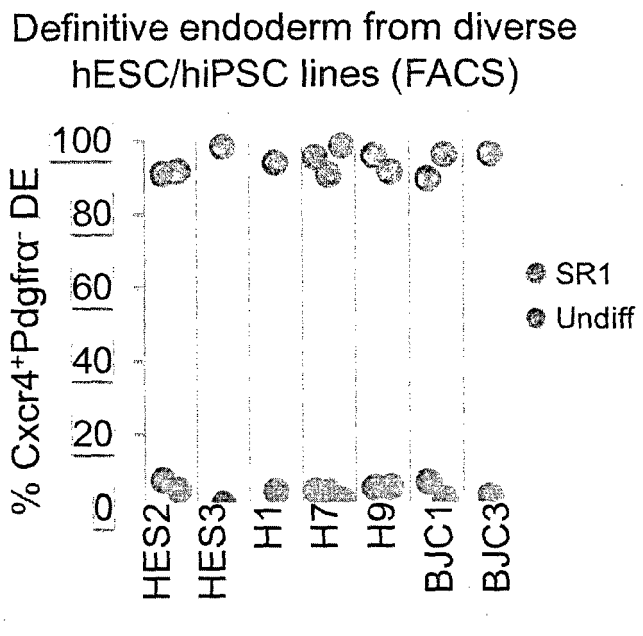
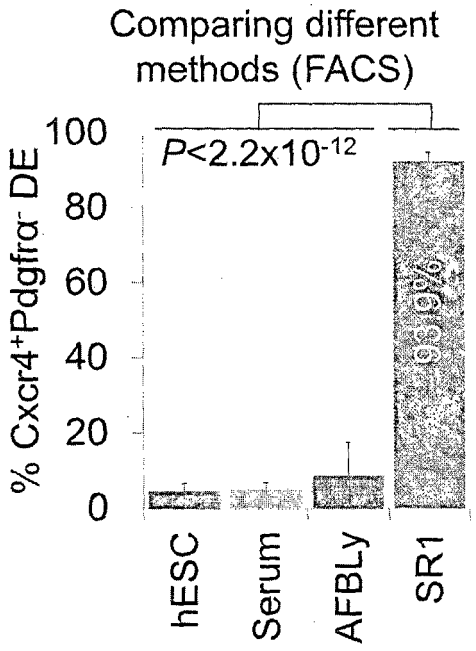

Day 0
Day 1
Day 3
hESC
APS
(ACP)
SR1
(AD)
AFBLy
Serum
Oct4
Nanog
Sox2
Prdm14
Rex1
Brachyury
Pdgfr?
Eomes
Mixl1
Wnt3
Fgf4
Fgf8
Sox17
Foxa2
Foxa1
Cer1
Hhex
Fzd8
Shisa2
Blimp1
Otx2
Foxf1
Foxc1
Lhx1
Irx3
Hand1
Bmp4
Zic3
Msx1
Isl1
Snai2
-3
0
+3

FIG. 7


SR1
Serum
AFBLy
Foxa2 Sox17
DAPI


Definitive endoderm from diverse hESC/hiPSC lines (FACS)
% Cxcr4+Pdgfra- DE
100
80
60
40
20
0
HES2
HES3
H1
H7
H9
BJC1
BJC3
SR1
Undiff


Comparing different methods (FACS)
% Cxcr4+Pdgfra- DE
P<2.2x10-12
100
80
60
40
20
0
hESC
Serum
AFBLy
SR1

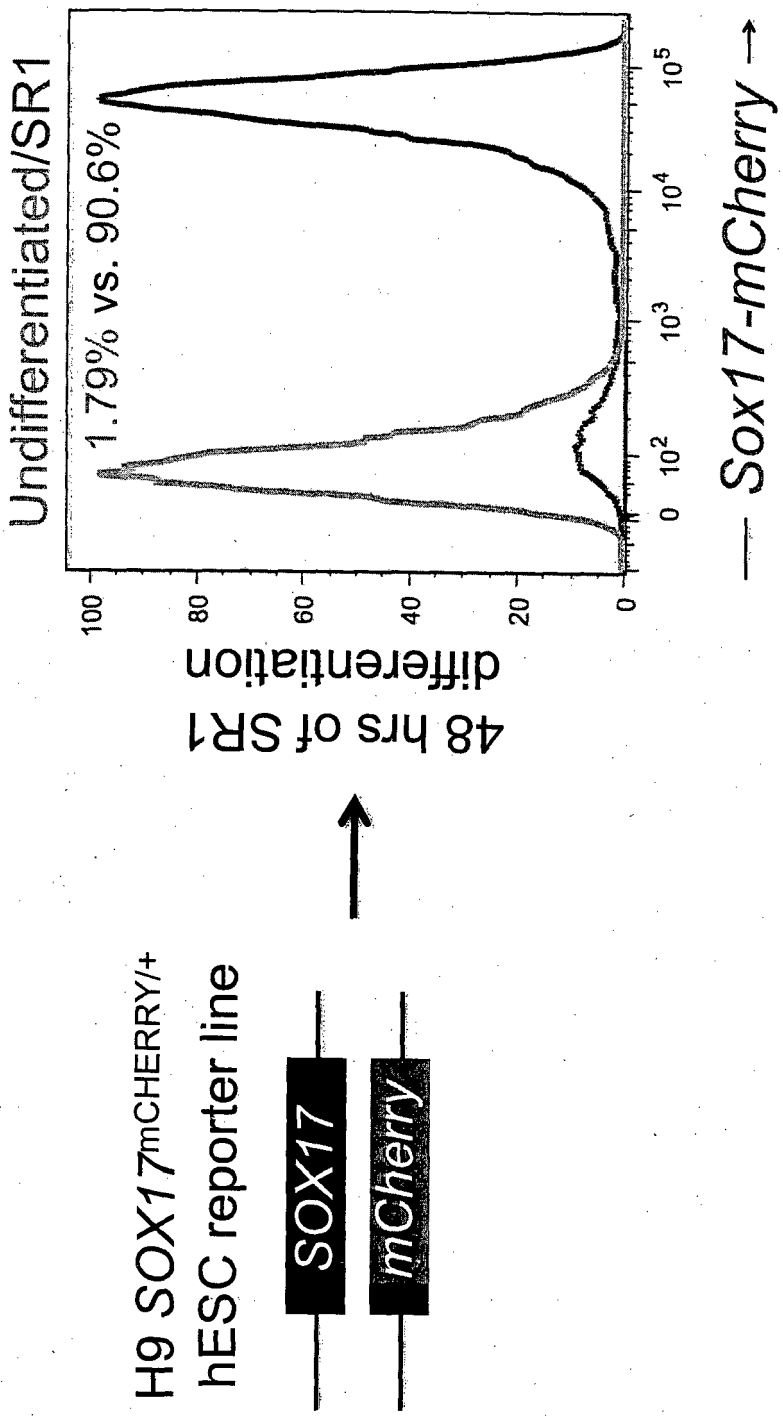

H9 SOX17mCHERRY/+
hESC reporter line
SOX17
mCherry
48 hrs of SR1
differentiation
Undifferentiated/SR1
1.79% vs. 90.6%
0
20
40
60
80
100
0
10^2
10^3
10^4
10^5
— Sox17-mCherry →

FIG. 8

FIG. 9

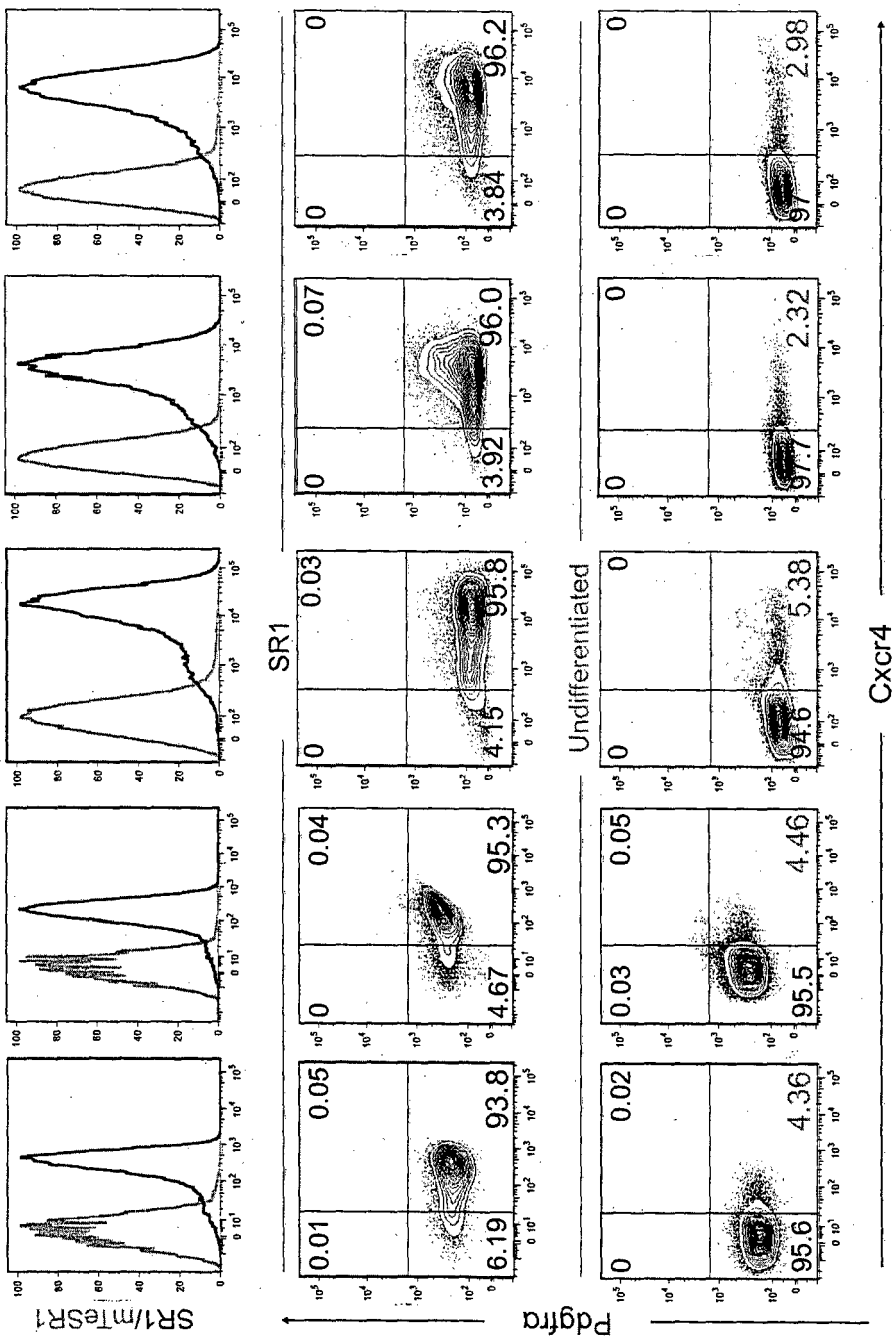

H1 hESC
H7 hESC
H9 hESC
BJC1 hiPSC
BJC3 hiPSC
SR1/mTeSR1
SR1
Undifferentiated
Pdgfra
Cxcr4

FIG. 10

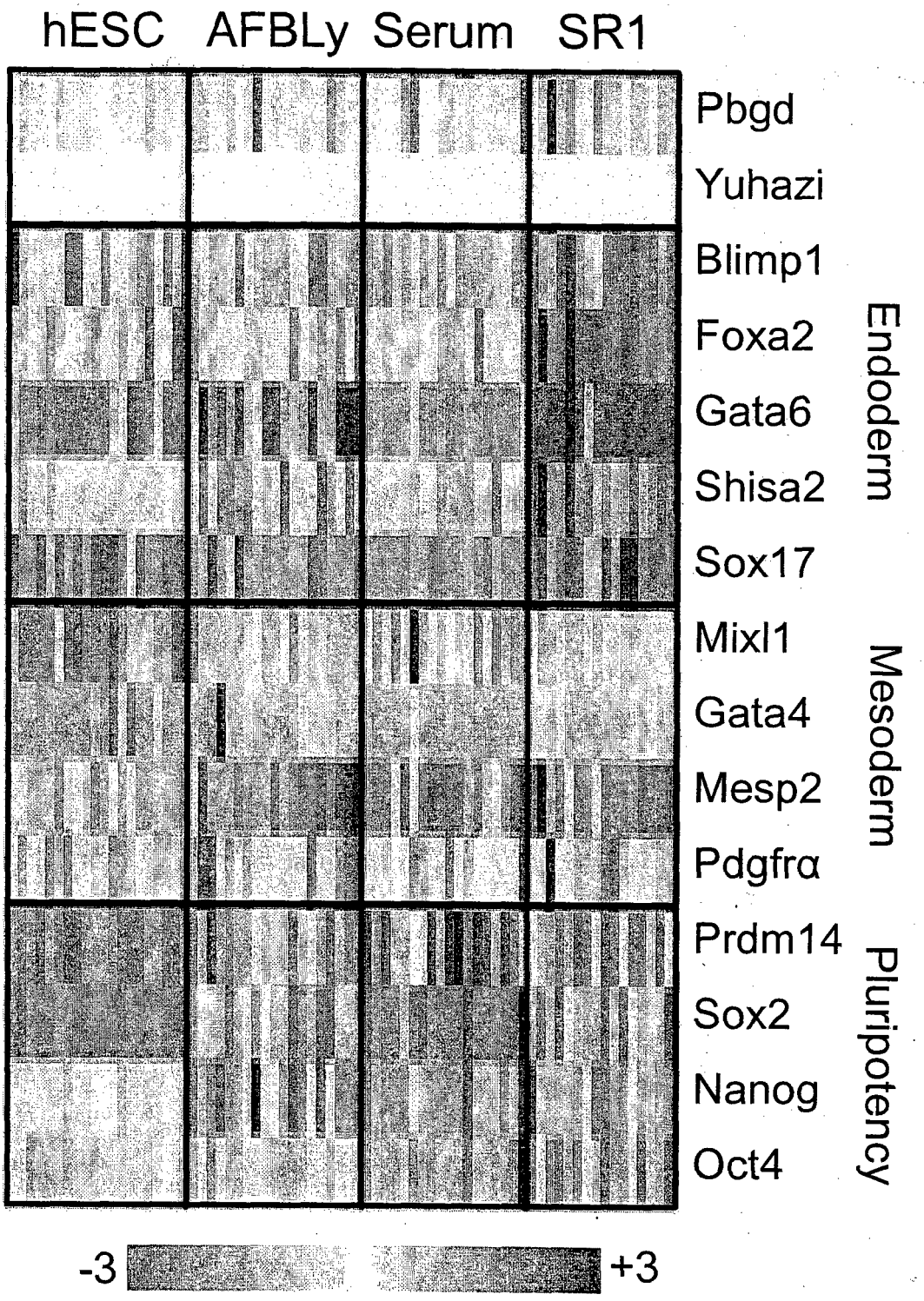

hESC
AFBLy
Serum
SR1
Pbgd
Yuhazi
Blimp1
Foxa2
Gata6
Shisa2
Sox17
Mixl1
Gata4
Mesp2
Pdgfrα
Prdm14
Sox2
Nanog
Oct4
Endoderm
Mesoderm
Pluripotency
-3
+3

FIG. 11

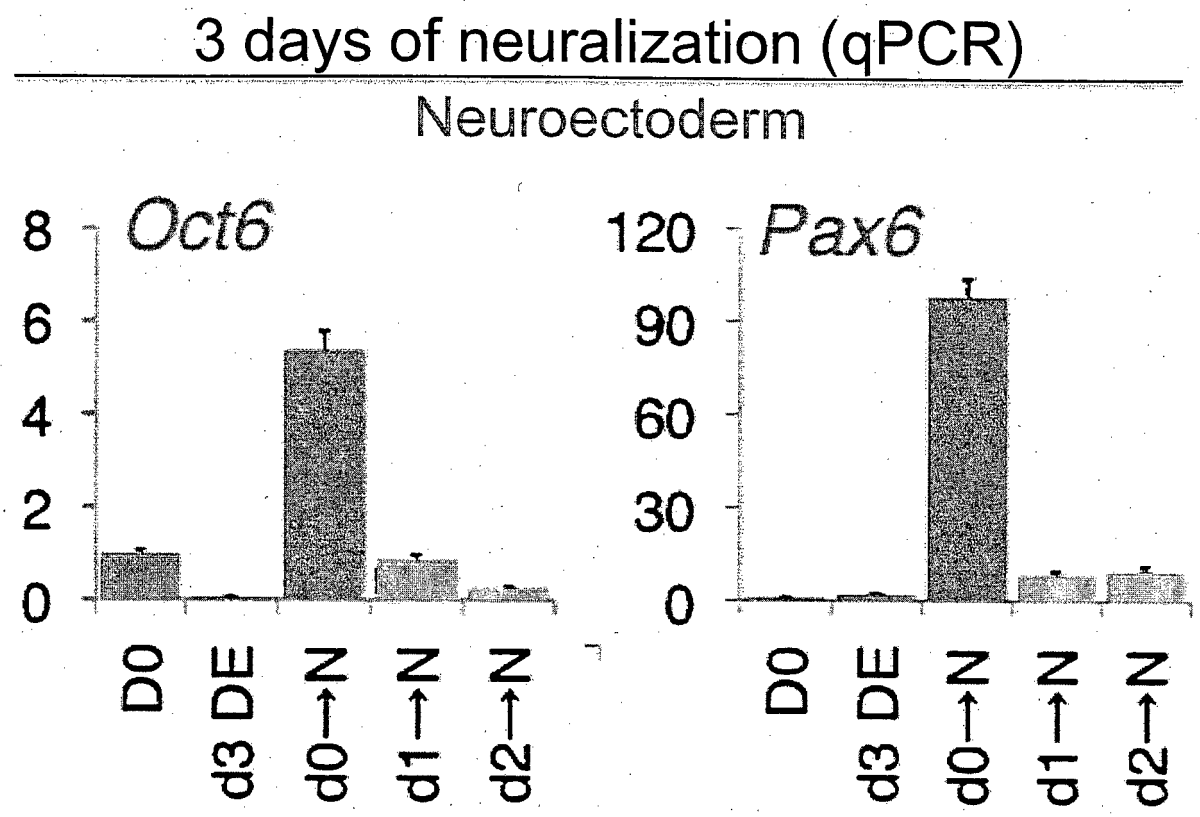
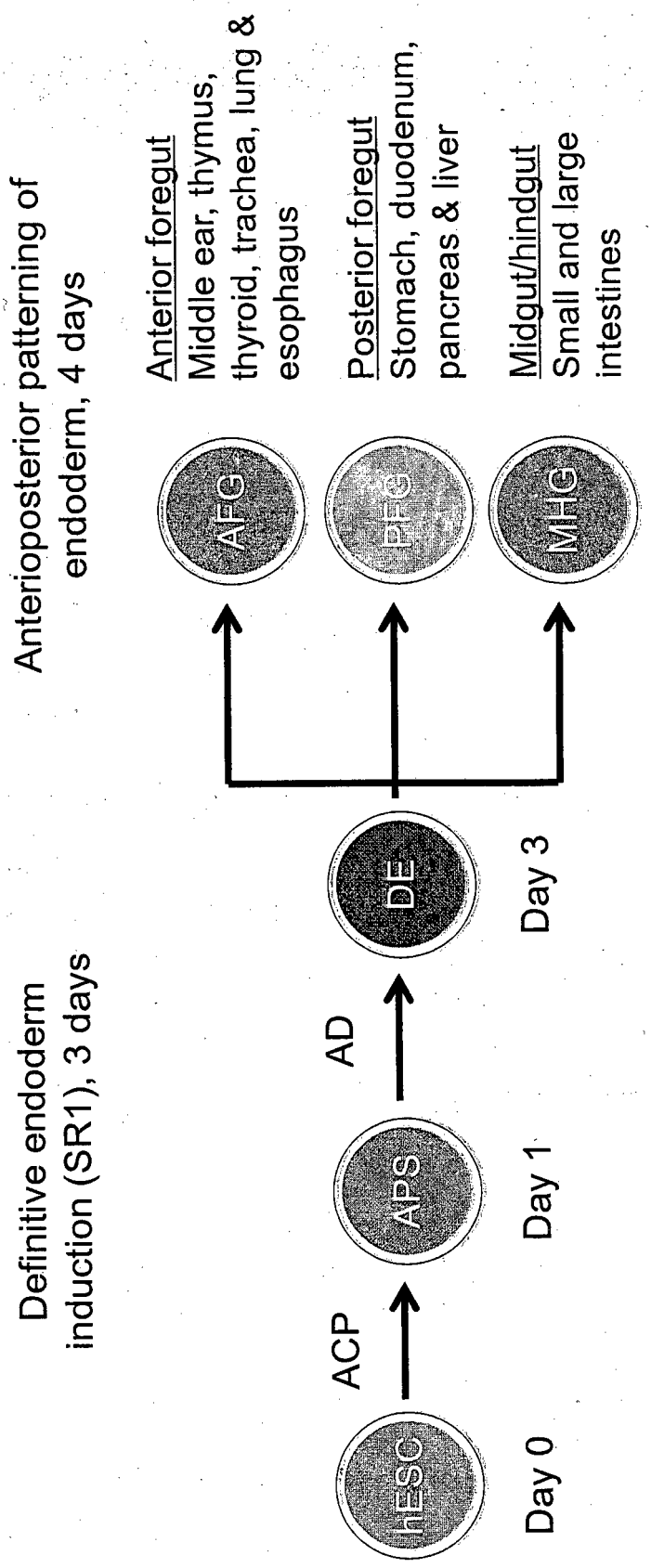

3 days of neuralization (qPCR)
Neuroectoderm
Oct6
8
6
4
2
0
D0
d3 DE
d0→N
d1→N
d2→N
Pax6
120
90
60
30
0
D0
d3 DE
d0→N
d1→N
d2→N

FIG. 12


hESC
Day 0
ACP
APS
Day 1
AD
DE
Day 3
Definitive endoderm induction (SR1), 3 days
Anterioposterior patterning of endoderm, 4 days
AFG
PFG
MHG
Anterior foregut
Middle ear, thymus, thyroid, trachea, lung & esophagus
Posterior foregut
Stomach, duodenum, pancreas & liver
Midgut/hindgut
Small and large intestines

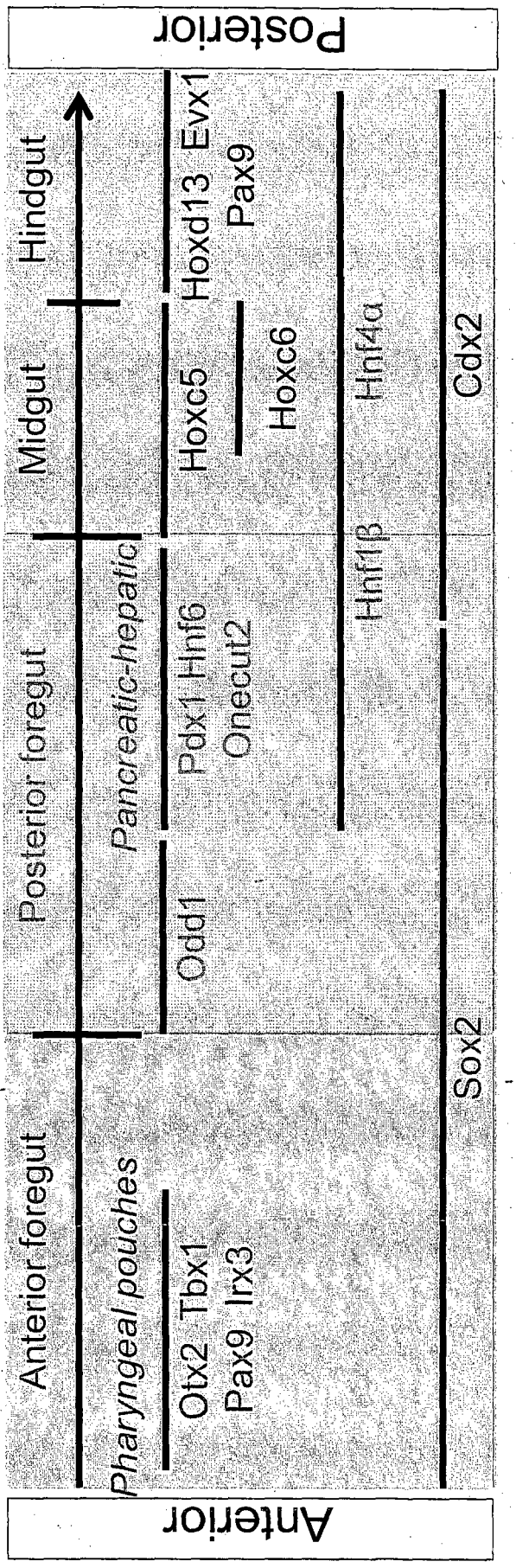

Anterior
Posterior
Anterior foregut
Posterior foregut
Midgut
Hindgut
Pharyngeal pouches
Pancreatic-hepatic
Otx2 Tbx1
Pax9 Irx3
Odd1
Pdx1 Hnf6
Onecut2
Hoxc5
Hoxc6
Hoxd13 Evx1
Pax9
Hnf1β
Hnf4α
Sox2
Cdx2

FIG. 13

FIG. 14

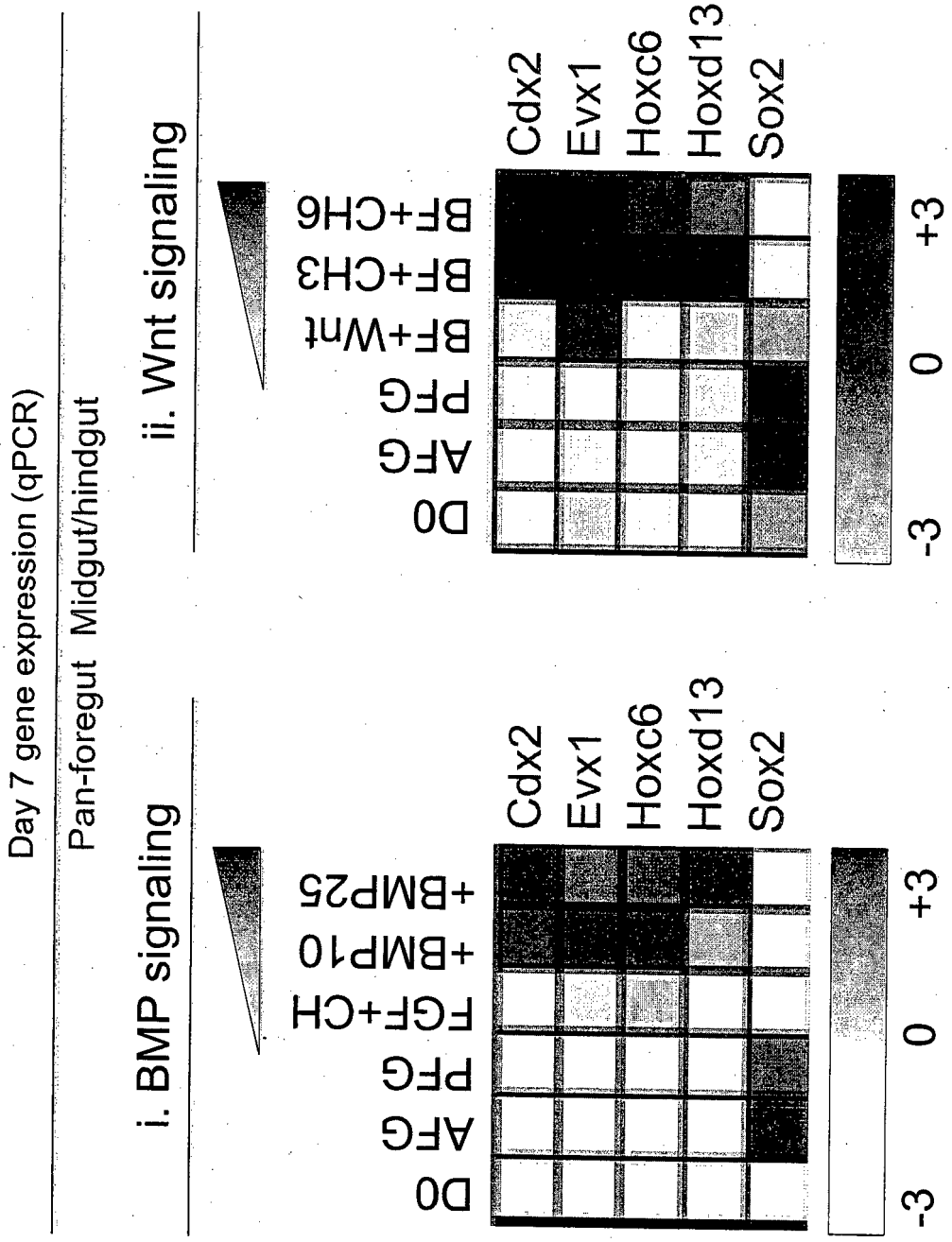

Day 7 gene expression (qPCR)
Pan-foregut Midgut/hindgut
i. BMP signaling
ii. Wnt signaling
Cdx2
Evx1
Hoxc6
Hoxd13
Sox2
D0
AFG
PFG
FGF+CH
+BMP10
+BMP25
BF+Wnt
BF+CH3
BF+CH6
-3
0
+3

FIG. 15

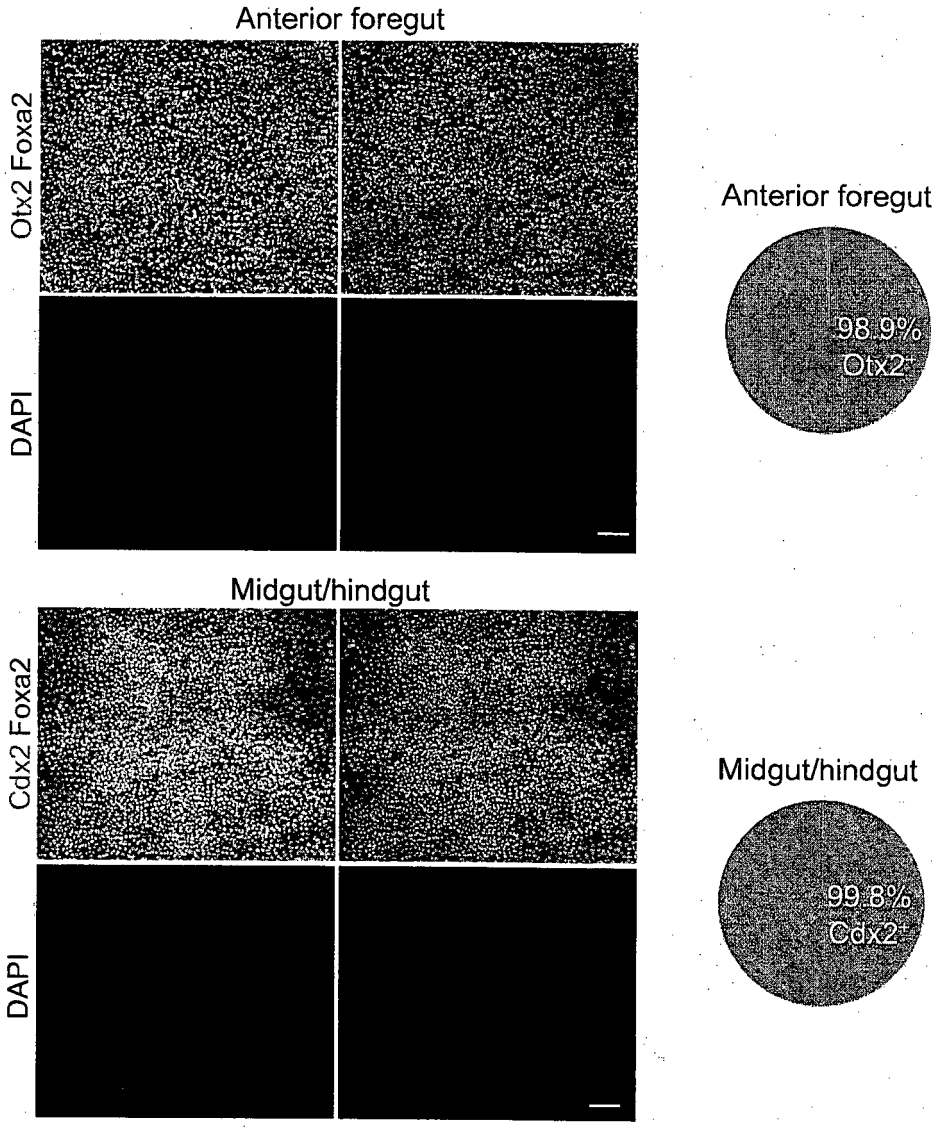

Anterior foregut
Otx2 Foxa2
DAPI
Anterior foregut
98.9%
Otx2+
Midgut/hindgut
Cdx2 Foxa2
DAPI
Midgut/hindgut
99.8%
Cdx2+

FIG. 16

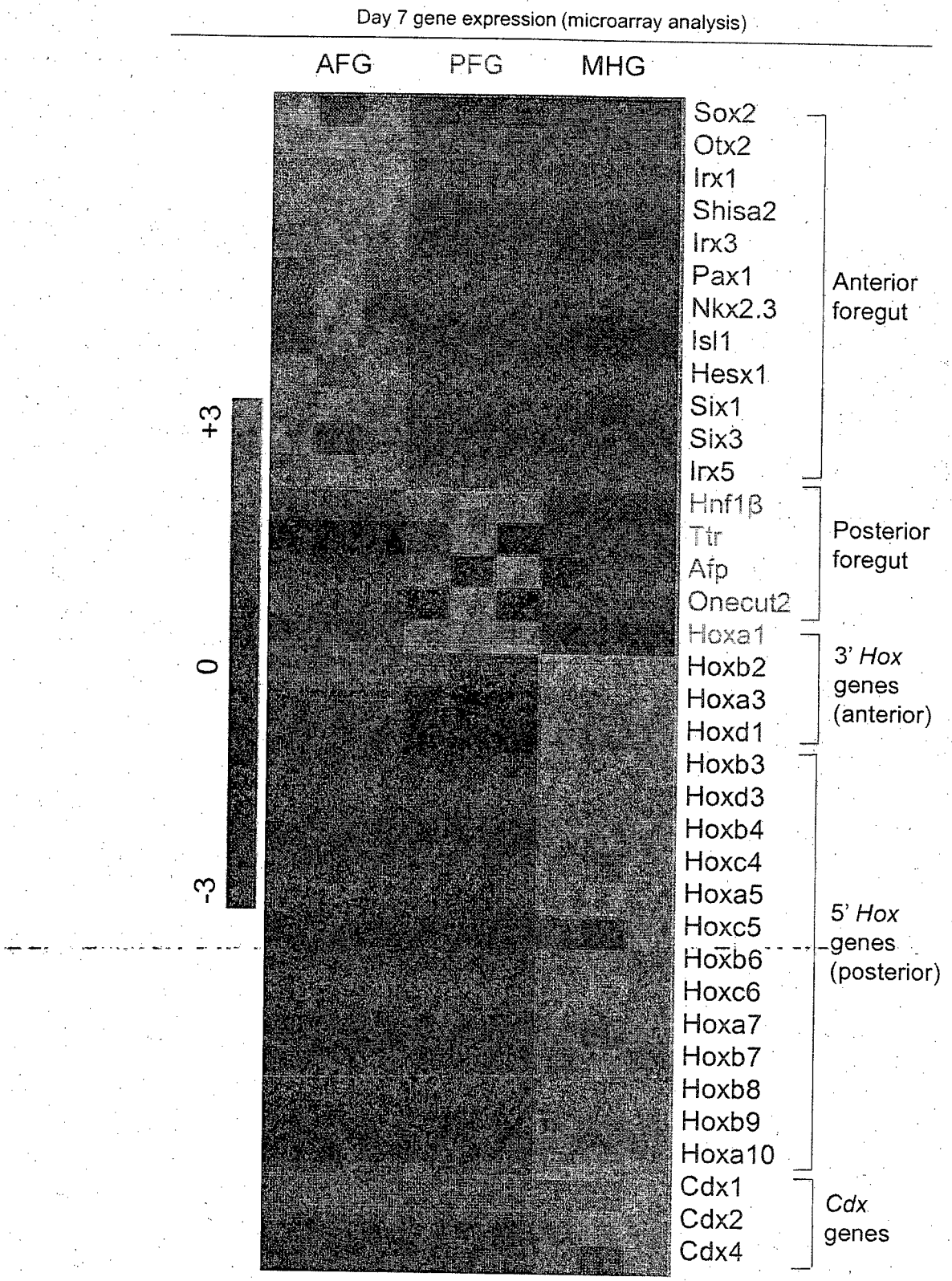

Day 7 gene expression (microarray analysis)
AFG
PFG
MHG
+3
0
-3
Sox2
Otx2
Irx1
Shisa2
Irx3
Pax1
Nkx2.3
Isl1
Hesx1
Six1
Six3
Irx5
Anterior foregut
Hnf1β
Ttr
Afp
Onecut2
Posterior foregut
Hoxa1
Hoxb2
Hoxa3
Hoxd1
3' Hox genes (anterior)
Hoxb3
Hoxd3
Hoxb4
Hoxc4
Hoxa5
Hoxc5
Hoxb6
Hoxc6
Hoxa7
Hoxb7
Hoxb8
Hoxb9
Hoxa10
5' Hox genes (posterior)
Cdx1
Cdx2
Cdx4
Cdx genes

FIG. 17

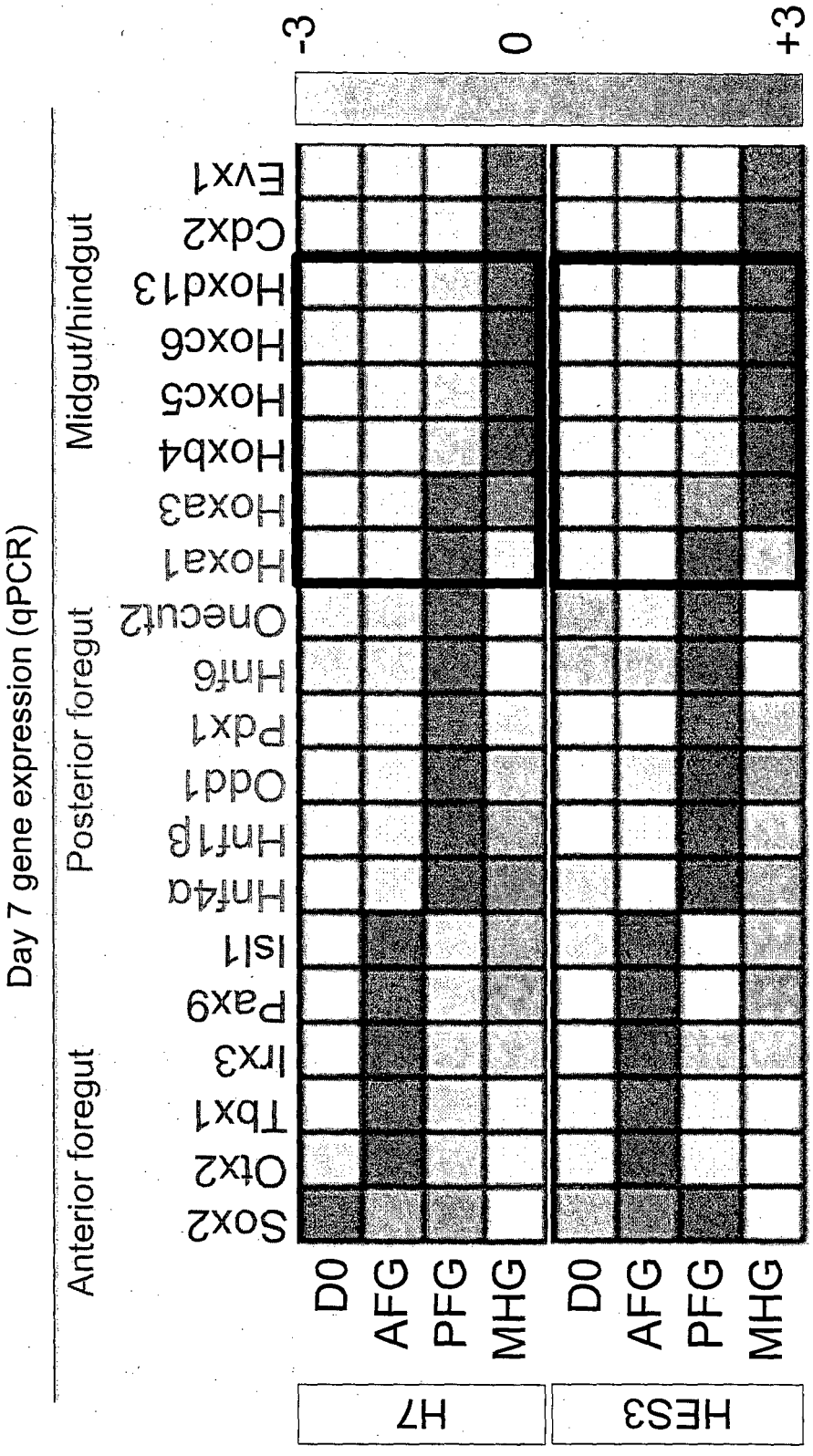

Day 7 gene expression (qPCR)
Anterior foregut
Posterior foregut
Midgut/hindgut
Sox2
Otx2
Tbx1
Irx3
Pax9
Isl1
Hnf4a
Hnf1β
Odd1
Pdx1
Hnf6
Onecut2
Hoxa1
Hoxa3
Hoxb4
Hoxc5
Hoxc6
Hoxd13
Cdx2
Evx1
-3
0
+3
H7
HES3
D0
AFG
PFG
MHG
D0
AFG
PFG
MHG

FIG. 18

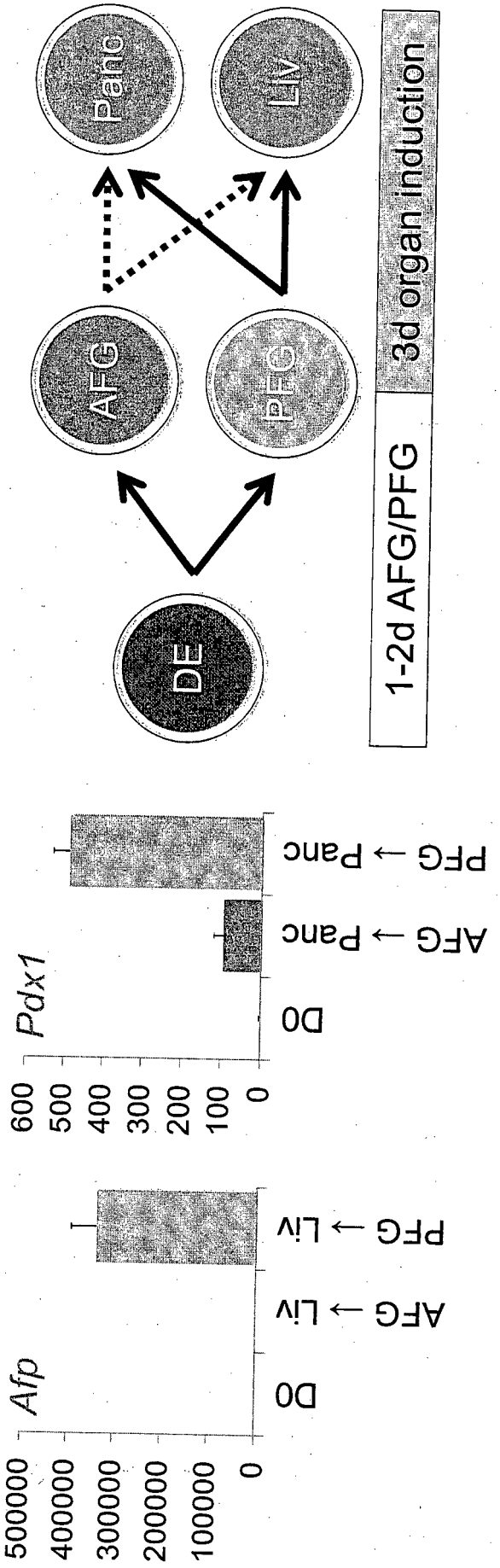

Panc
Liv
AFG
PFG
DE
1-2d AFG/PFG
3d organ induction
Pdx1
600
500
400
300
200
100
0
D0
AFG → Panc
PFG → Panc
Afp
500000
400000
300000
200000
100000
0
D0
AFG → Liv
PFG → Liv

FIG. 19

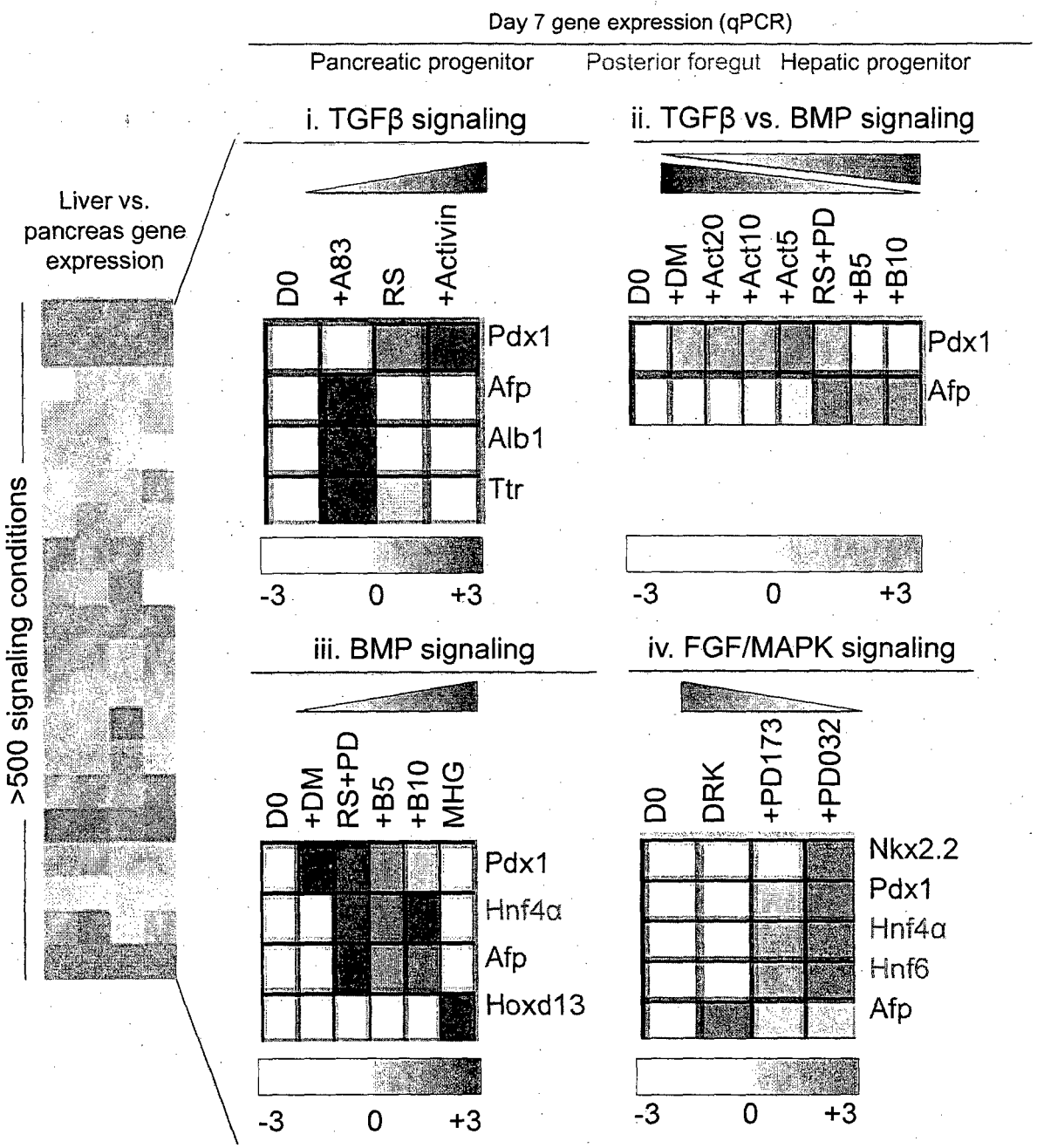
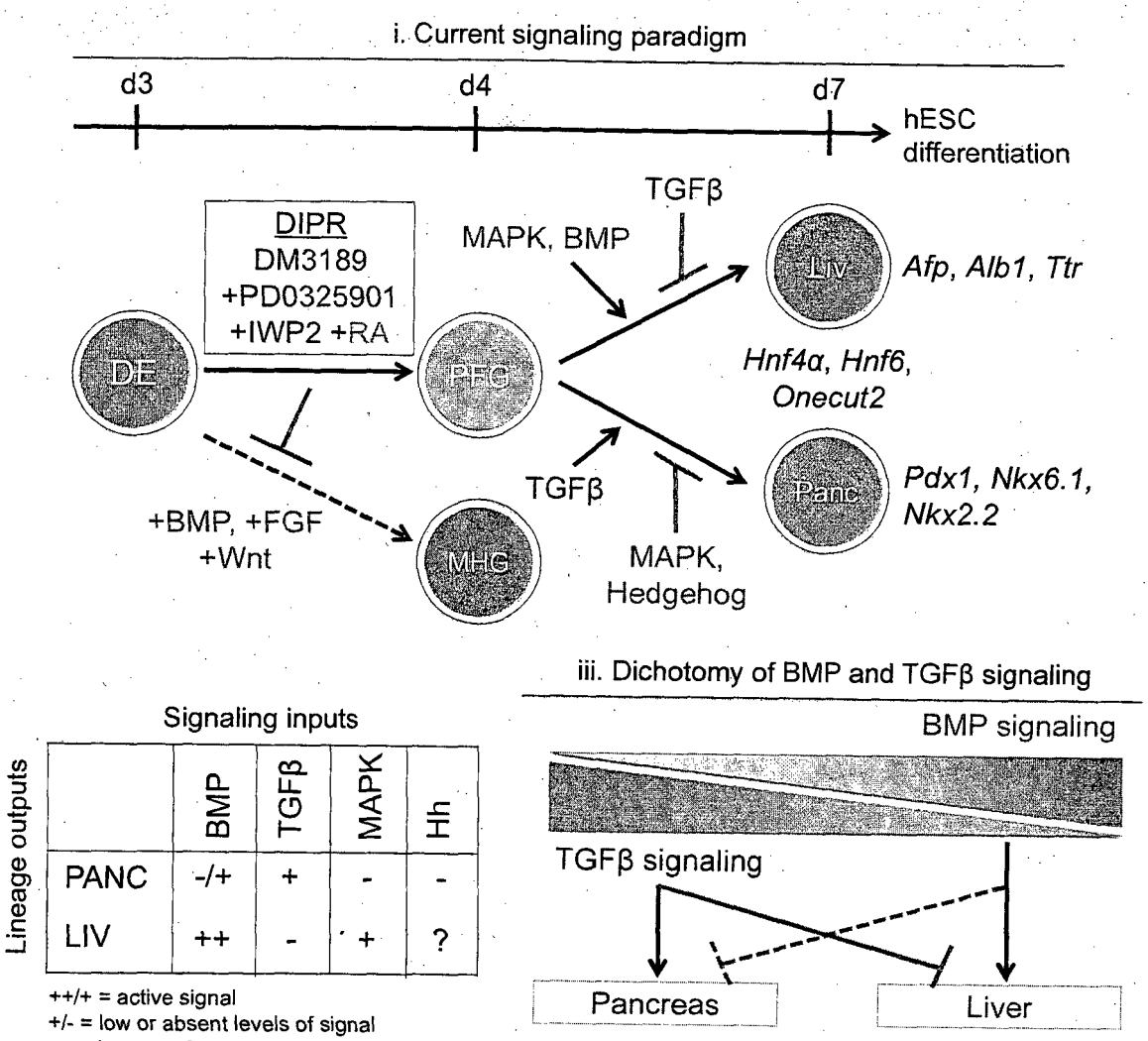

Day 7 gene expression (qPCR)
Pancreatic progenitor
Posterior foregut
Hepatic progenitor
Liver vs. pancreas gene expression
>500 signaling conditions
i. TGFβ signaling
D0
+A83
RS
+Activin
Pdx1
Afp
Alb1
Ttr
-3
0
+3
ii. TGFβ vs. BMP signaling
D0
+DM
+Act20
+Act10
+Act5
RS+PD
+B5
+B10
Pdx1
Afp
-3
0
+3
iii. BMP signaling
D0
+DM
RS+PD
+B5
+B10
MHG
Pdx1
Hnf4α
Afp
Hoxd13
-3
0
+3
iv. FGF/MAPK signaling
D0
DRK
+PD173
+PD032
Nkx2.2
Pdx1
Hnf4α
Hnf6
Afp
-3
0
+3

FIG. 20




Signaling inputs

| Lineage outputs | BMP | TGFβ | MAPK | Hh |
|---|---|---|---|---|
| PANC | -/+ | + | - | - |
| LIV | ++ | - | + | ? |

++/+ = active signal
+/- = low or absent levels of signal
− = absence of signal

FIG. 21

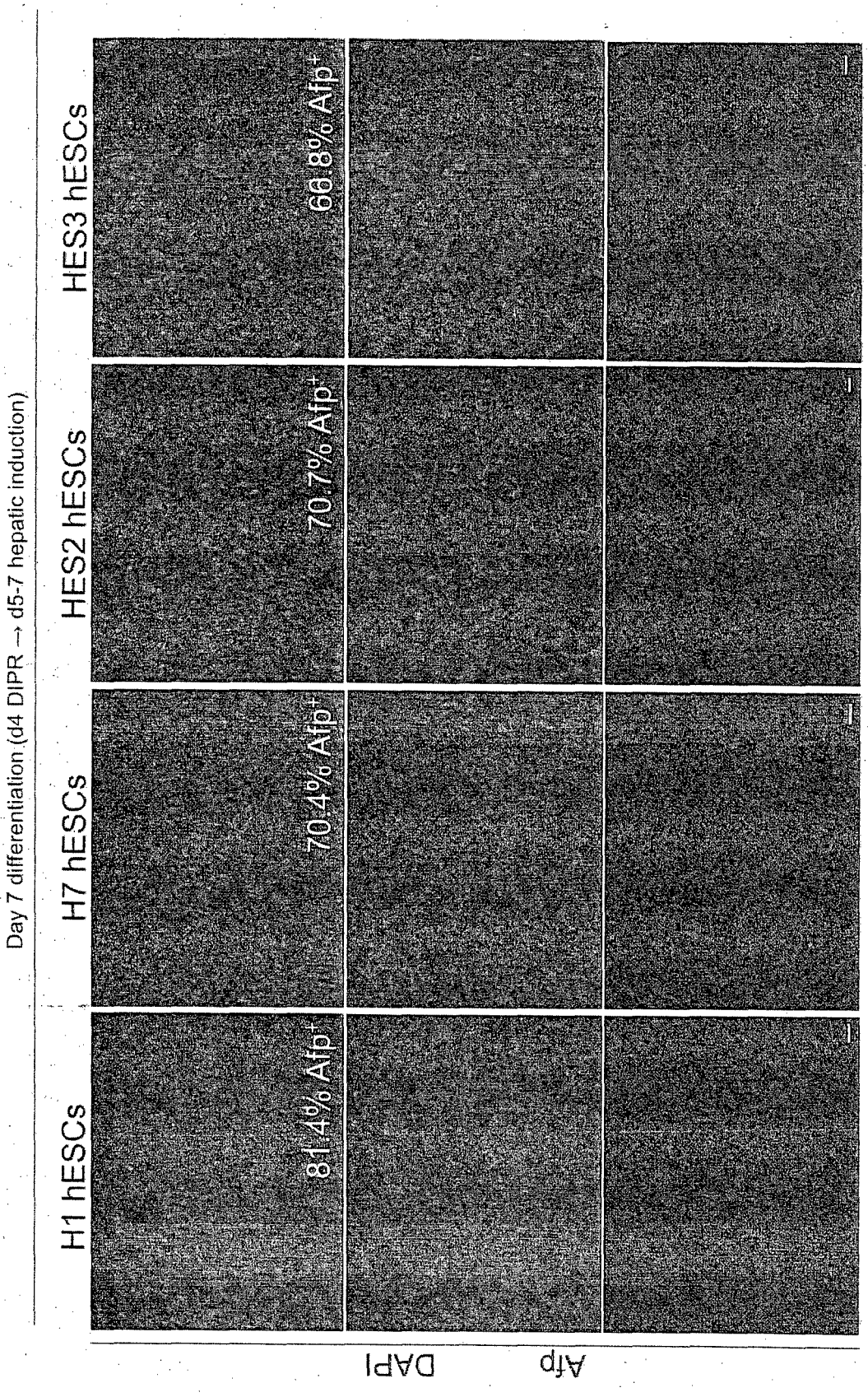

Day 7 differentiation (d4 DIPR → d5-7 hepatic induction)
H1 hESCs
H7 hESCs
HES2 hESCs
HES3 hESCs
81.4% Afp+
70.4% Afp+
70.7% Afp+
66.8% Afp+
Afp
DAPI

FIG. 22

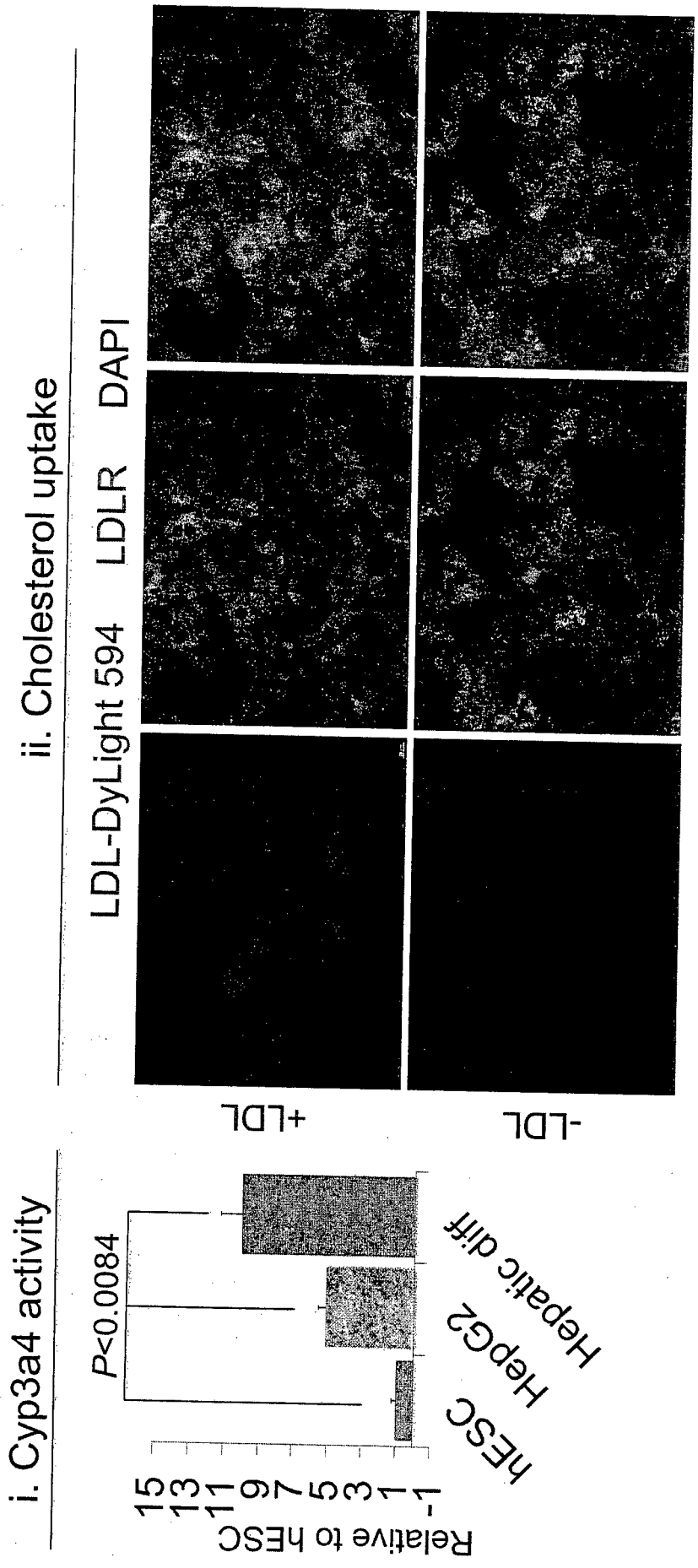

i. Cyp3a4 activity
P<0.0084
Relative to hESC
15
13
11
9
7
5
3
1
-1
hESC
HepG2
Hepatic diff
ii. Cholesterol uptake
LDL-DyLight 594 LDLR DAPI
+LDL
-LDL

FIG. 23

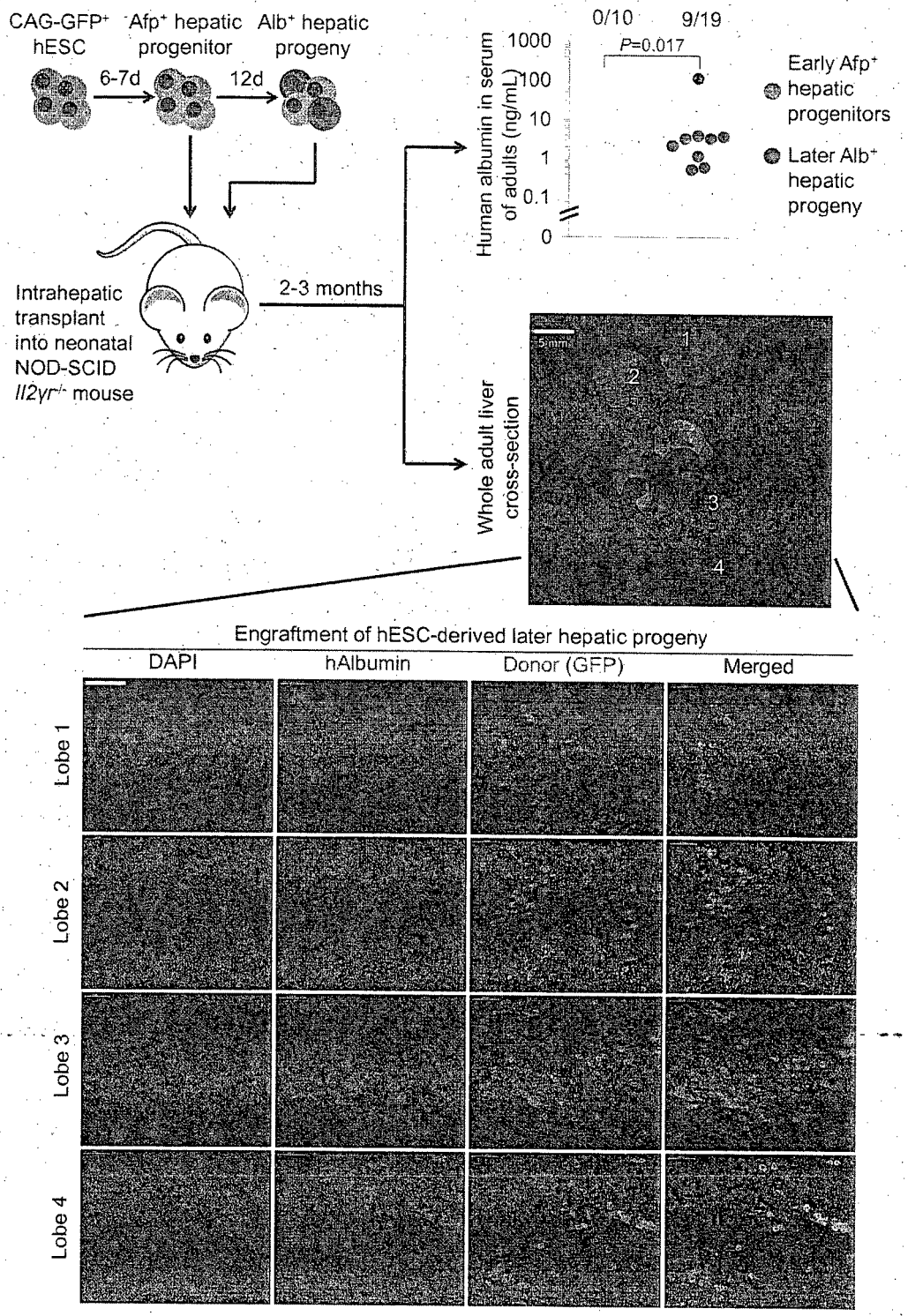

CAG-GFP+ hESC
Afp+ hepatic progenitor
Alb+ hepatic progeny
6-7d
12d
Intrahepatic transplant into neonatal NOD-SCID Il2γr-/- mouse
2-3 months
Human albumin in serum of adults (ng/mL)
1000
100
10
1
0.1
0
0/10
9/19
P=0.017
Early Afp+ hepatic progenitors
Later Alb+ hepatic progeny
Whole adult liver cross-section
5mm
1
2
3
4
Engraftment of hESC-derived later hepatic progeny
DAPI
hAlbumin
Donor (GFP)
Merged
Lobe 1
Lobe 2
Lobe 3
Lobe 4

FIG. 24

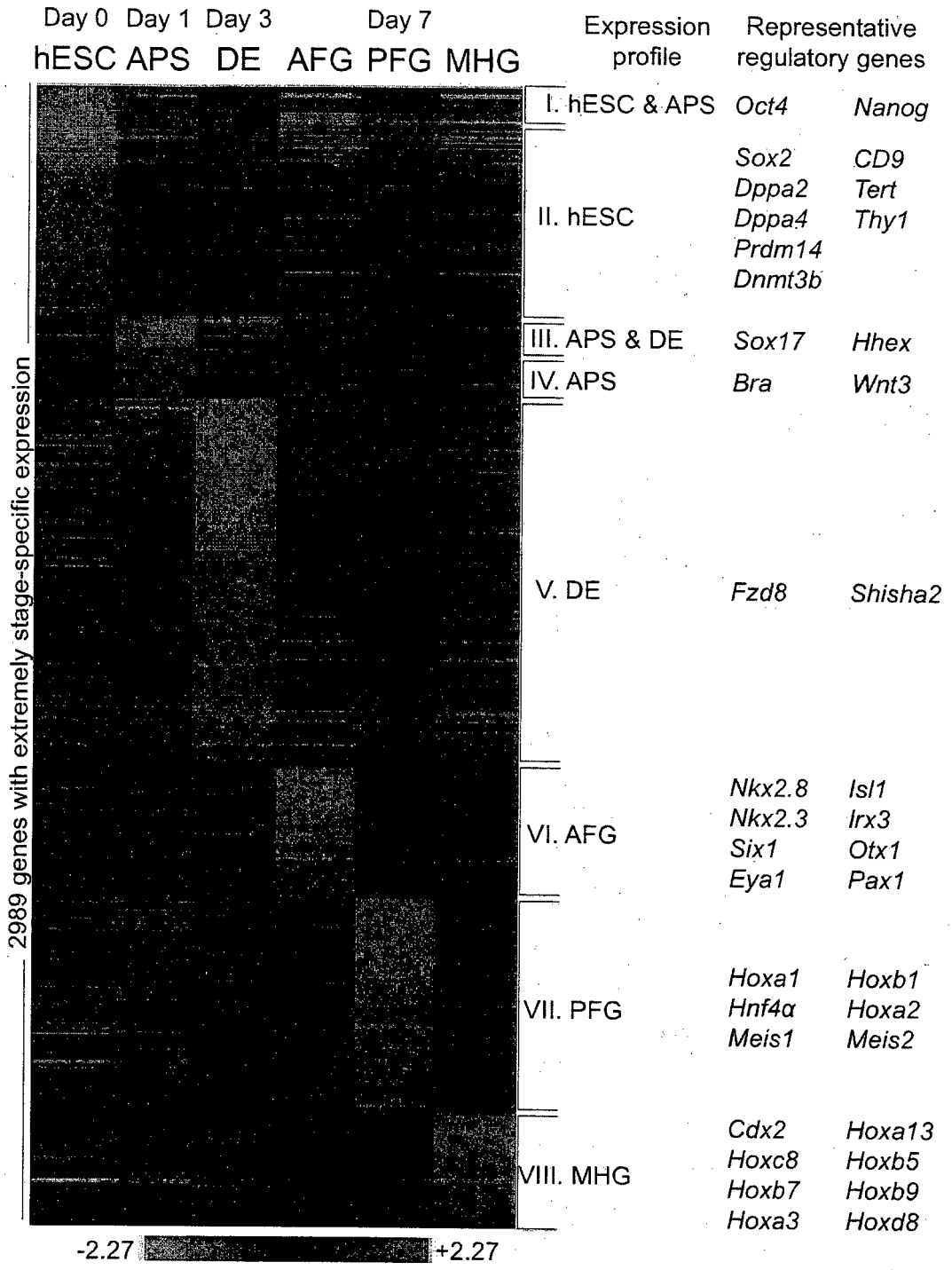

Day 0 Day 1 Day 3 Day 7
hESC APS DE AFG PFG MHG
2989 genes with extremely stage-specific expression
-2.27
+2.27
Expression profile
Representative regulatory genes
I. hESC & APS
Oct4
Nanog
II. hESC
Sox2
Dppa2
Dppa4
Prdm14
Dnmt3b
CD9
Tert
Thy1
III. APS & DE
Sox17
Hhex
IV. APS
Bra
Wnt3
V. DE
Fzd8
Shisha2
VI. AFG
Nkx2.8
Nkx2.3
Six1
Eya1
Isl1
Irx3
Otx1
Pax1
VII. PFG
Hoxa1
Hnf4α
Meis1
Hoxb1
Hoxa2
Meis2
VIII. MHG
Cdx2
Hoxc8
Hoxb7
Hoxa3
Hoxa13
Hoxb5
Hoxb9
Hoxd8

FIG. 25

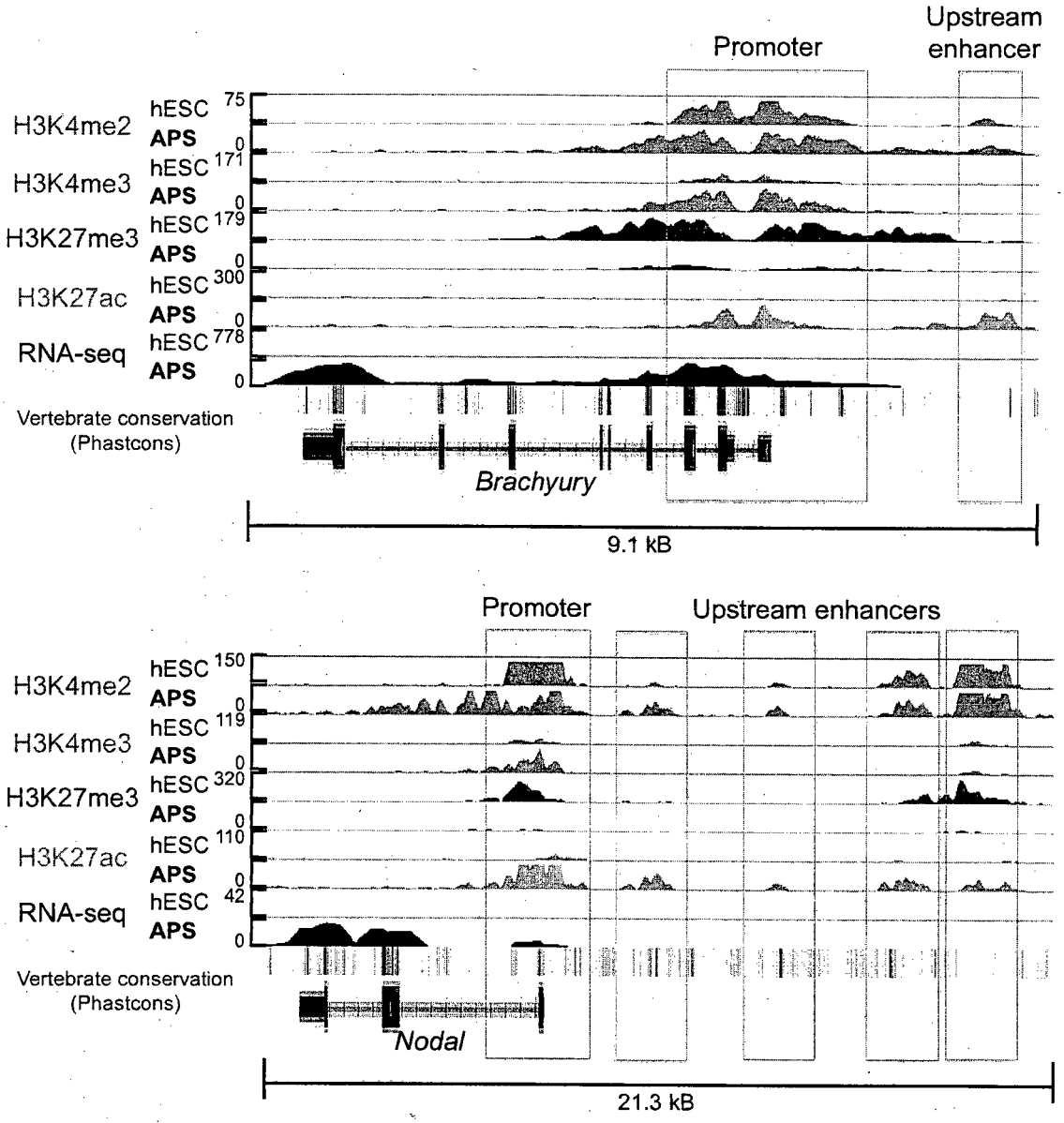

Promoter
Upstream enhancer
H3K4me2
hESC 75
APS 0
H3K4me3
hESC 171
APS 0
H3K27me3
hESC 179
APS 0
H3K27ac
hESC 300
APS 0
RNA-seq
hESC 778
APS 0
Vertebrate conservation (Phastcons)
Brachyury
9.1 kB
Promoter
Upstream enhancers
H3K4me2
hESC 150
APS 0
H3K4me3
hESC 119
APS 0
H3K27me3
hESC 320
APS 0
H3K27ac
hESC 110
APS 0
RNA-seq
hESC 42
APS 0
Vertebrate conservation (Phastcons)
Nodal
21.3 kB

FIG. 26

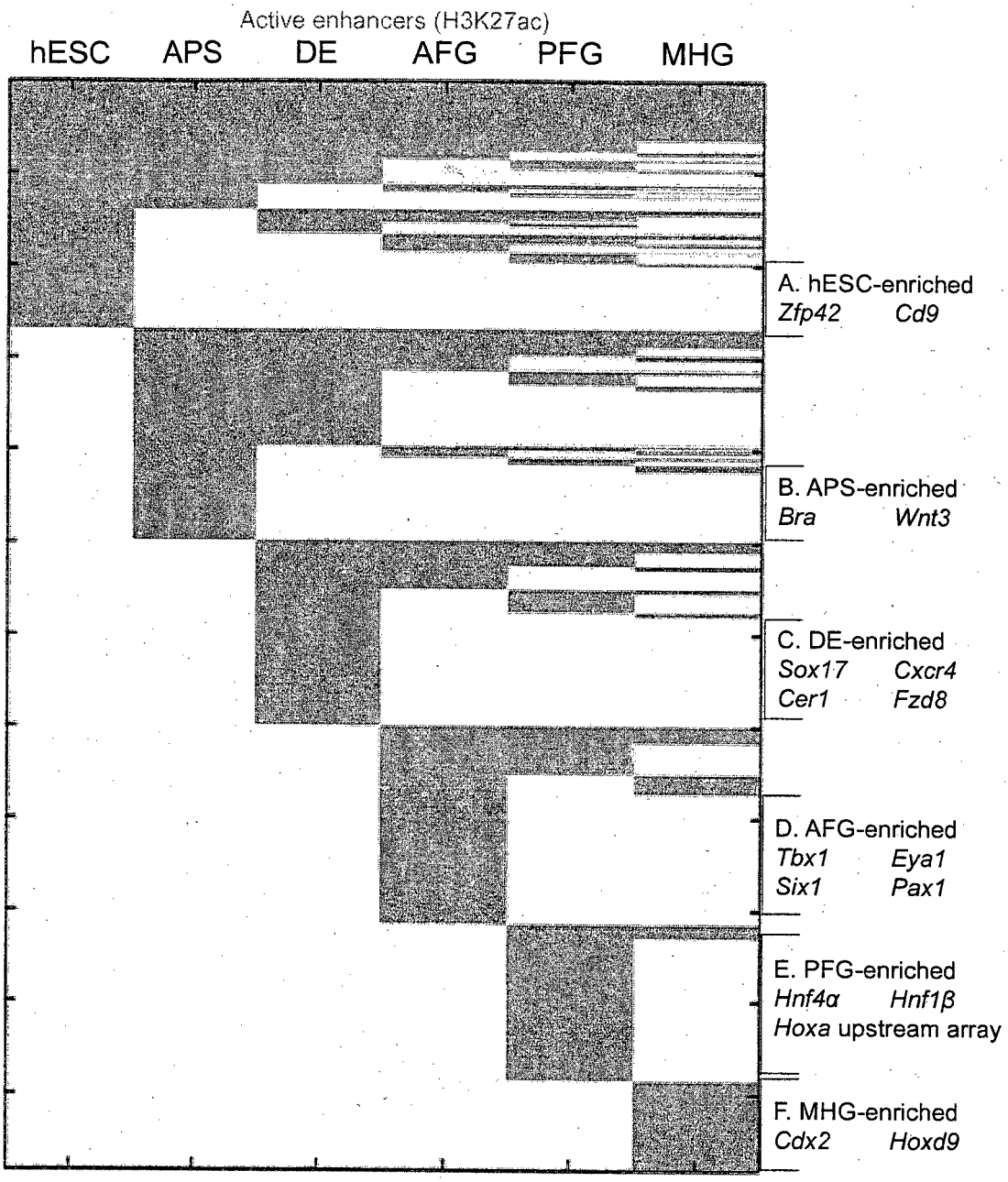

Active enhancers (H3K27ac)
hESC
APS
DE
AFG
PFG
MHG
A. hESC-enriched
Zfp42 Cd9
B. APS-enriched
Bra Wnt3
C. DE-enriched
Sox17 Cxcr4
Cer1 Fzd8
D. AFG-enriched
Tbx1 Eya1
Six1 Pax1
E. PFG-enriched
Hnf4α Hnf1β
Hoxa upstream array
F. MHG-enriched
Cdx2 Hoxd9

FIG. 27

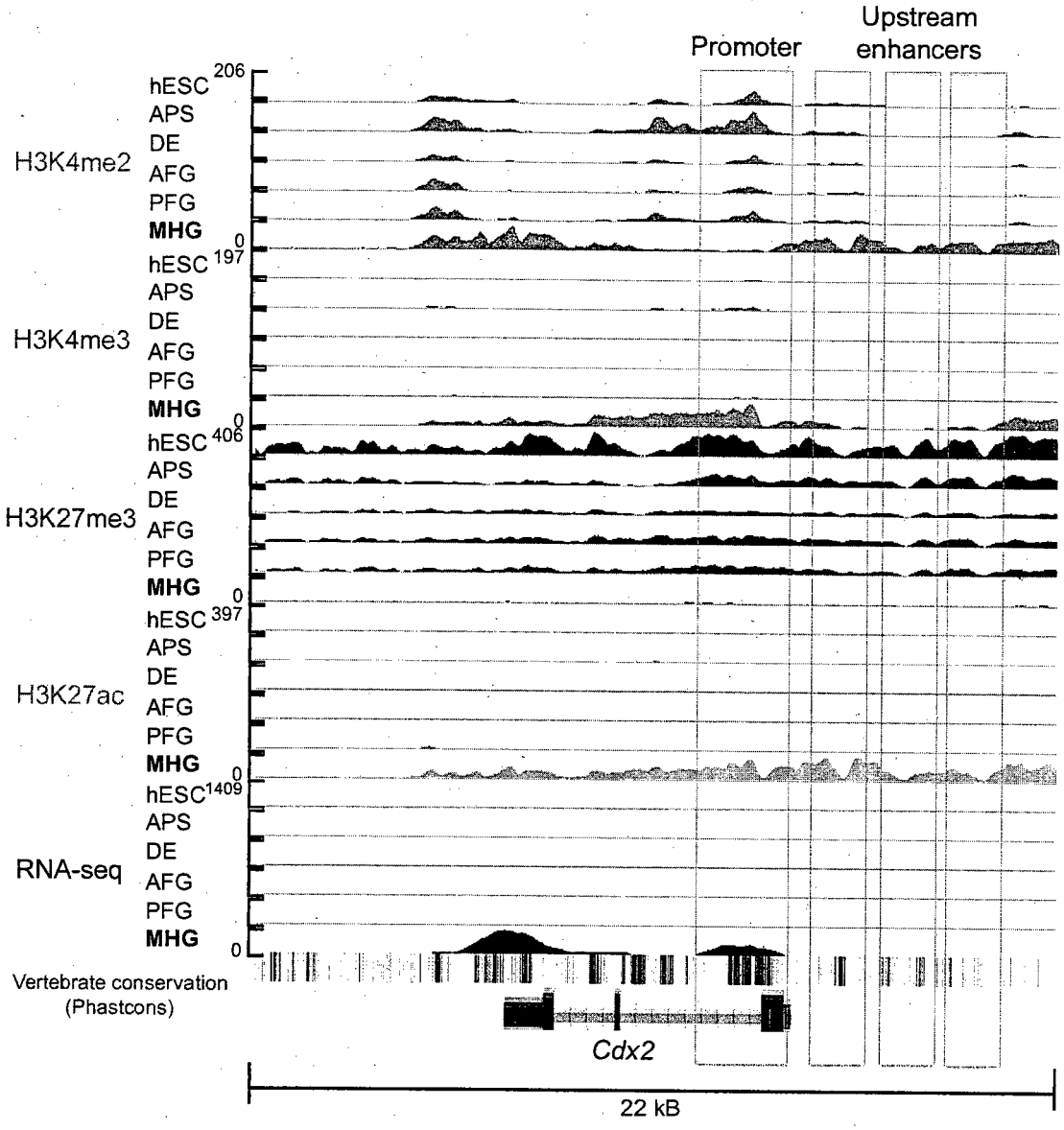

Promoter
Upstream enhancers
H3K4me2
H3K4me3
H3K27me3
H3K27ac
RNA-seq
hESC
APS
DE
AFG
PFG
MHG
206
197
406
397
1409
0
Vertebrate conservation (Phastcons)
Cdx2
22 kB

FIG. 28

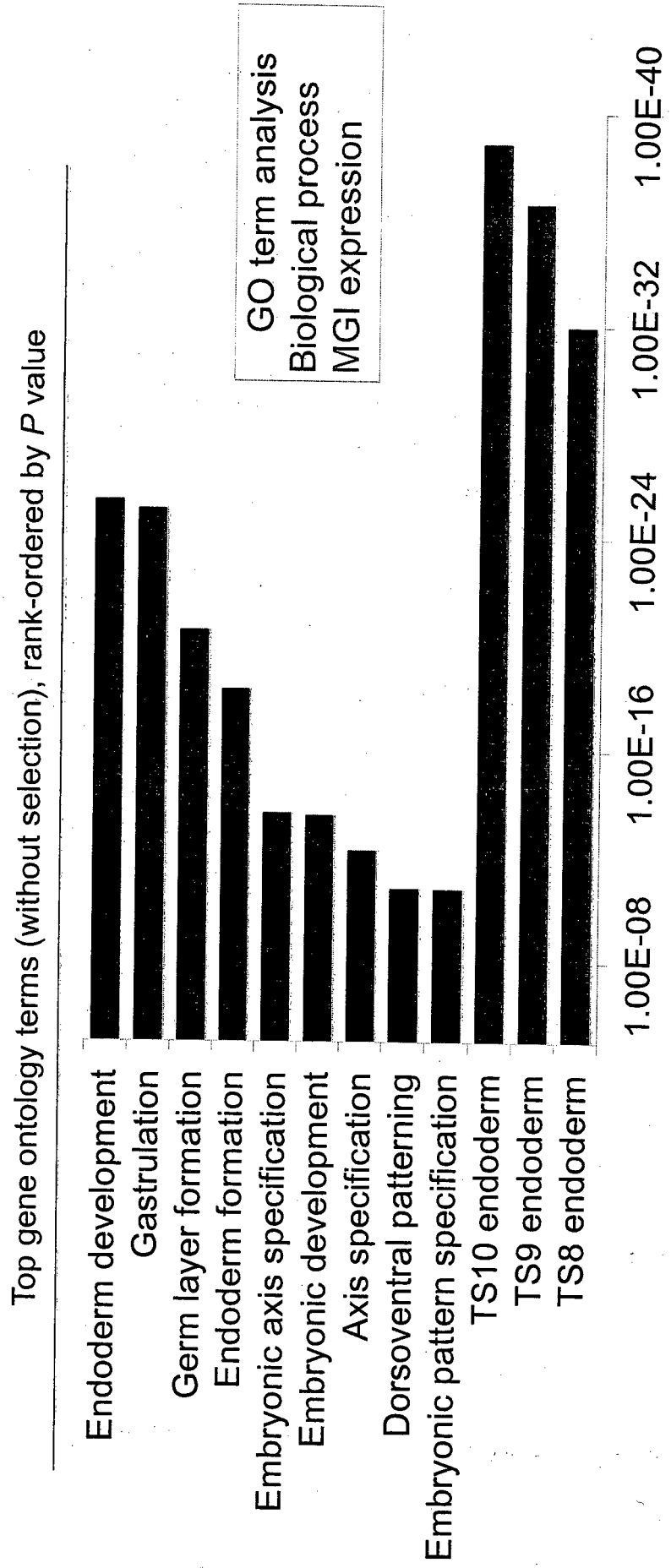

Top gene ontology terms (without selection), rank-ordered by P value
GO term analysis
Biological process
MGI expression
Endoderm development
Gastrulation
Germ layer formation
Endoderm formation
Embryonic axis specification
Embryonic development
Axis specification
Dorsoventral patterning
Embryonic pattern specification
TS10 endoderm
TS9 endoderm
TS8 endoderm
1.00E-08
1.00E-16
1.00E-24
1.00E-32
1.00E-40
Binomial P value

FIG. 29

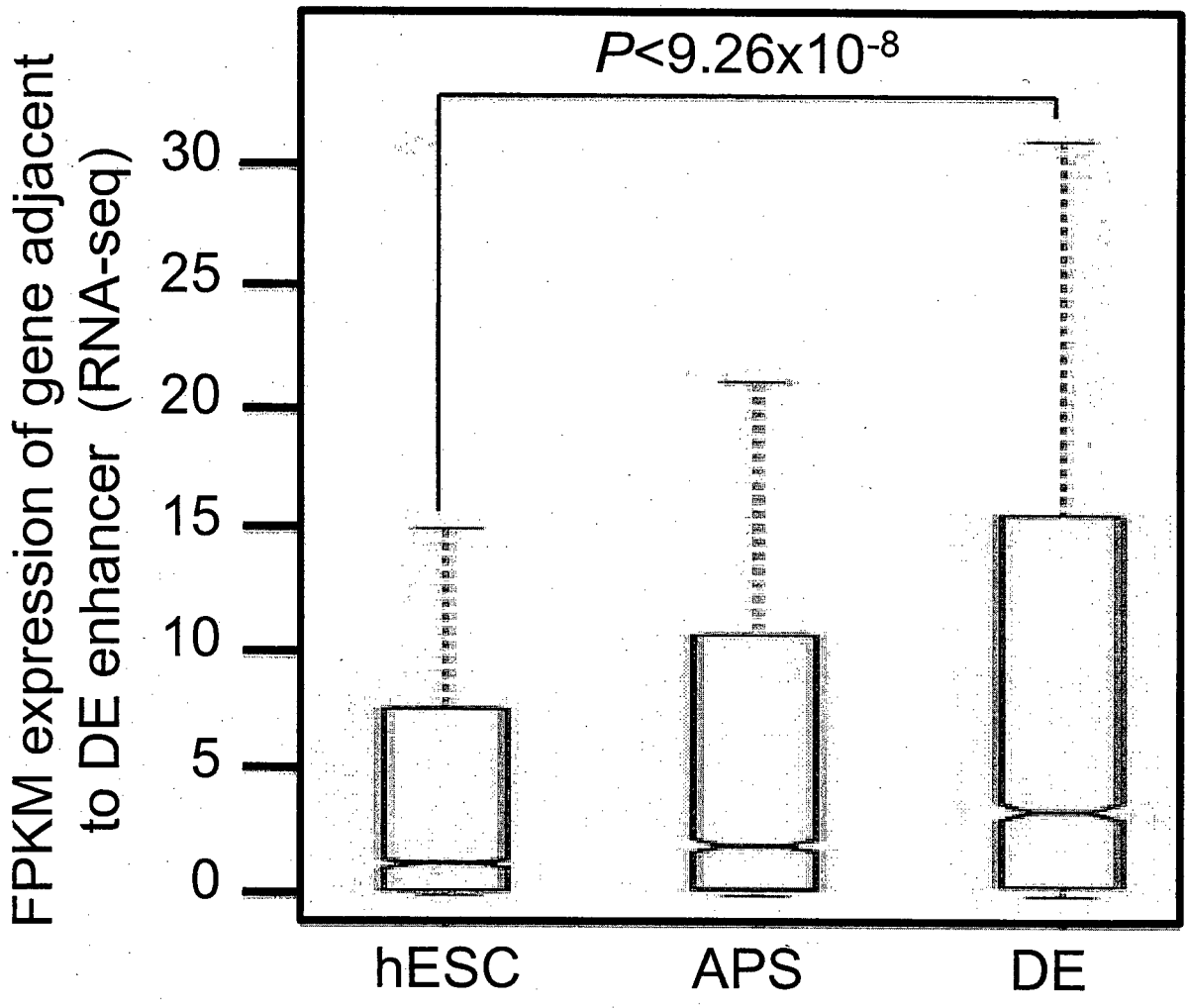

P<9.26x10-8
FPKM expression of gene adjacent to DE enhancer (RNA-seq)
30
25
20
15
10
5
0
hESC
APS
DE

FIG. 30

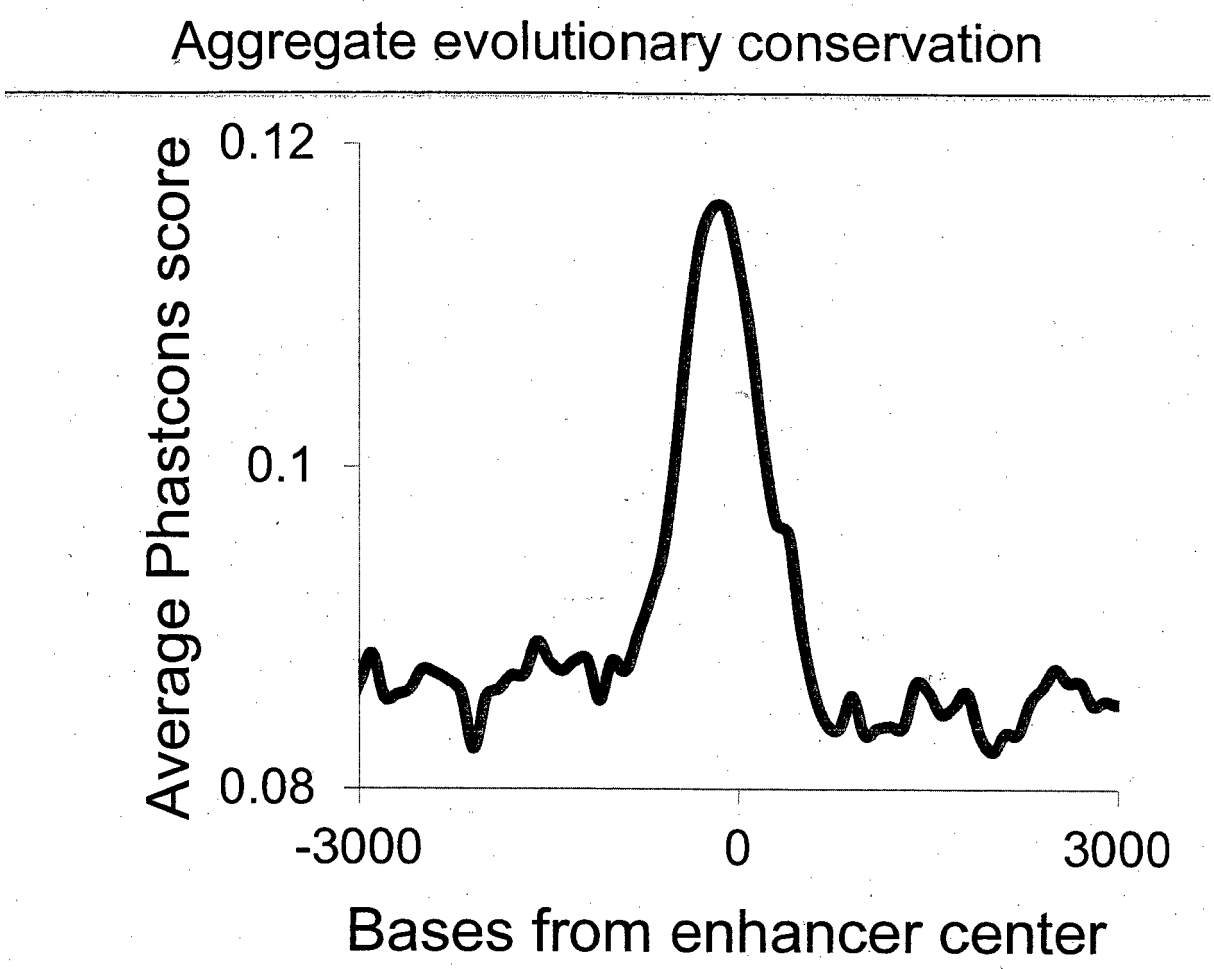

Aggregate evolutionary conservation
Average Phastcons score
0.12
0.1
0.08
-3000
0
3000
Bases from enhancer center

FIG. 31

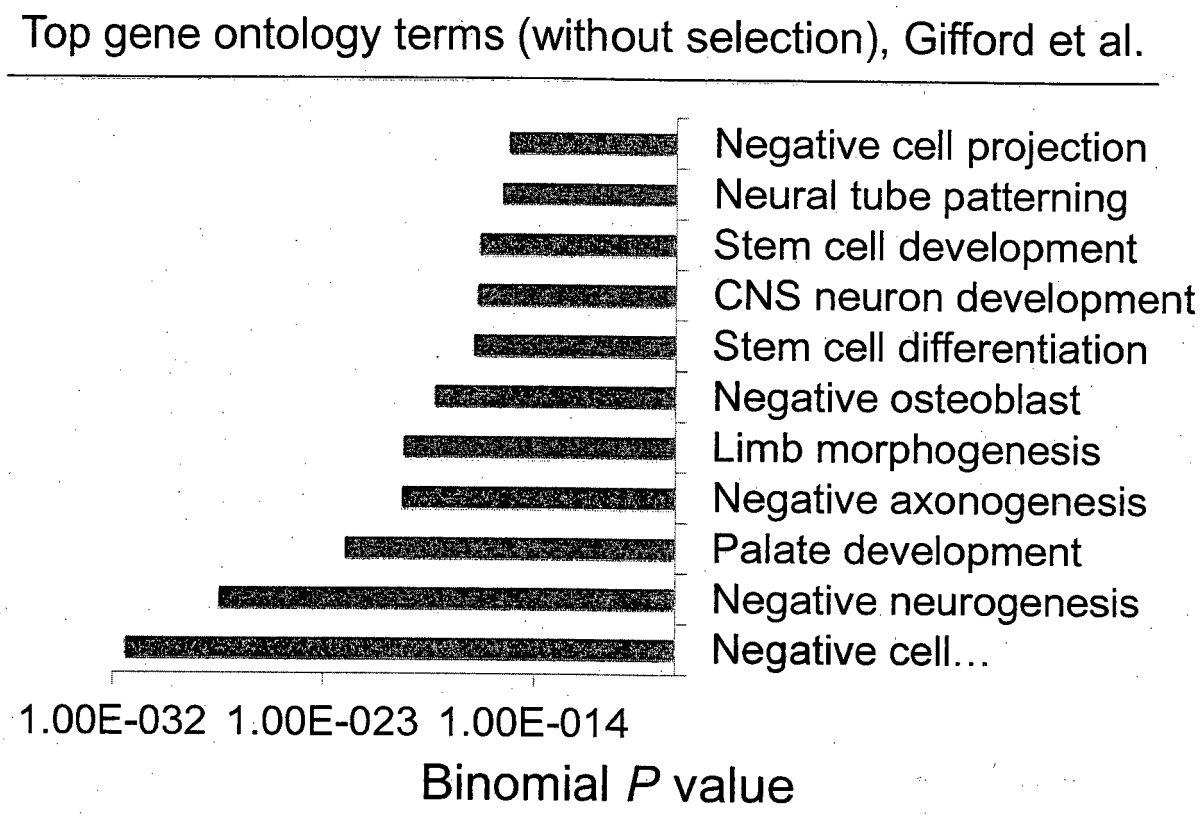

Top gene ontology terms (without selection), Gifford et al.
Negative cell projection
Neural tube patterning
Stem cell development
CNS neuron development
Stem cell differentiation
Negative osteoblast
Limb morphogenesis
Negative axonogenesis
Palate development
Negative neurogenesis
Negative cell...
1.00E-032
1.00E-023
1.00E-014
Binomial P value

FIG. 32

Within top 15 enriched motifs, rank-ordered by *P* value



Smad2/3
($P<10^{-197}$, $Q\approx0$)



Foxh1
($P<10^{-61}$, $Q\approx0$)



Eomes
($P<10^{-73}$, $Q\approx0$)



Foxa2
($P<10^{-59}$, $Q\approx0$)

FIG. 33

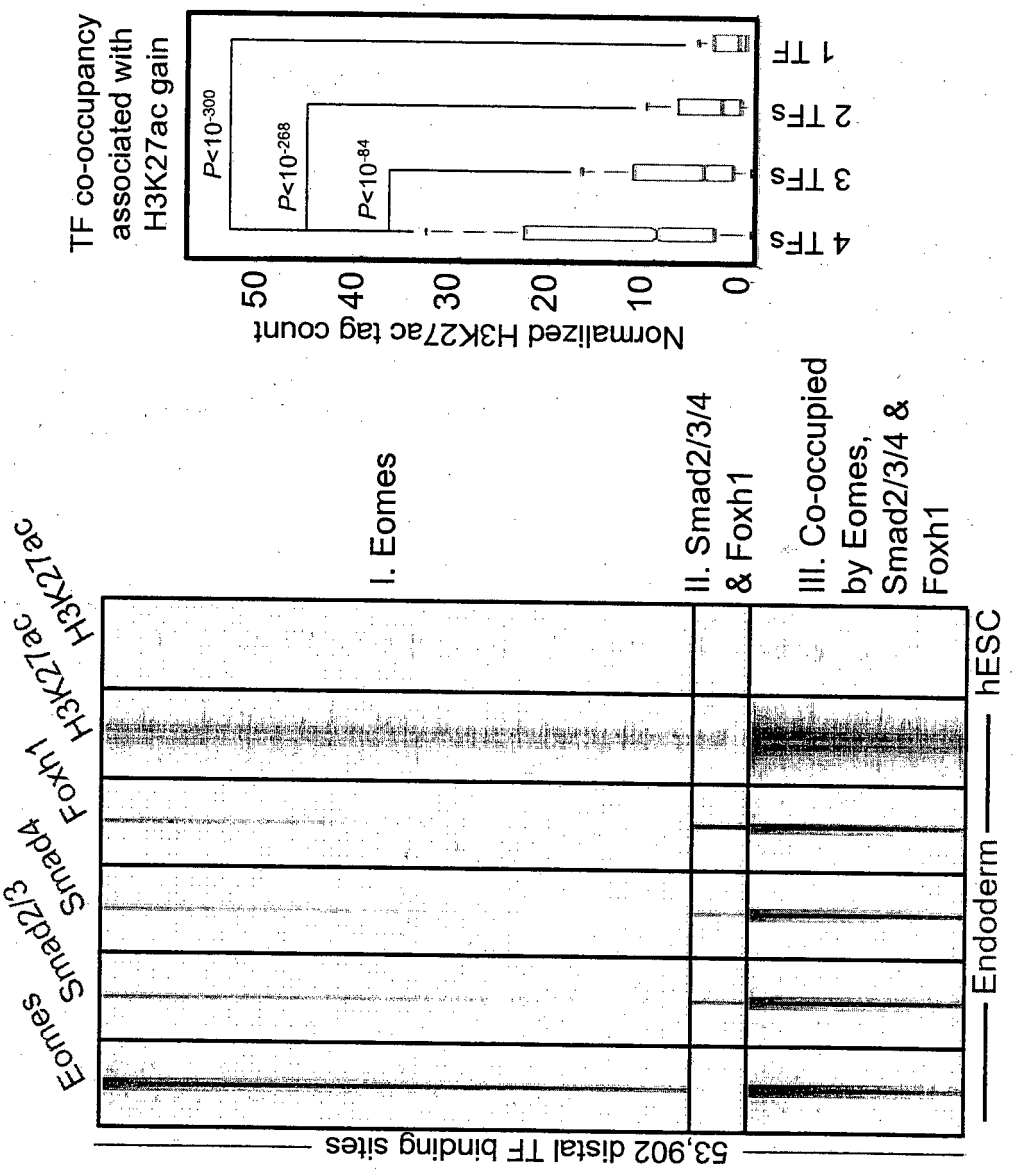

TF co-occupancy associated with H3K27ac gain
P<10-300
P<10-268
P<10-84
4 TFs
3 TFs
2 TFs
1 TF
Normalized H3K27ac tag count
0
10
20
30
40
50
I. Eomes
II. Smad2/3/4 & Foxh1
III. Co-occupied by Eomes, Smad2/3/4 & Foxh1
Eomes
Smad2/3
Smad4
Foxh1
H3K4me1
H3K27ac
53,902 distal TF binding sites
Endoderm
hESC

FIG. 34

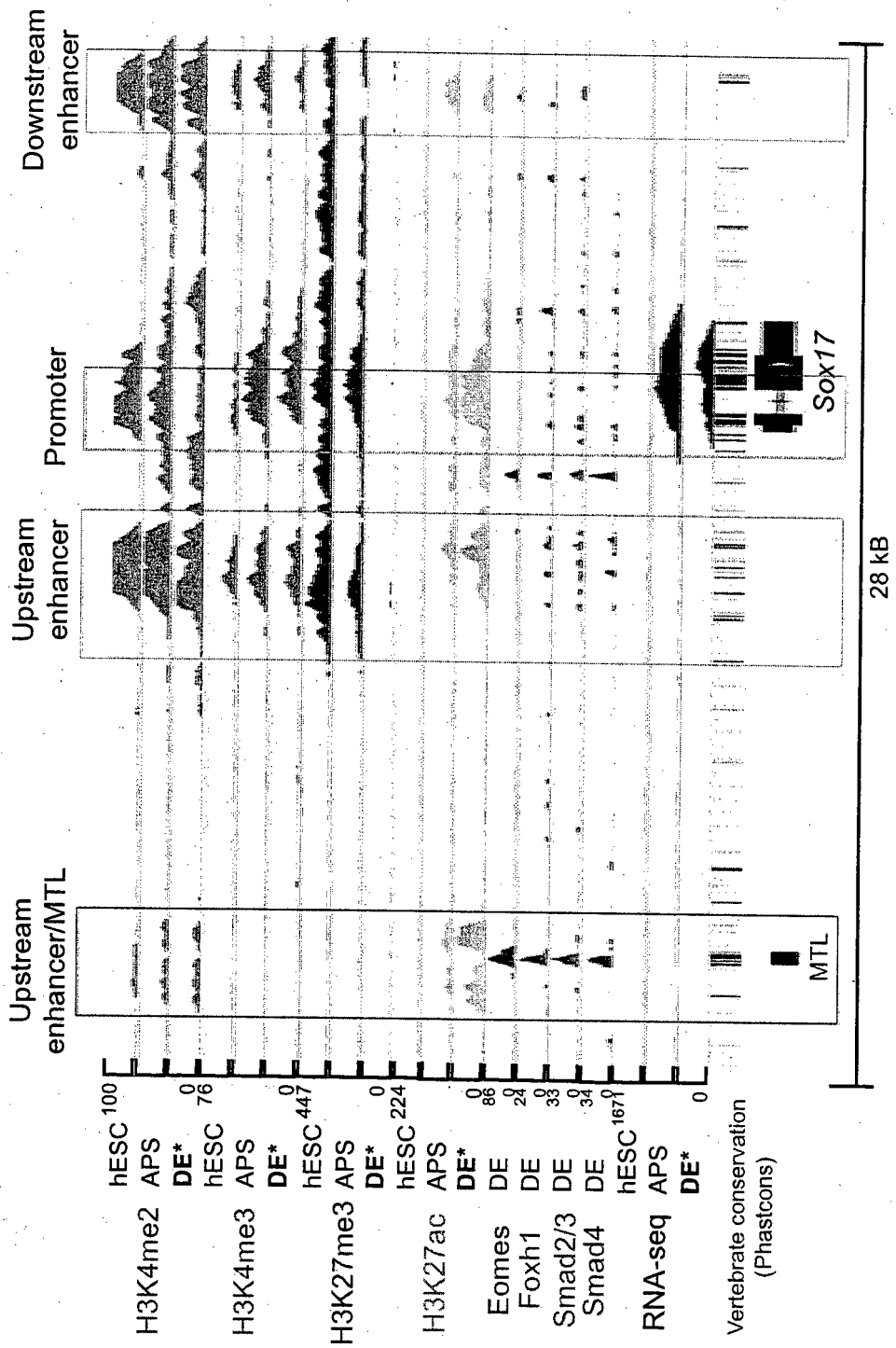
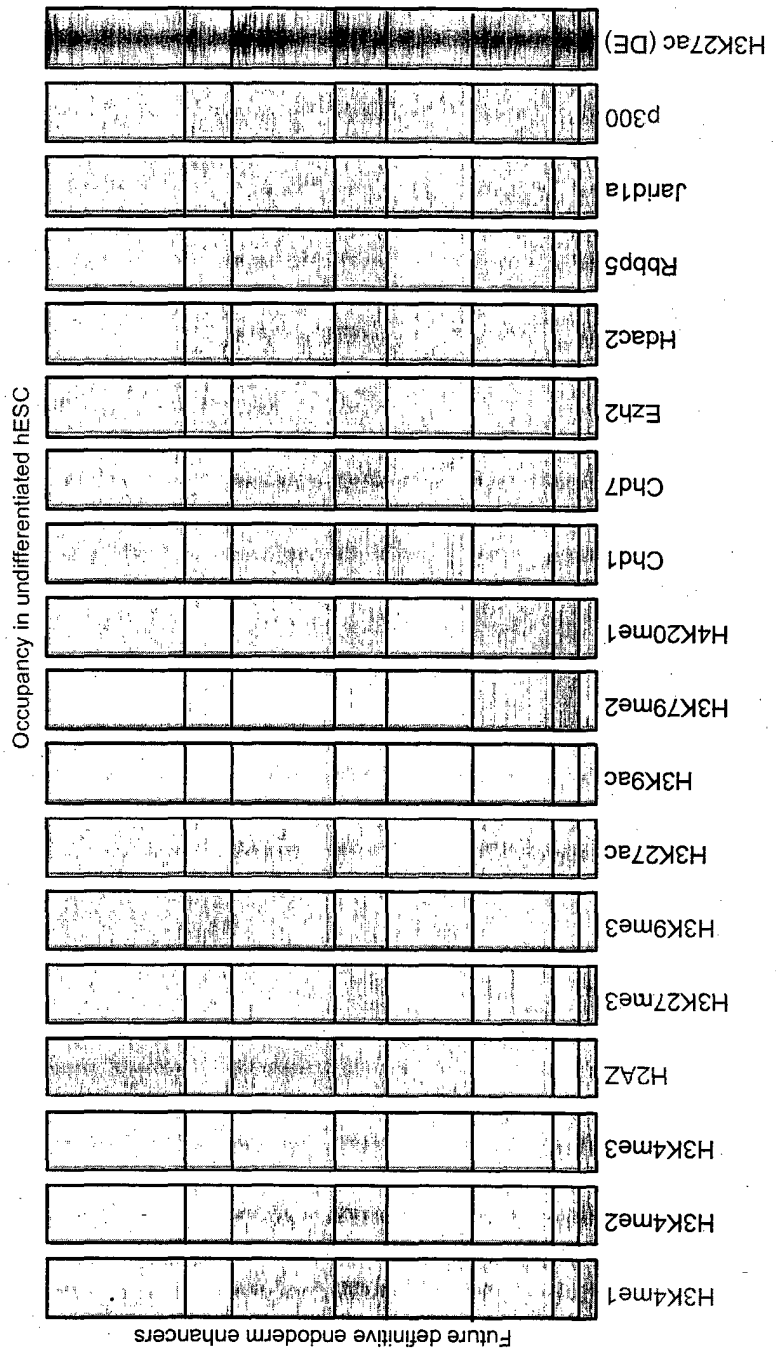

Upstream enhancer/MTL
Upstream enhancer
Promoter
Downstream enhancer
MTL
Sox17
28 kB
H3K4me2
H3K4me3
H3K27me3
H3K27ac
Eomes
Foxh1
Smad2/3
Smad4
RNA-seq
hESC
APS
DE*
DE
Vertebrate conservation (Phastcons)

FIG. 35


Cluster 1: H2AZ alone
Cluster 2: H3K9me3 alone
Cluster 3: H2AZ and H3K4me1
Cluster 4: H3K4me1, with low H2AZ, H3K27me3 and p300 (poised)
Cluster 5: Largely barren (latent)
Cluster 6: Low H4K20me1
Cluster 7: H3K79me2 and H4K20me1
Cluster 8: H3K4me1, with low H2AZ, H3K27me3 and p300 (poised)
H3K27ac (DE)
p300
Jarid1a
Rbbp5
Hdac2
Ezh2
Chd7
Chd1
H4K20me1
H3K79me2
H3K9ac
H3K27ac
H3K9me3
H3K27me3
H2AZ
H3K4me3
H3K4me2
H3K4me1
Occupancy in undifferentiated hESC
Future definitive endoderm enhancers

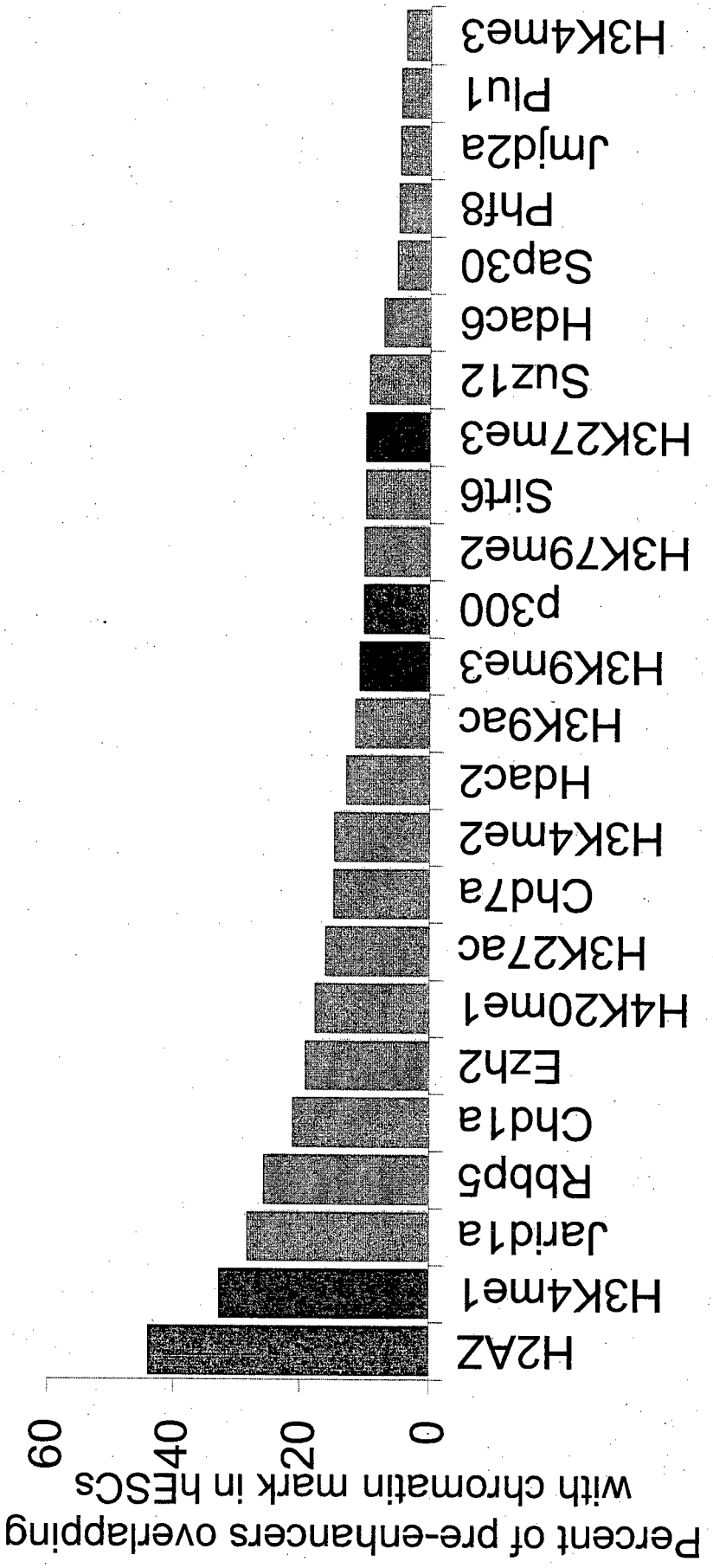

Percent of pre-enhancers overlapping with chromatin mark in hESCs
0
20
40
60
H2AZ
H3K4me1
Jarid1a
Rbbp5
Chd1a
Ezh2
H4K20me1
H3K27ac
Chd7a
H3K4me2
Hdac2
H3K9ac
H3K9me3
p300
H3K79me2
Sirt6
H3K27me3
Suz12
Hdac6
Sap30
Ph!8
Jmjd2a
Plu1
H3K4me3

FIG. 36

FIG. 37

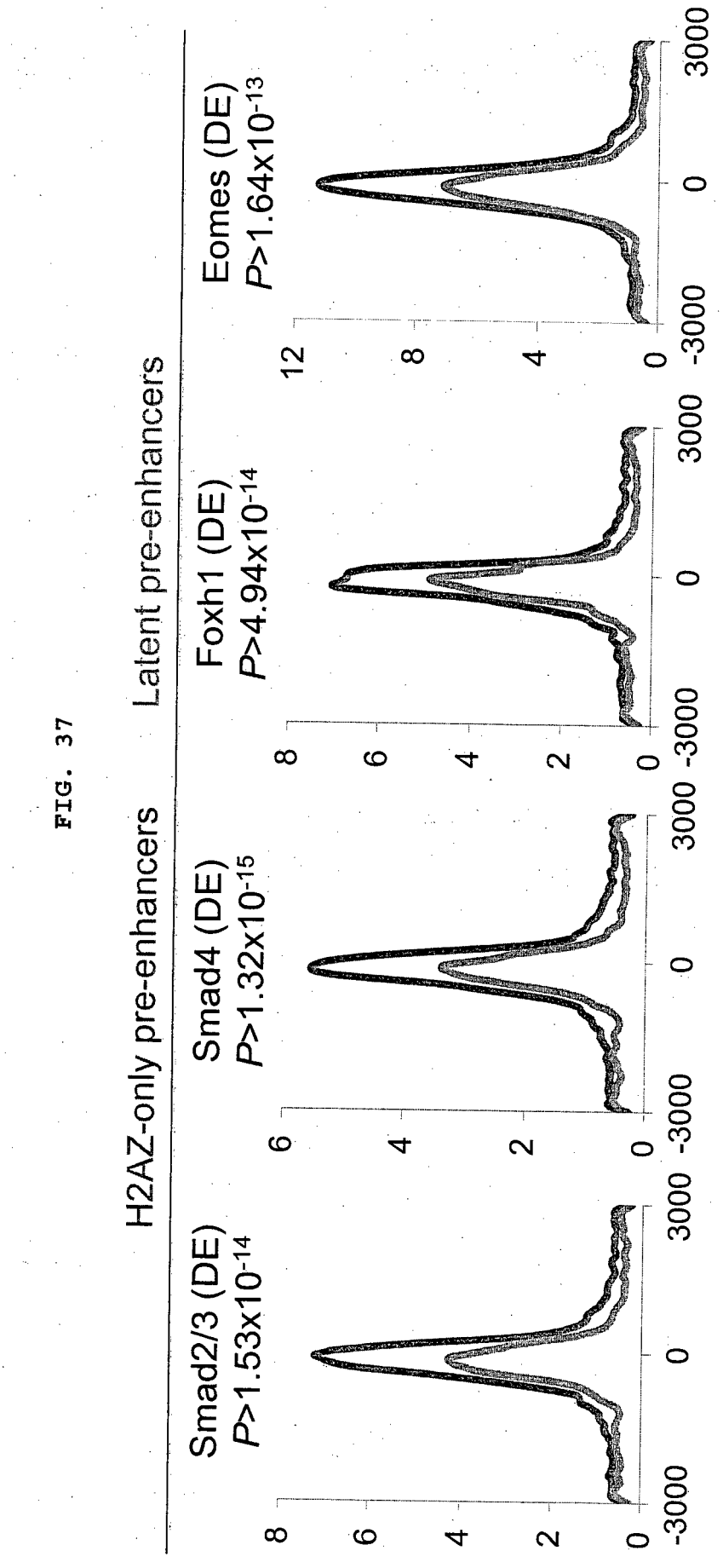

H2AZ-only pre-enhancers
Latent pre-enhancers
Smad2/3 (DE)
P>1.53x10-14
Smad4 (DE)
P>1.32x10-15
Foxh1 (DE)
P>4.94x10-14
Eomes (DE)
P>1.64x10-13
-3000
0
3000

FIG. 38

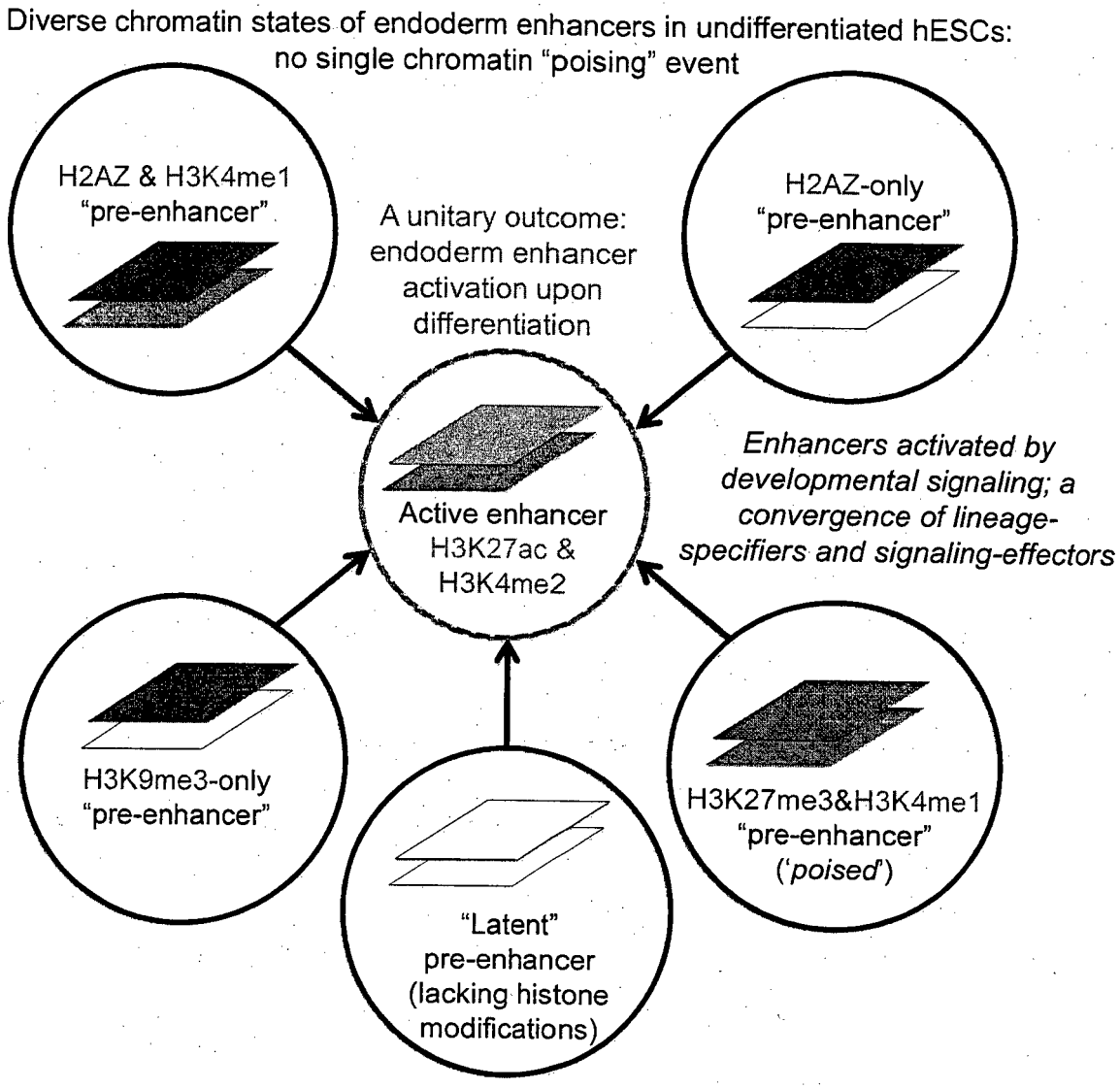

Diverse chromatin states of endoderm enhancers in undifferentiated hESCs: no single chromatin "poising" event
H2AZ & H3K4me1 "pre-enhancer"
A unitary outcome: endoderm enhancer activation upon differentiation
H2AZ-only "pre-enhancer"
Active enhancer H3K27ac & H3K4me2
Enhancers activated by developmental signaling; a convergence of lineage-specifiers and signaling-effectors
H3K9me3-only "pre-enhancer"
H3K27me3&H3K4me1 "pre-enhancer" ('poised')
"Latent" pre-enhancer (lacking histone modifications)

FIG. 39

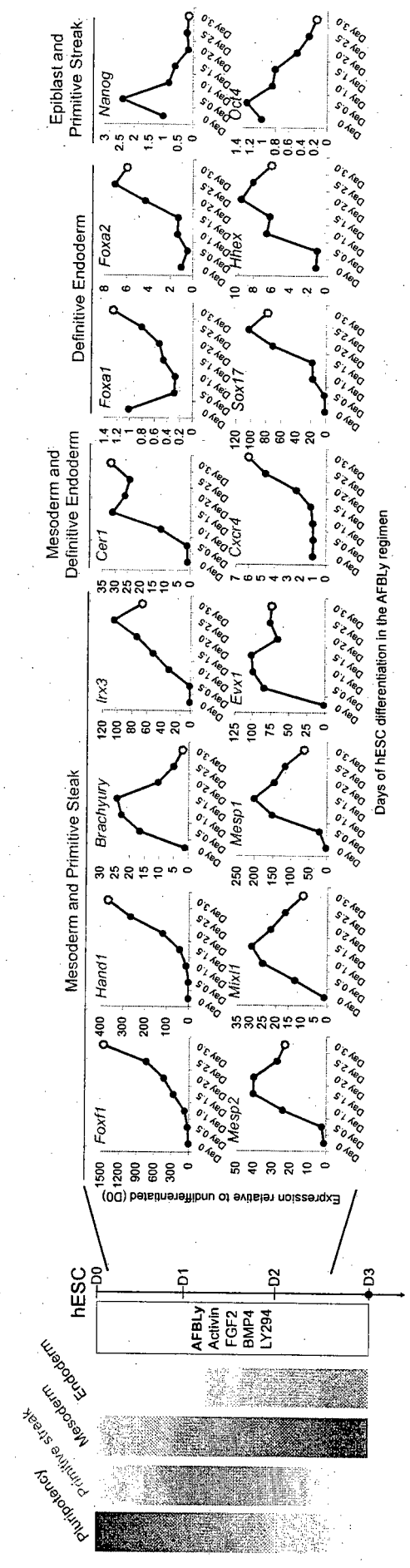
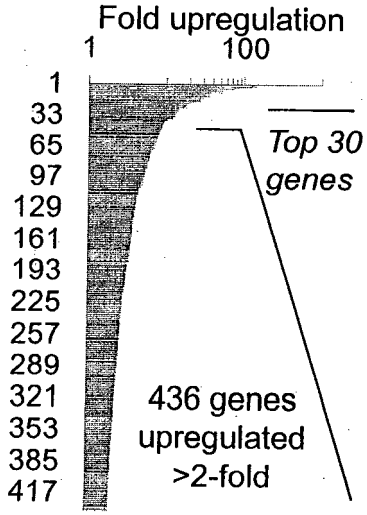

Pluripotency
Primitive streak
Mesoderm
Endoderm
hESC
D0
D1
D2
D3
AFBLy
Activin
FGF2
BMP4
LY294
Expression relative to undifferentiated (D0)
Mesoderm and Primitive Steak
Foxf1
Hand1
Brachyury
Irx3
Mesp2
Mixl1
Mesp1
Evx1
Mesoderm and Definitive Endoderm
Cer1
Cxcr4
Definitive Endoderm
Foxa1
Foxa2
Sox17
Hhex
Epiblast and Primitive Streak
Nanog
Oct4
Day 0
Day 0.5
Day 1.0
Day 1.5
Day 2.0
Day 2.5
Day 3.0
Days of hESC differentiation in the AFBLy regimen

FIG. 40




Top 30 genes upregulated by day 3 AFBLy differentiation versus undifferentiated hESCs (Touboul et al., 2010)

| Rank | Gene name | Fold change | Rank | Gene name | Fold change | Rank | Gene name | Fold change |
|---|---|---|---|---|---|---|---|---|
| 1 | HAND1 | 146.30 | 11 | GRP | 35.63 | 21 | BMP2 | 18.25 |
| 2 | CER1 | 140.21 | 12 | MFAP4 | 35.34 | 22 | GSC | 17.91 |
| 3 | GRP | 122.55 | 13 | AGTRL1 | 34.41 | 23 | SNAI2 | 17.43 |
| 4 | LHX1 | 85.59 | 14 | PTHR1 | 31.40 | 24 | LZTS1 | 17.28 |
| 5 | FOXC1 | 72.66 | 15 | CXorf6 | 29.29 | 25 | COL13A1 | 16.54 |
| 6 | LEFTY2 | 53.34 | 16 | CYP26A1 | 28.34 | 26 | SOX17 | 14.74 |
| 7 | MIXL1 | 52.05 | 17 | IRX3 | 26.99 | 27 | SMAD6 | 14.53 |
| 8 | MESP1 | 44.61 | 18 | EOMES | 26.66 | 28 | HAK | 14.06 |
| 9 | FGF17 | 38.37 | 19 | TMOD1 | 22.22 | 29 | NLF2 | 14.04 |
| 10 | CYP26A1 | 35.91 | 20 | FAM89A | 19.65 | 30 | PCDH17 | 13.48 |

**Red** = known mesodermal expression | Blue = definitive endoderm-exclusive

FIG. 41

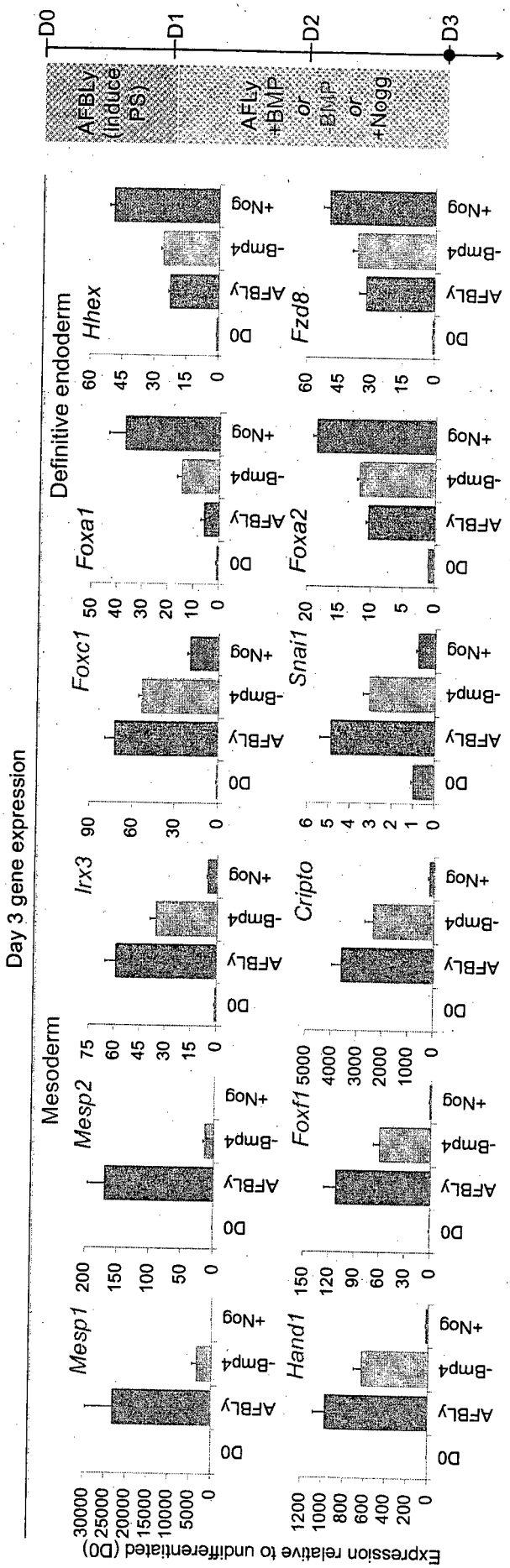

D0
D1
D2
D3
AFBLy (Induce PS)
AFLy +BMP or BMP or +Nogg
Day 3 gene expression
Mesoderm
Definitive endoderm
Mesp1
Mesp2
Irx3
Foxc1
Foxa1
Hhex
Hand1
Foxf1
Cripto
Snai1
Foxa2
Fzd8
D0
AFBLy
-Bmp4
+Nog
Expression relative to undifferentiated (D0)

FIG. 42

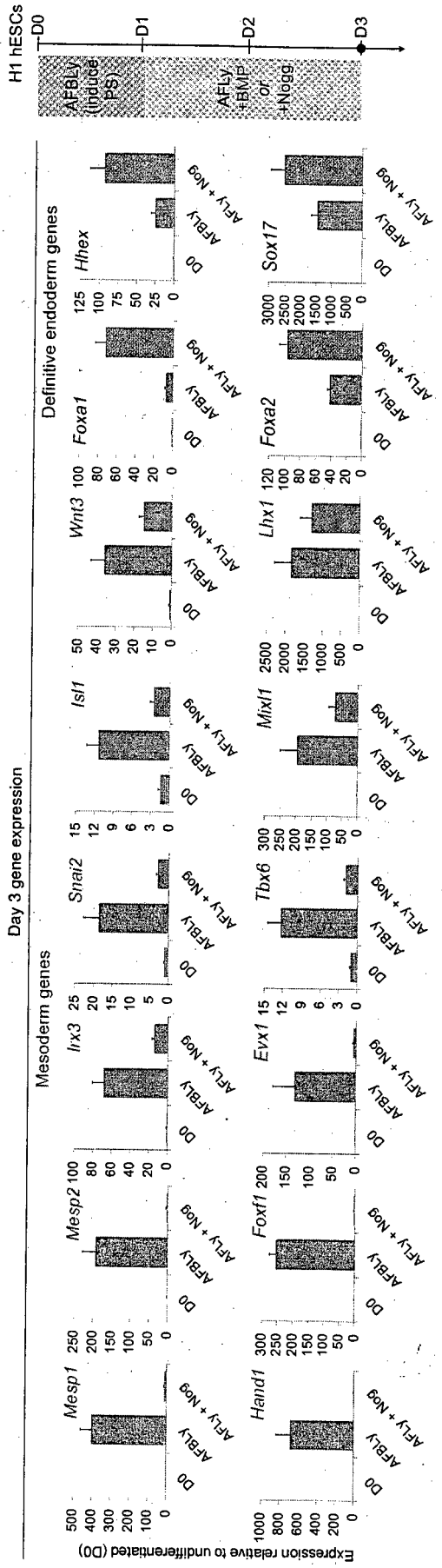

H1 hESCs
D0
D1
D2
D3
AFBLy (Induce PS)
AFLy +BMP or +Nogg
Day 3 gene expression
Mesoderm genes
Definitive endoderm genes
Expression relative to undifferentiated (D0)
Mesp1
Mesp2
Irx3
Snai2
Isl1
Wnt3
Foxa1
Hhex
Hand1
Foxf1
Evx1
Tbx6
Mixl1
Lhx1
Foxa2
Sox17
D0
AFBLy
AFLy + Nog

FIG. 43

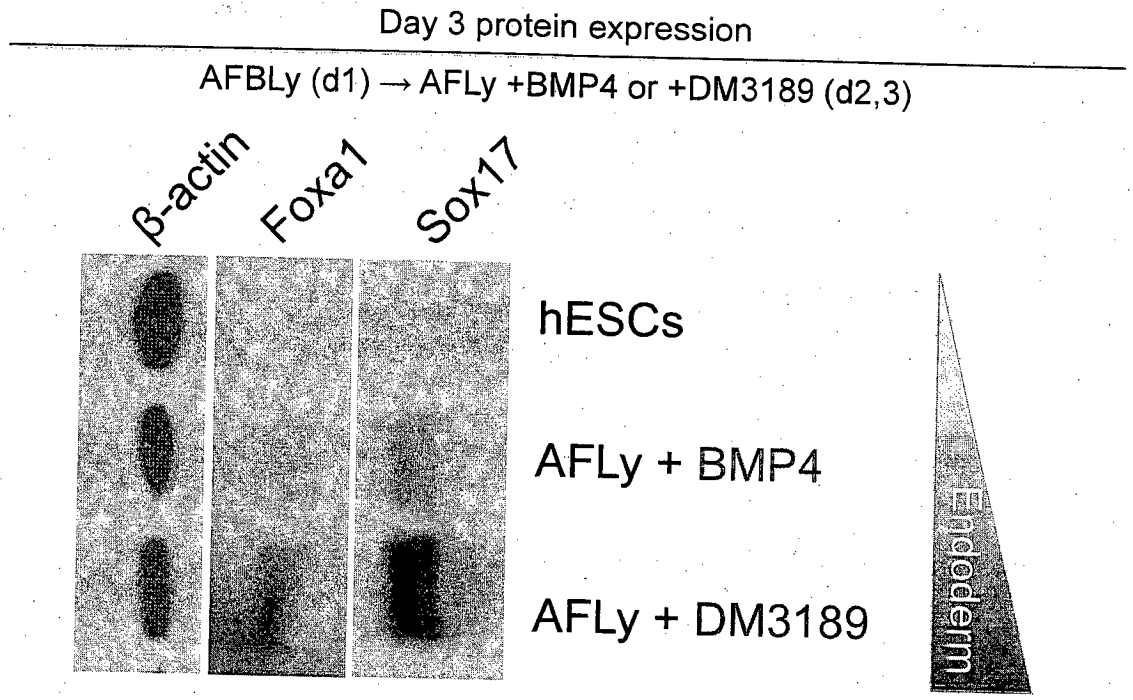

Day 3 protein expression
AFBLy (d1) → AFLy +BMP4 or +DM3189 (d2,3)
β-actin
Foxa1
Sox17
hESCs
AFLy + BMP4
AFLy + DM3189
Endoderm

FIG. 44

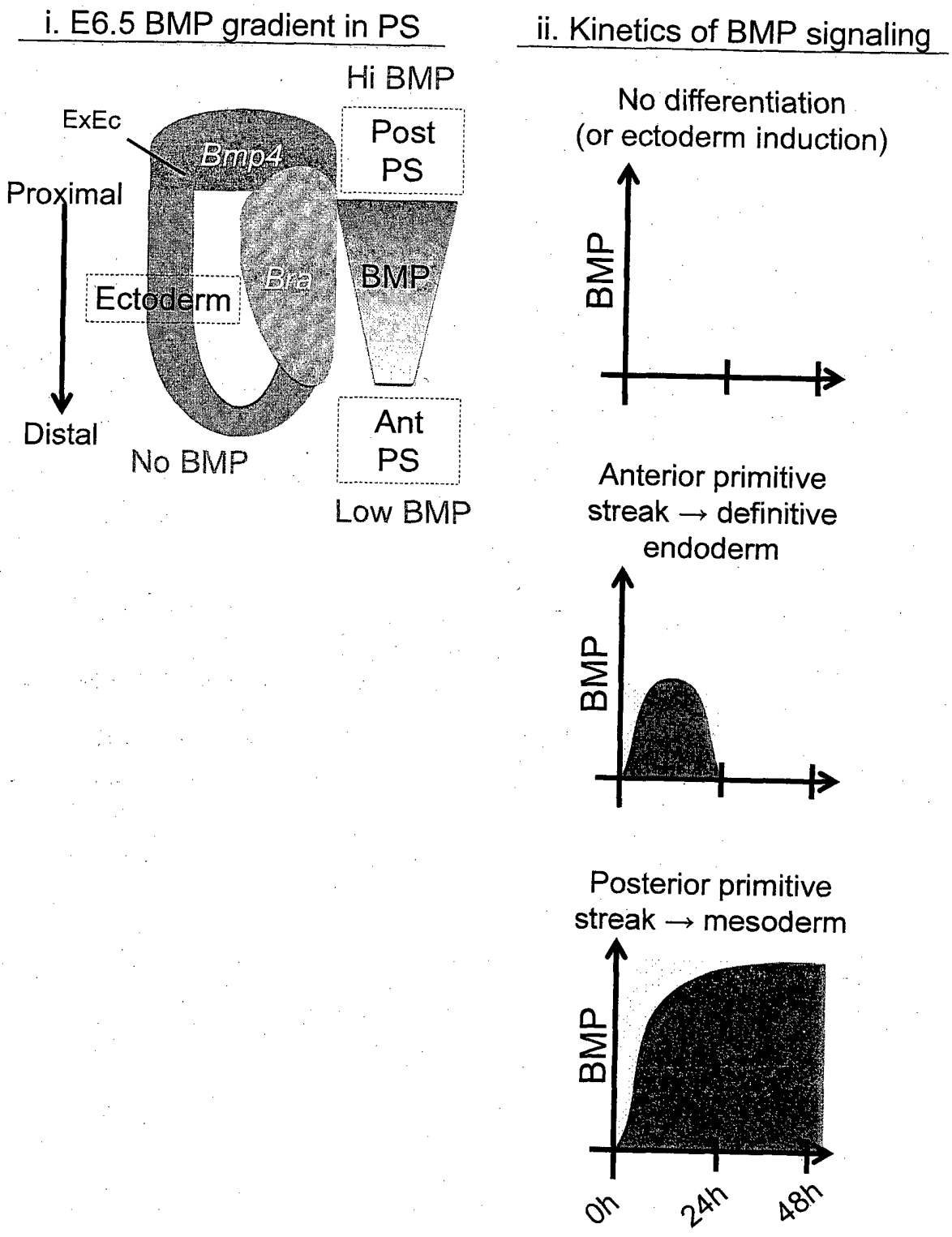
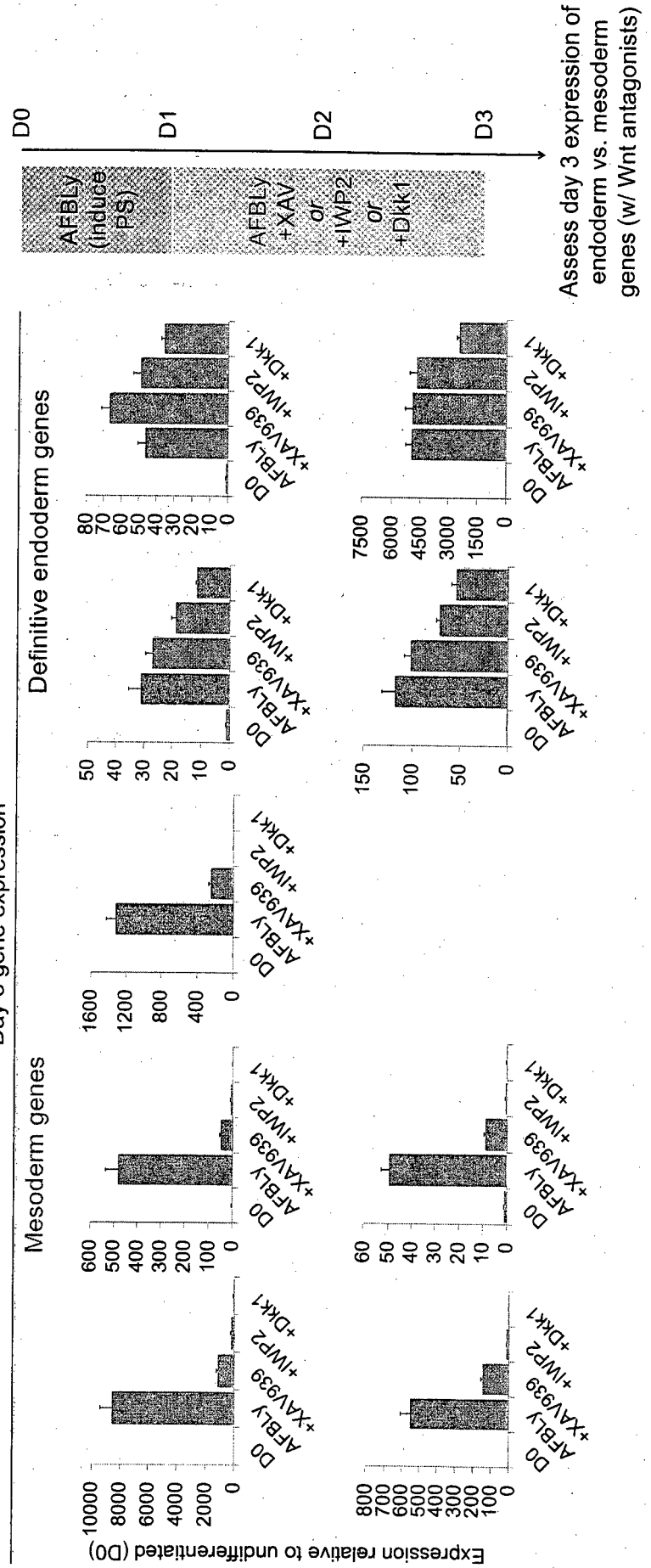

i. E6.5 BMP gradient in PS
Hi BMP
ExEc
Bmp4
Post PS
Proximal
Bra
BMP
Ectoderm
Distal
No BMP
Ant PS
Low BMP
ii. Kinetics of BMP signaling
No differentiation (or ectoderm induction)
BMP
Anterior primitive streak → definitive endoderm
BMP
Posterior primitive streak → mesoderm
BMP
0h
24h
48h

FIG. 45


H7 hESCs
D0
D1
D2
D3
AFBLy (induce PS)
AFBLy +XAV or +IWP2 or +Dkk1
Assess day 3 expression of endoderm vs. mesoderm genes (w/ Wnt antagonists)
Day 3 gene expression
Mesoderm genes
Definitive endoderm genes
Expression relative to undifferentiated (D0)
D0
AFBLy +XAV939 +IWP2 +Dkk1

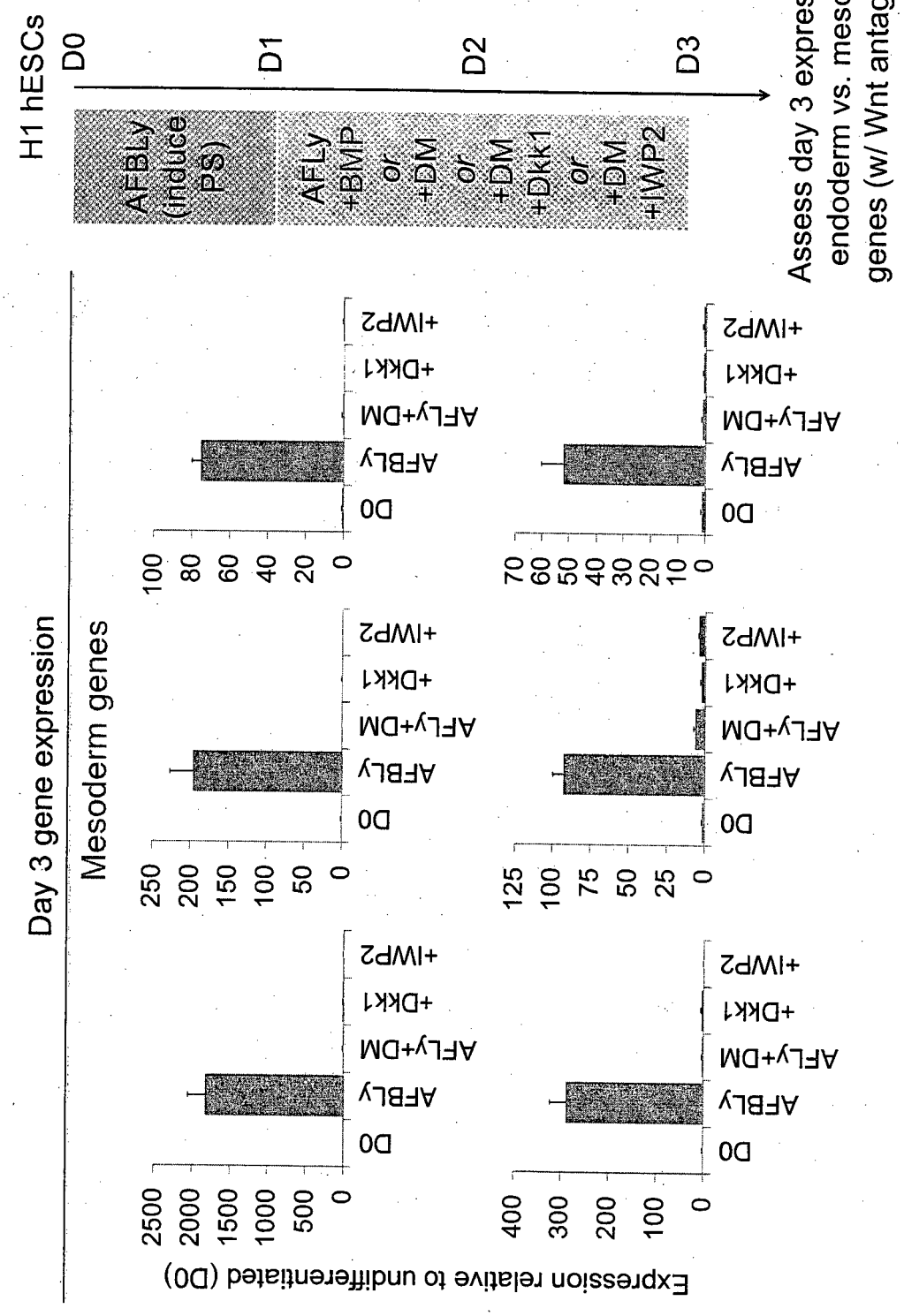

H1 hESCs
D0
D1
D2
D3
AFBLy (induce PS)
AFLy +BMP or +DM or +DM +Dkk1 or +DM +IWP2
Assess day 3 expression of endoderm vs. mesoderm genes (w/ Wnt antagonists)
Day 3 gene expression
Mesoderm genes
Expression relative to undifferentiated (D0)
D0
AFBLy
AFLy+DM
+Dkk1
+IWP2

FIG. 46

FIG. 47

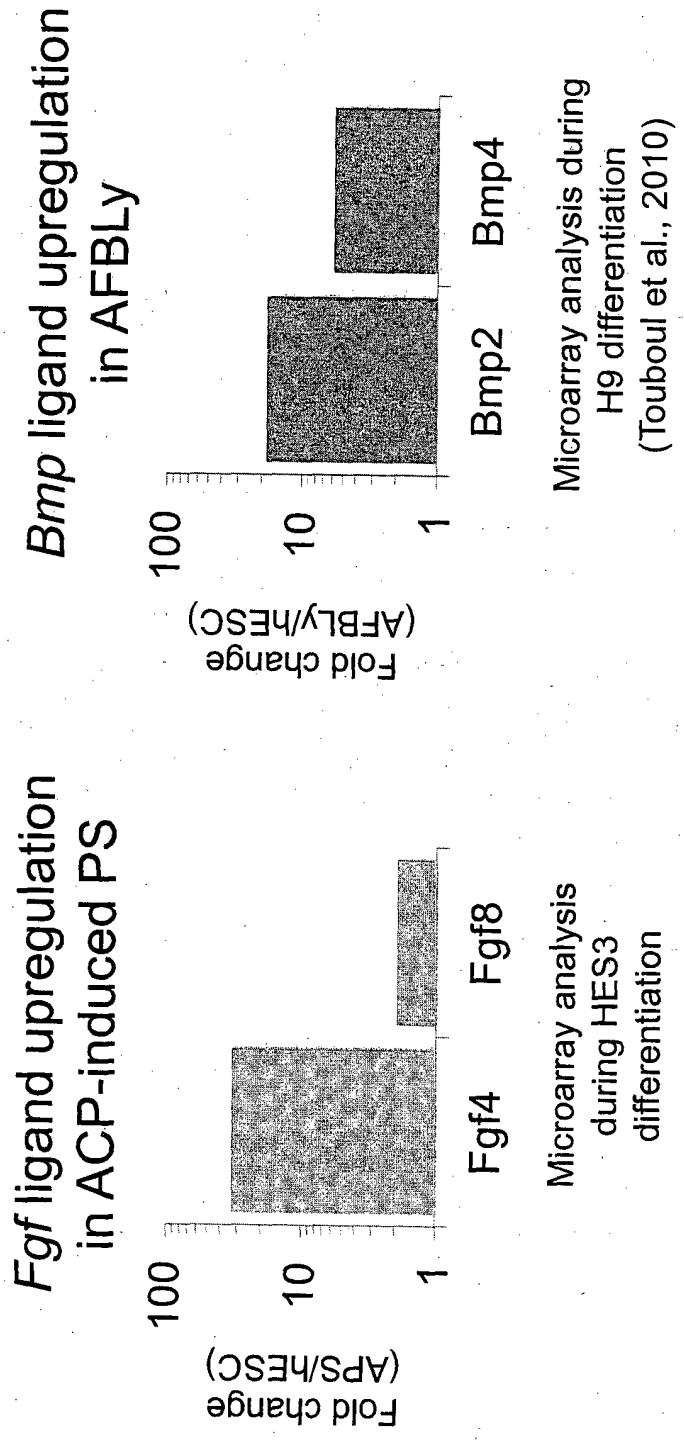

Fgf ligand upregulation in ACP-induced PS
Fold change (APS/hESC)
100
10
1
Fgf4
Fgf8
Microarray analysis during HES3 differentiation
Bmp ligand upregulation in AFBLy
Fold change (AFBLy/hESC)
100
10
1
Bmp2
Bmp4
Microarray analysis during H9 differentiation (Touboul et al., 2010)

FIG. 48

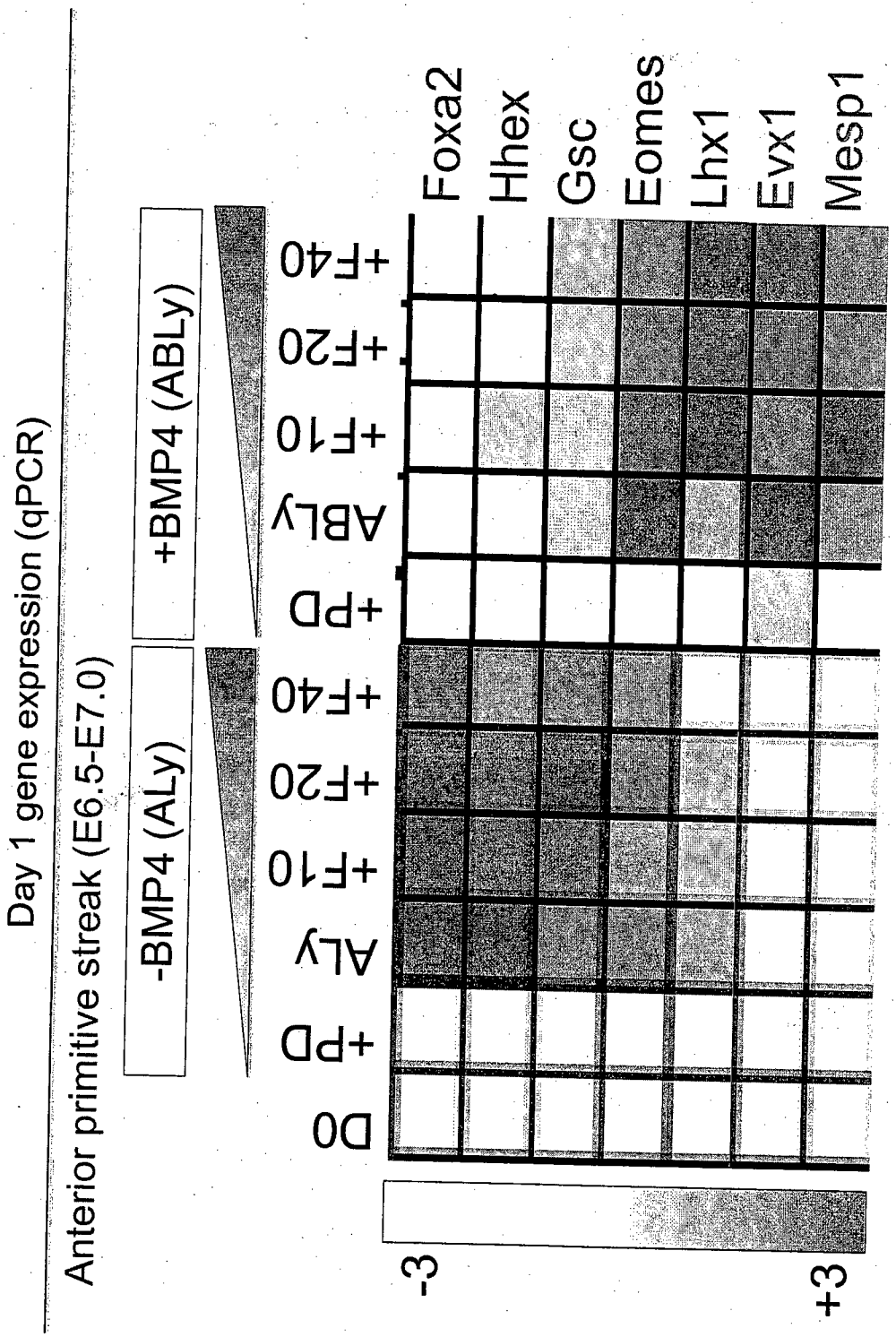

Day 1 gene expression (qPCR)
Anterior primitive streak (E6.5-E7.0)
-BMP4 (ALy)
+BMP4 (ABLy)
D0
+PD
ALy
+F10
+F20
+F40
+PD
ABLy
+F10
+F20
+F40
Foxa2
Hhex
Gsc
Eomes
Lhx1
Evx1
Mesp1
-3
+3

FIG. 49

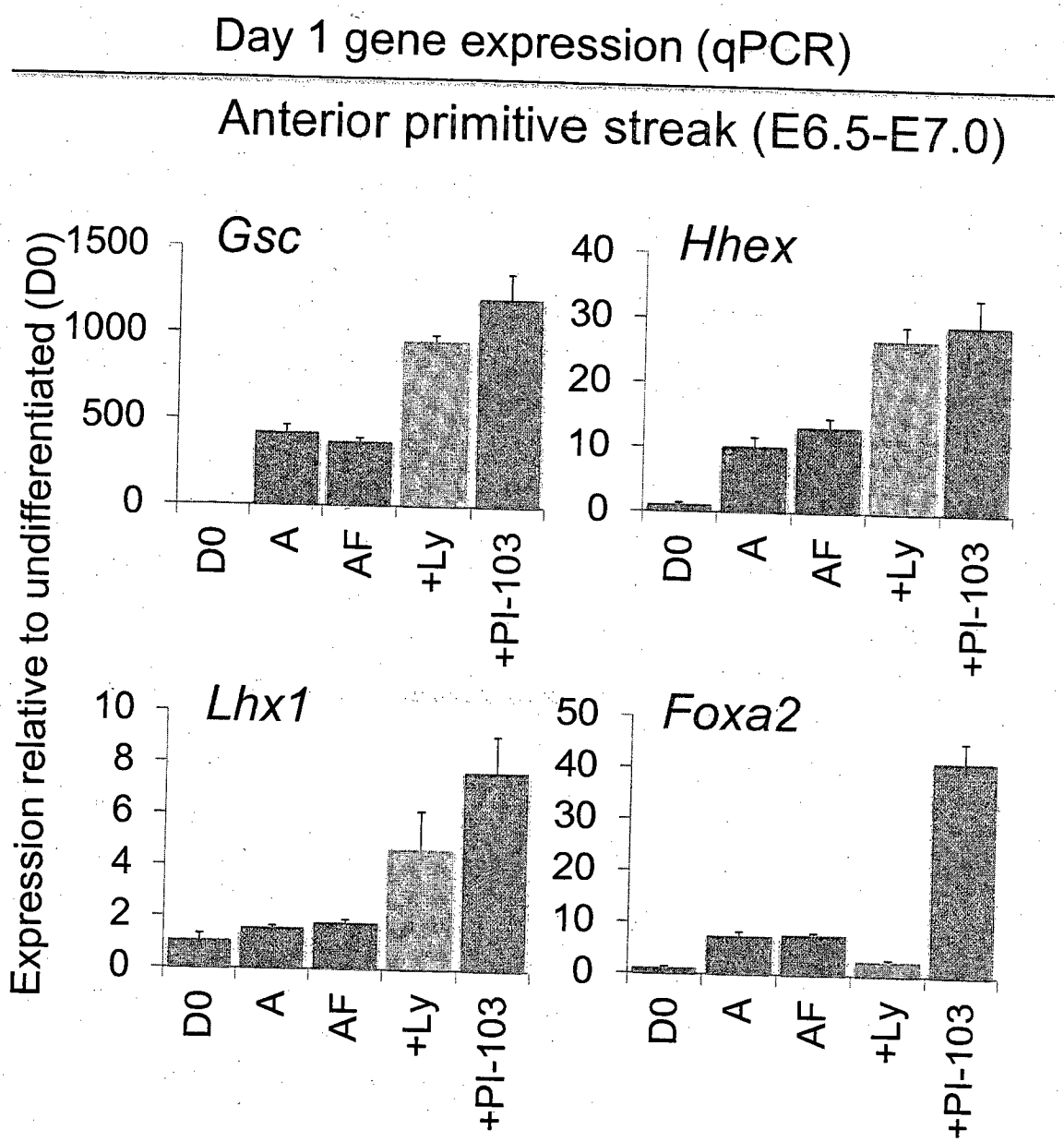
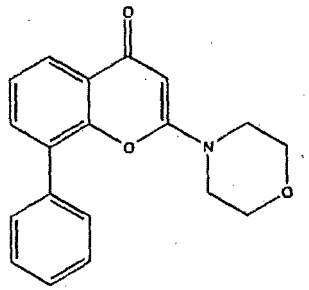

Day 1 gene expression (qPCR)
Anterior primitive streak (E6.5-E7.0)
Expression relative to undifferentiated (D0)
Gsc
Hhex
Lhx1
Foxa2
D0
A
AF
+Ly
+PI-103

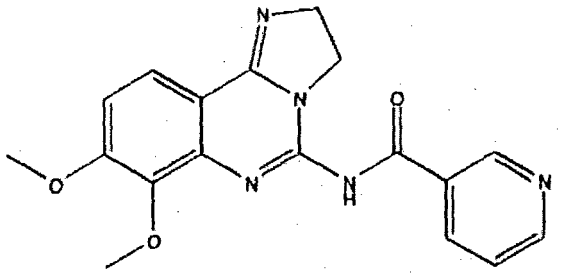
FIG. 50



**LY294002**

PI3K $IC_{50}$ ≈ 1-2 μM

Also binds to and/or inhibits mTORC, CK2, GSK3β, PLK1, PIM1, PIM3, and HIPK2

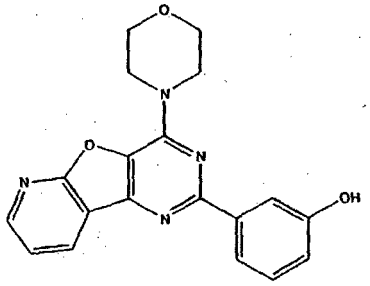


**PIK-90**

PI3K $IC_{50}$ = 11-18 nM (p110α/p110γ)

Does not inhibit mTORC1, CK2, or GSK3β

Also weakly inhibits ATM (490 nM) and DNA-PK (64 nM)



**PI-103**

PI3K $IC_{50}$ = 8 nM (p110α)

Does not inhibit CK2 or GSK3β

Also weakly inhibits ATR (850 nM), ATM (920 nM), DNA-PK (2 nM), mTORC1 (20 nM), mTORC2 (83 nM)

FIG. 51

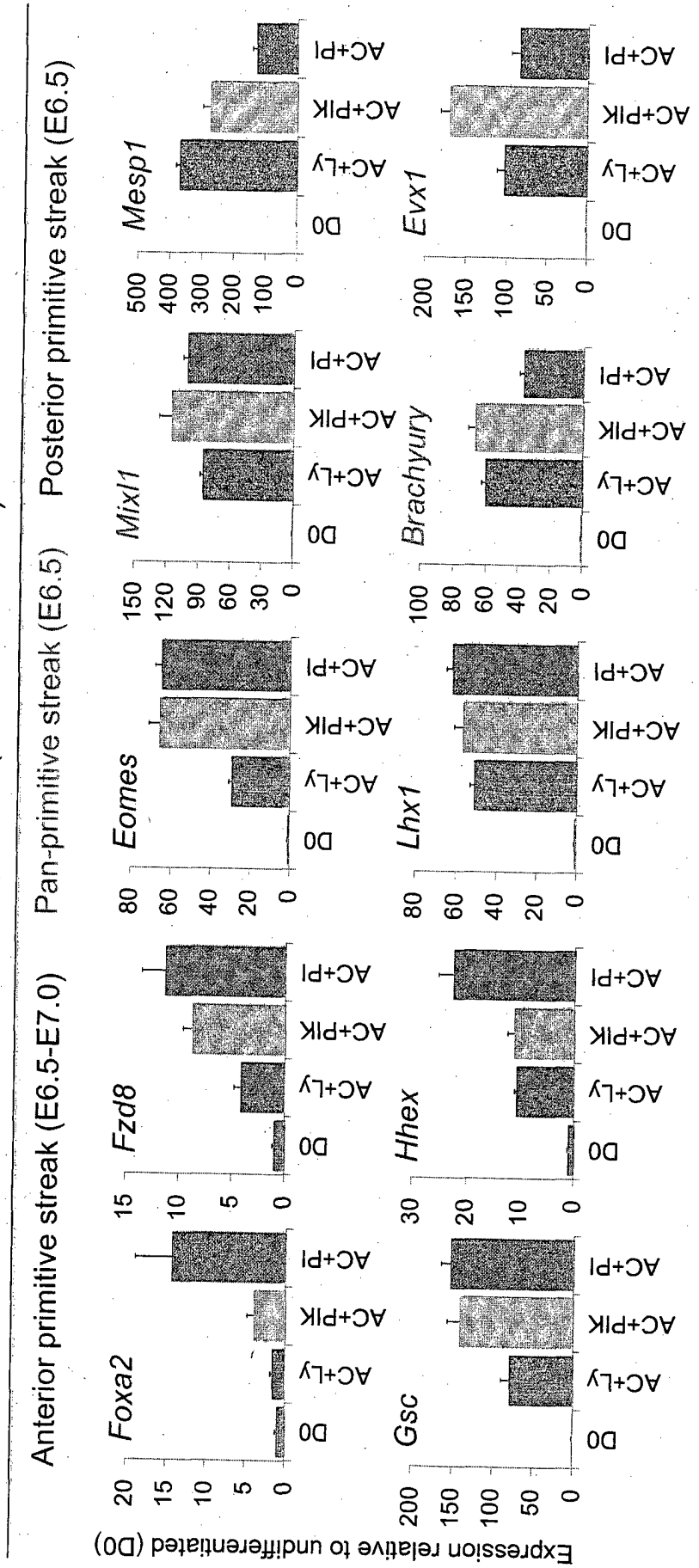

Day 1 gene expression (AC + PI3K inhibitor)
Anterior primitive streak (E6.5-E7.0)
Pan-primitive streak (E6.5)
Posterior primitive streak (E6.5)
Expression relative to undifferentiated (D0)
Foxa2
Fzd8
Eomes
Mixl1
Mesp1
Gsc
Hhex
Lhx1
Brachyury
Evx1
D0
AC+Ly
AC+PIK
AC+PI

FIG. 52

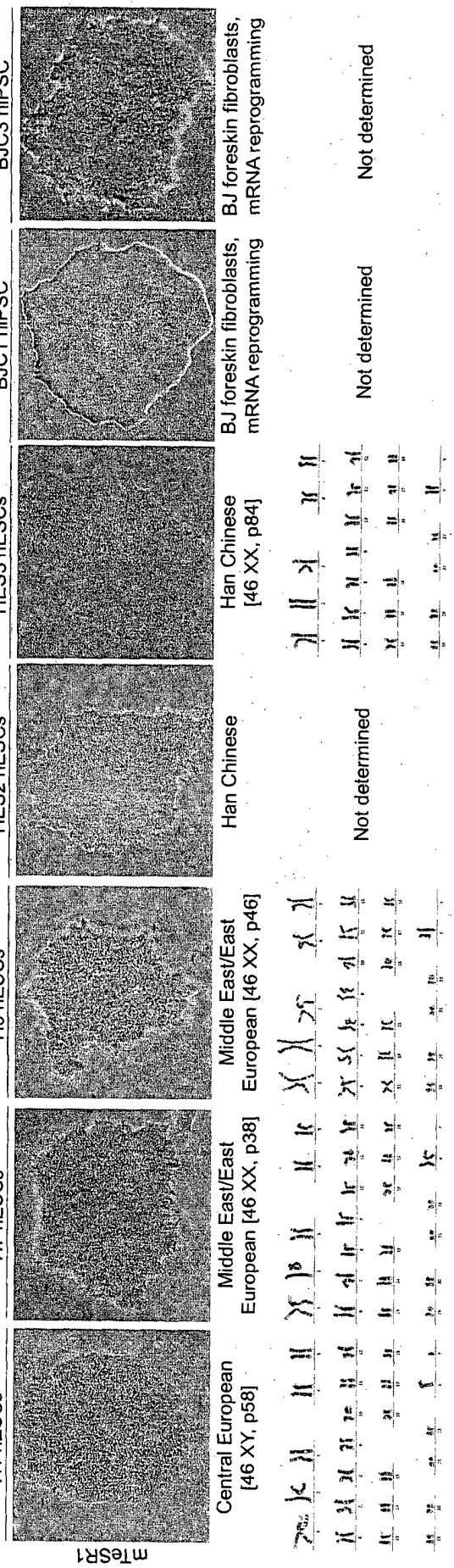

H1 hESCs
H7 hESCs
H9 hESCs
HES2 hESCs
HES3 hESCs
BJC1 hiPSC
BJC3 hiPSC
mTeSR1
Central European [46 XY, p58]
Middle East/East European [46 XX, p38]
Middle East/East European [46 XX, p46]
Han Chinese
Han Chinese [46 XX, p84]
BJ foreskin fibroblasts, mRNA reprogramming
BJ foreskin fibroblasts, mRNA reprogramming
Not determined
Not determined
Not determined

FIG. 53

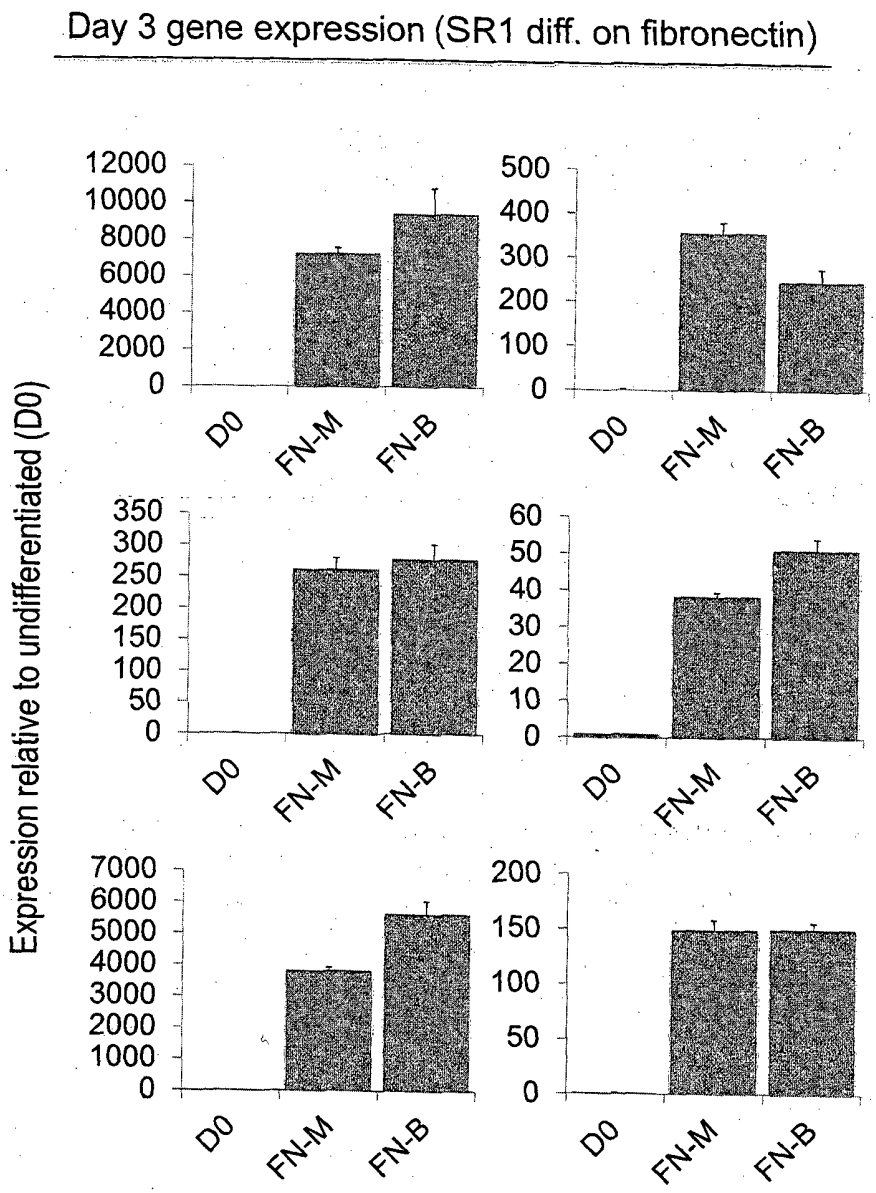

Day 3 gene expression (SR1 diff. on fibronectin)
Expression relative to undifferentiated (D0)
12000
10000
8000
6000
4000
2000
0
500
400
300
200
100
350
300
250
200
150
100
50
60
50
40
30
20
10
7000
6000
5000
4000
3000
2000
1000
200
150
100
50
D0
FN-M
FN-B

FIG. 54

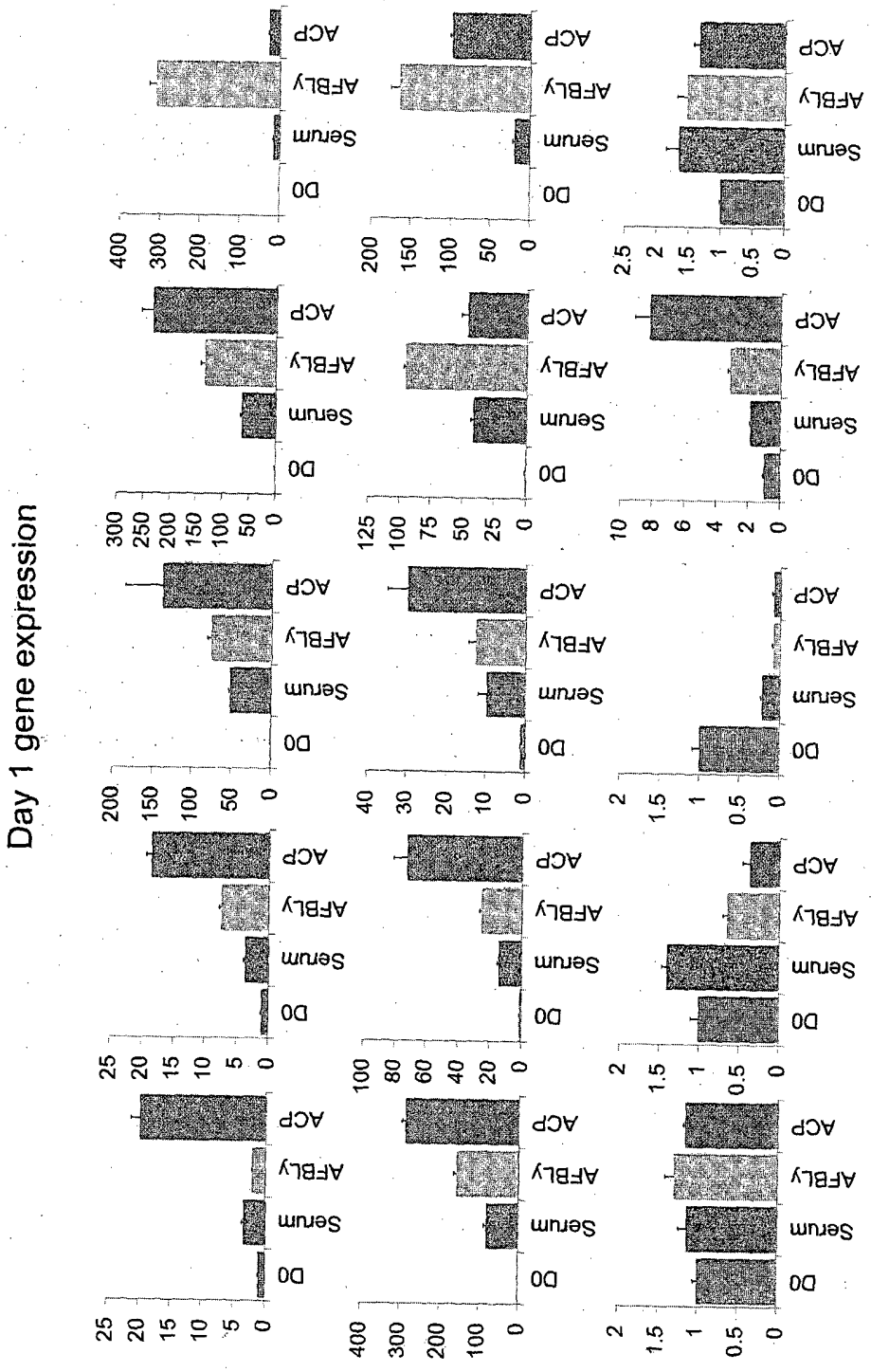
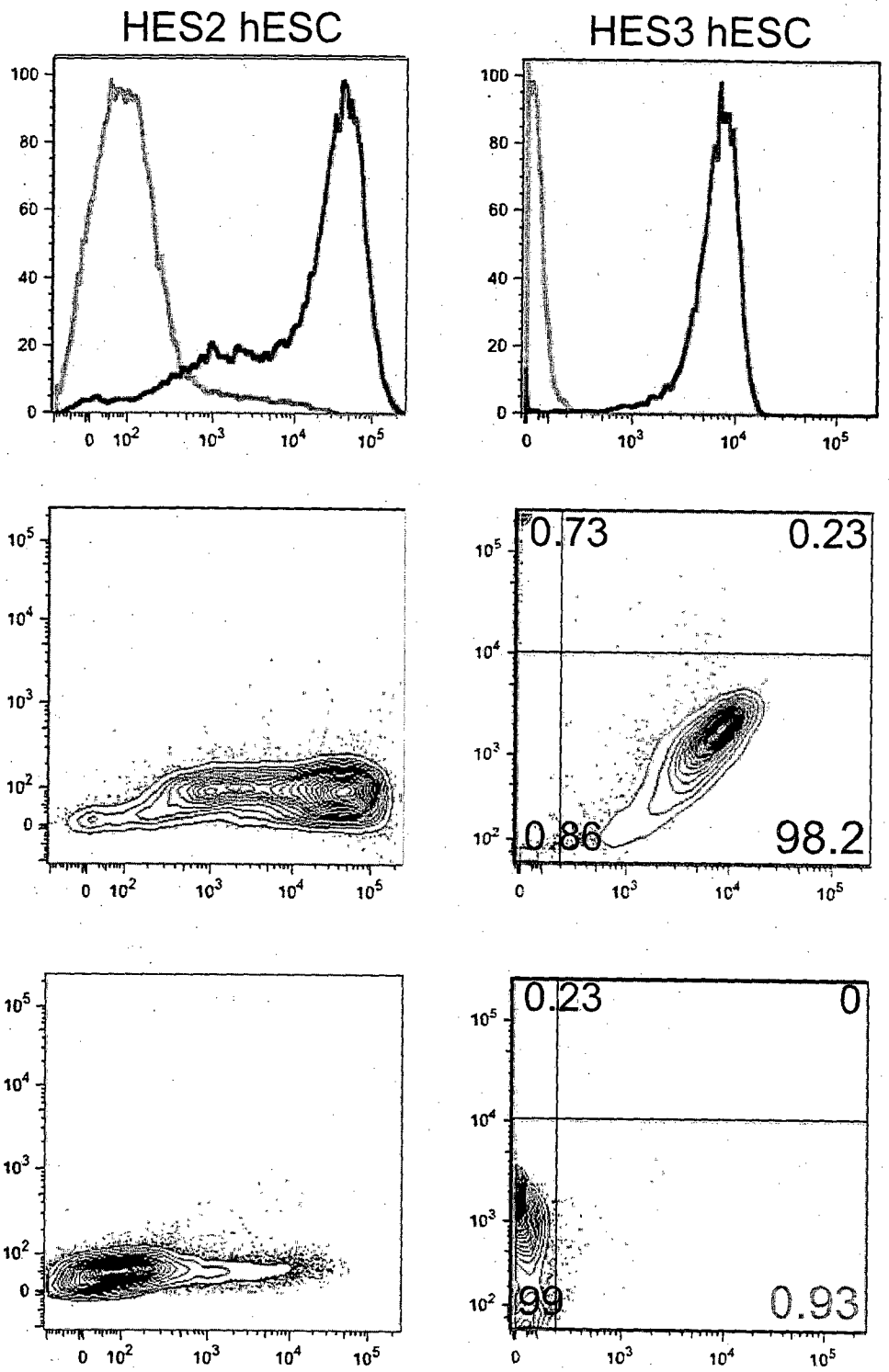


FIG. 55



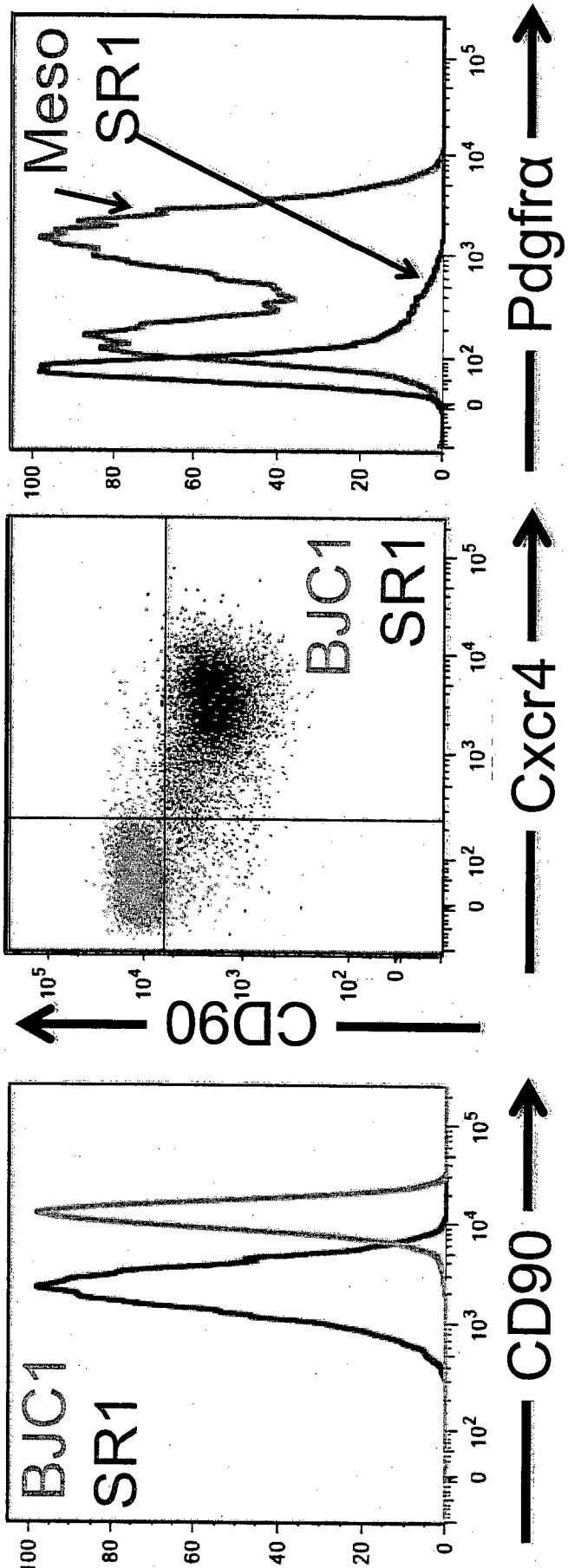



FIG. 56

FIG. 57

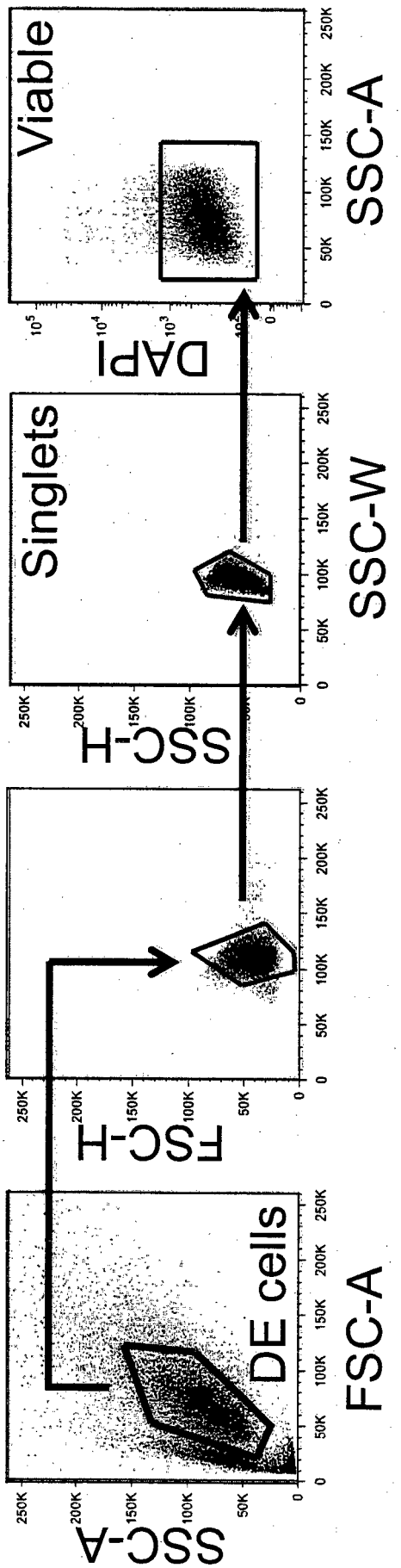


FIG. 58 (a), (b) and (c)

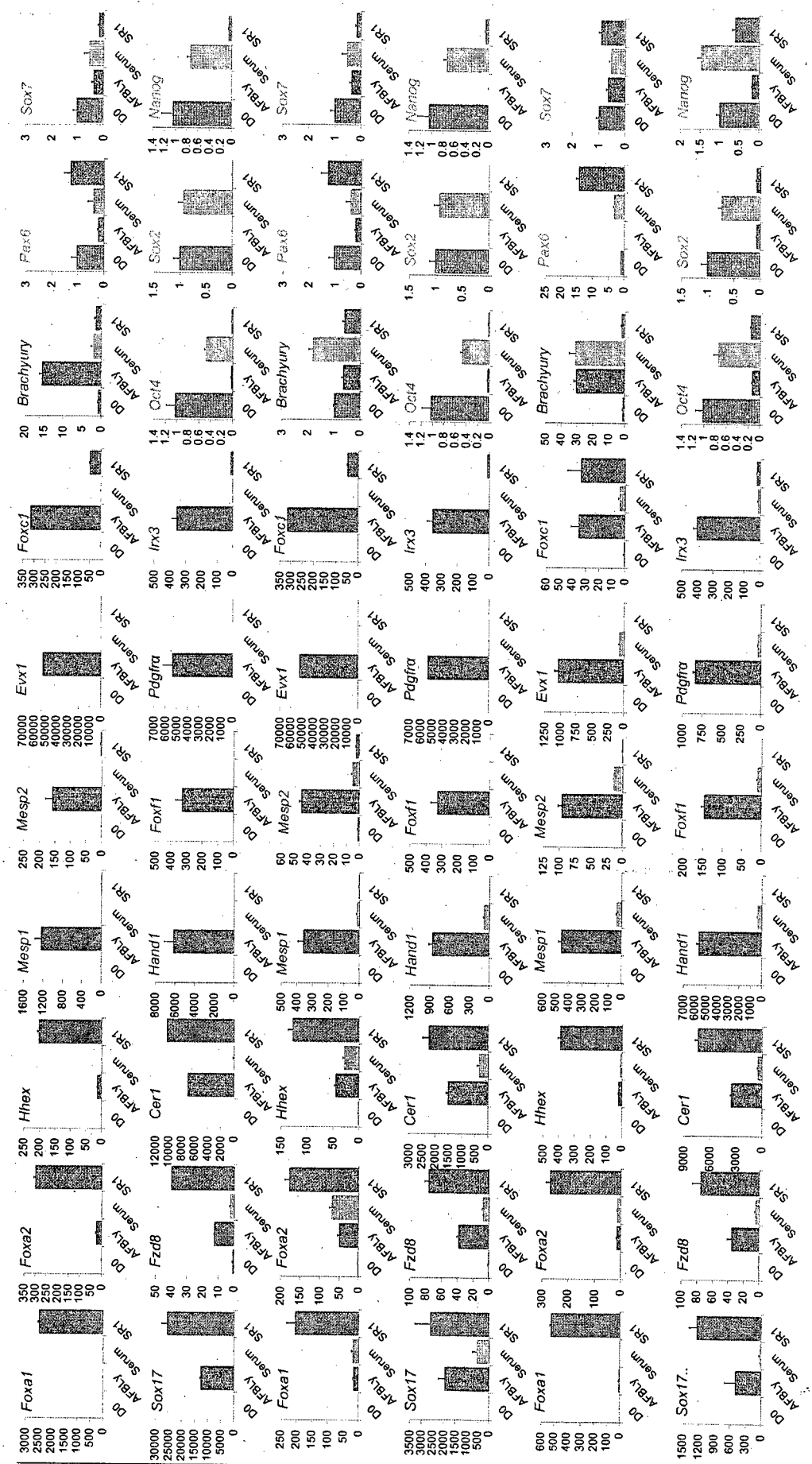


FIG. 58 (d), (e) and (f)

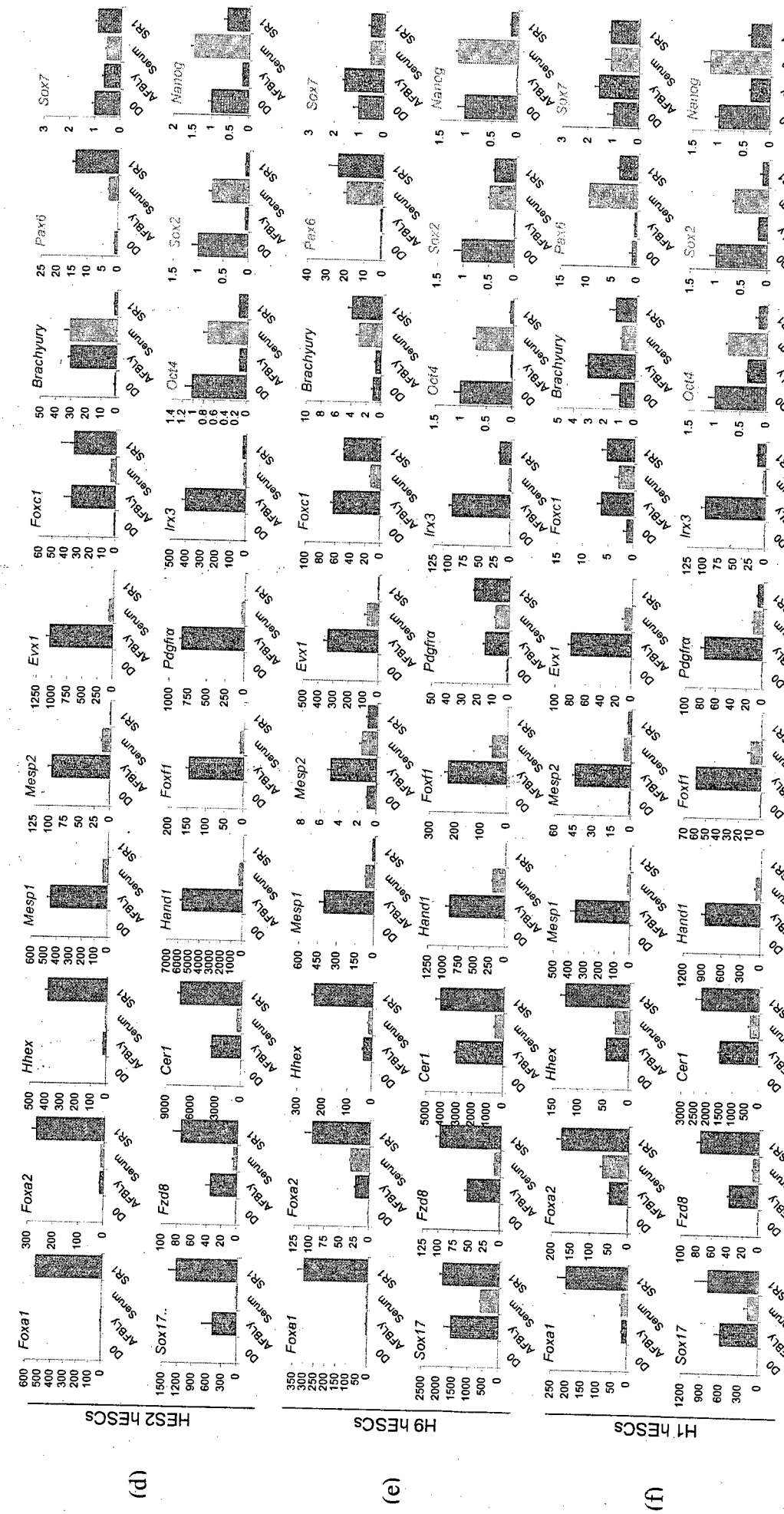


FIG. 59

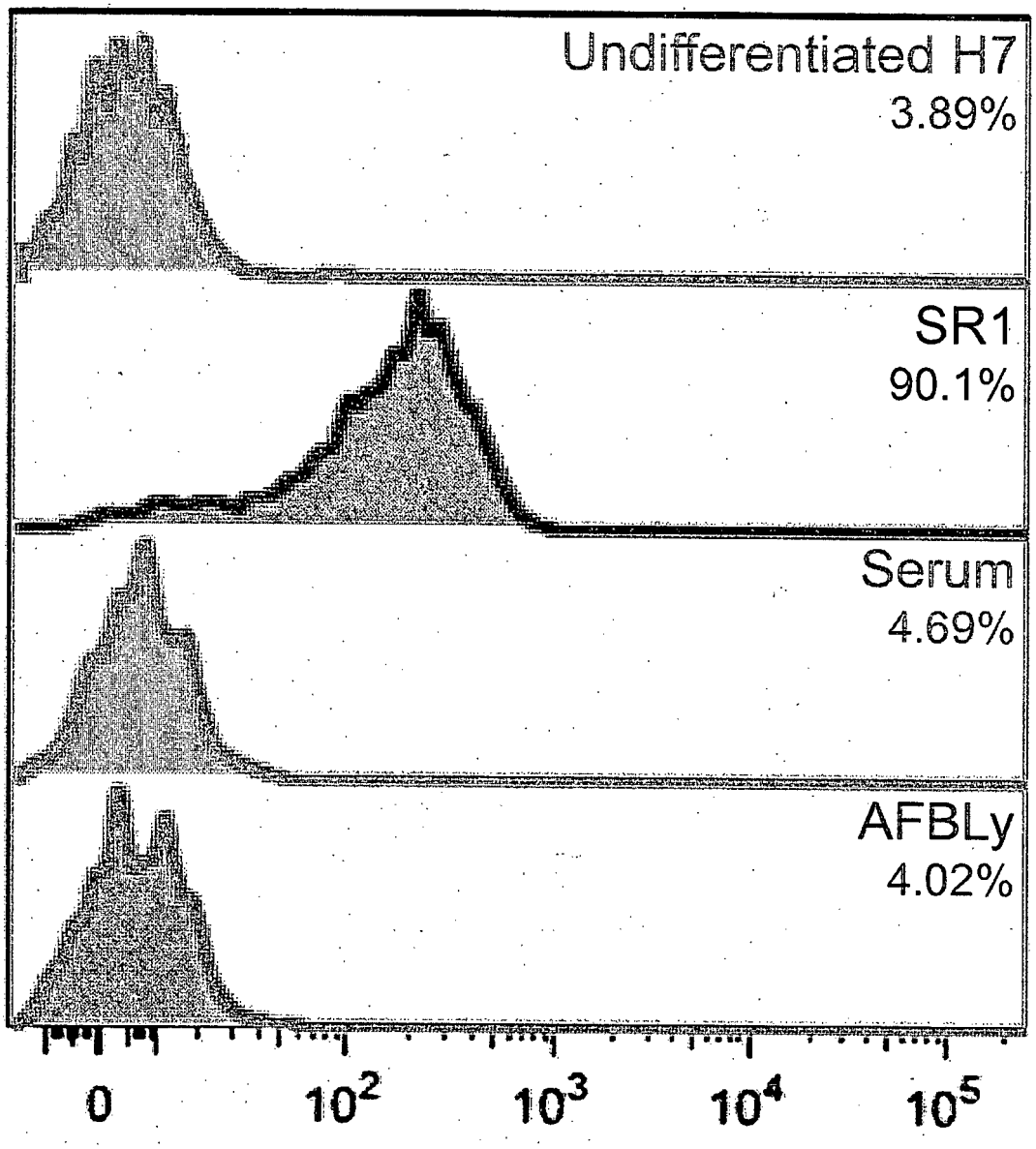
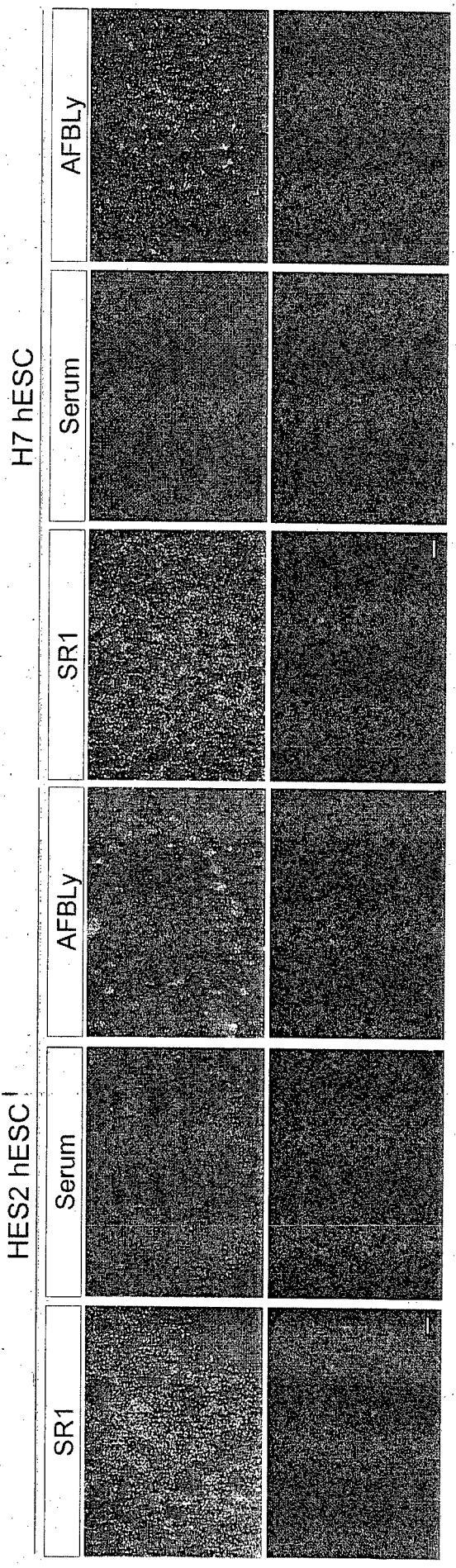

Undifferentiated H7
3.89%
SR1
90.1%
Serum
4.69%
AFBLy
4.02%
0
10^2
10^3
10^4
10^5

FIG. 60



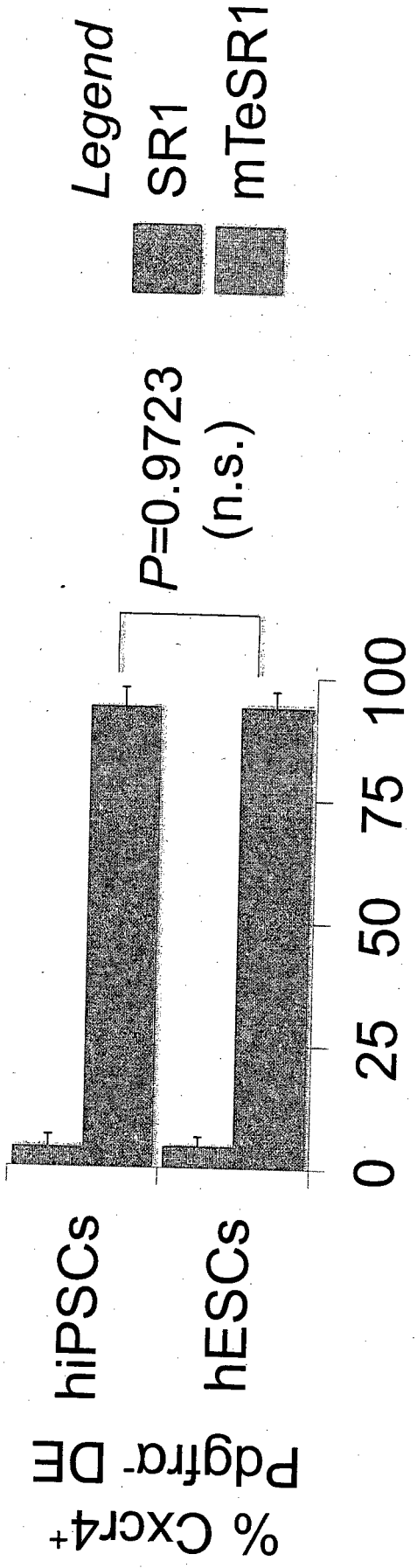

Legend
SR1
mTeSR1
P=0.9723
(n.s.)
% Cxcr4+
Pdgfra- DE
0
25
50
75
100
hiPSCs
hESCs

FIG. 61

FIG. 62

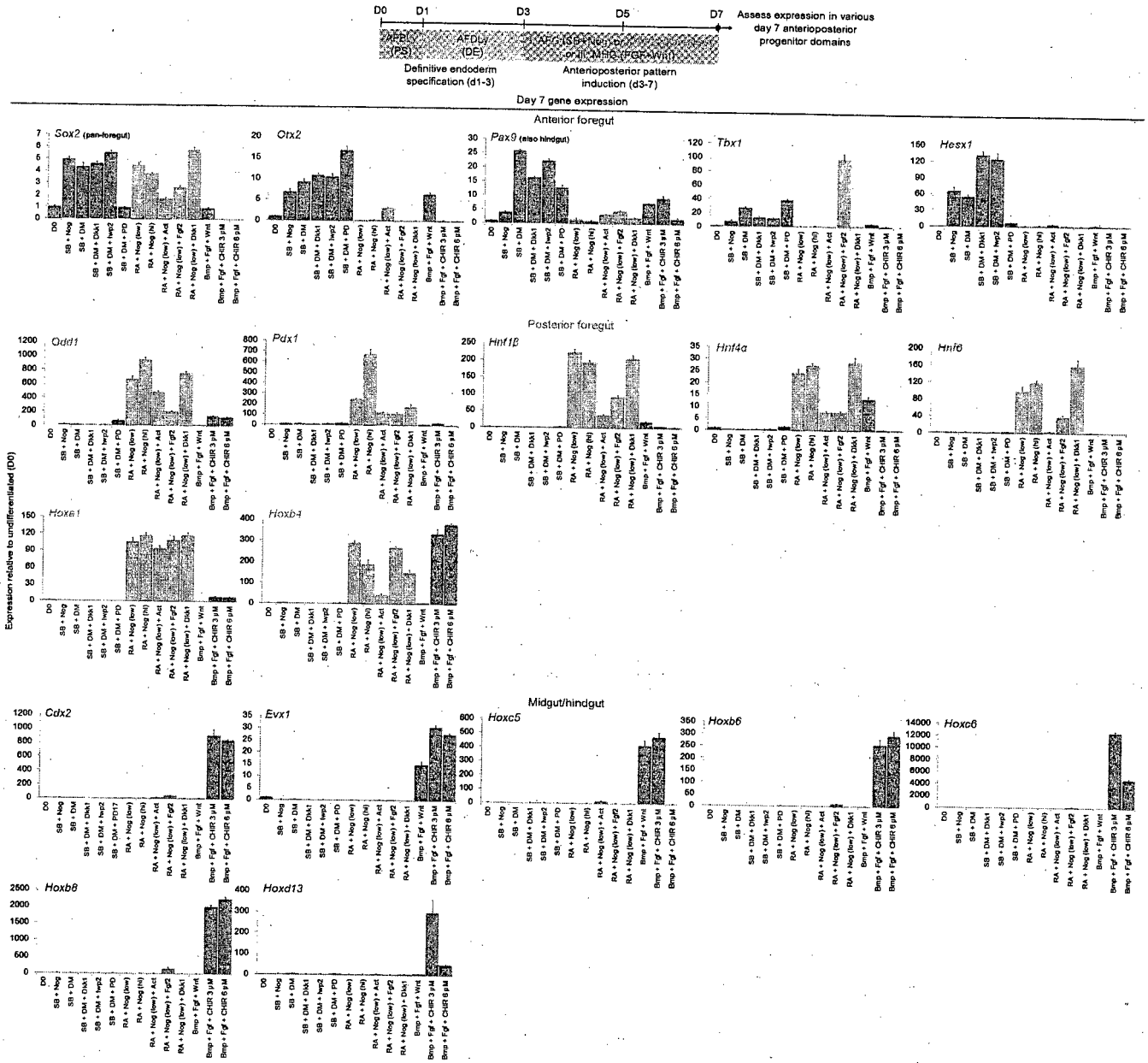

D0
D1
D3
D5
D7
Assess expression in various day 7 anterioposterior progenitor domains
AFDL (PS)
AFDL (DE)
+ Activin/Nodal
+ FGF
+ Wnt
Definitive endoderm specification (d1-3)
Anterioposterior pattern induction (d3-7)
Day 7 gene expression
Anterior foregut
Sox2 (pan-foregut)
Otx2
Pax9 (also hindgut)
Tbx1
Hesx1
Posterior foregut
Odd1
Pdx1
Hnf1β
Hnf4a
Hnf6
Hoxa1
Hoxb4
Expression relative to undifferentiated (D0)
Midgut/hindgut
Cdx2
Evx1
Hoxc5
Hoxb6
Hoxc6
Hoxb8
Hoxd13

**FIG. 63**

Day 7 gene expression (d4 DIPR → d5-7 conditions)

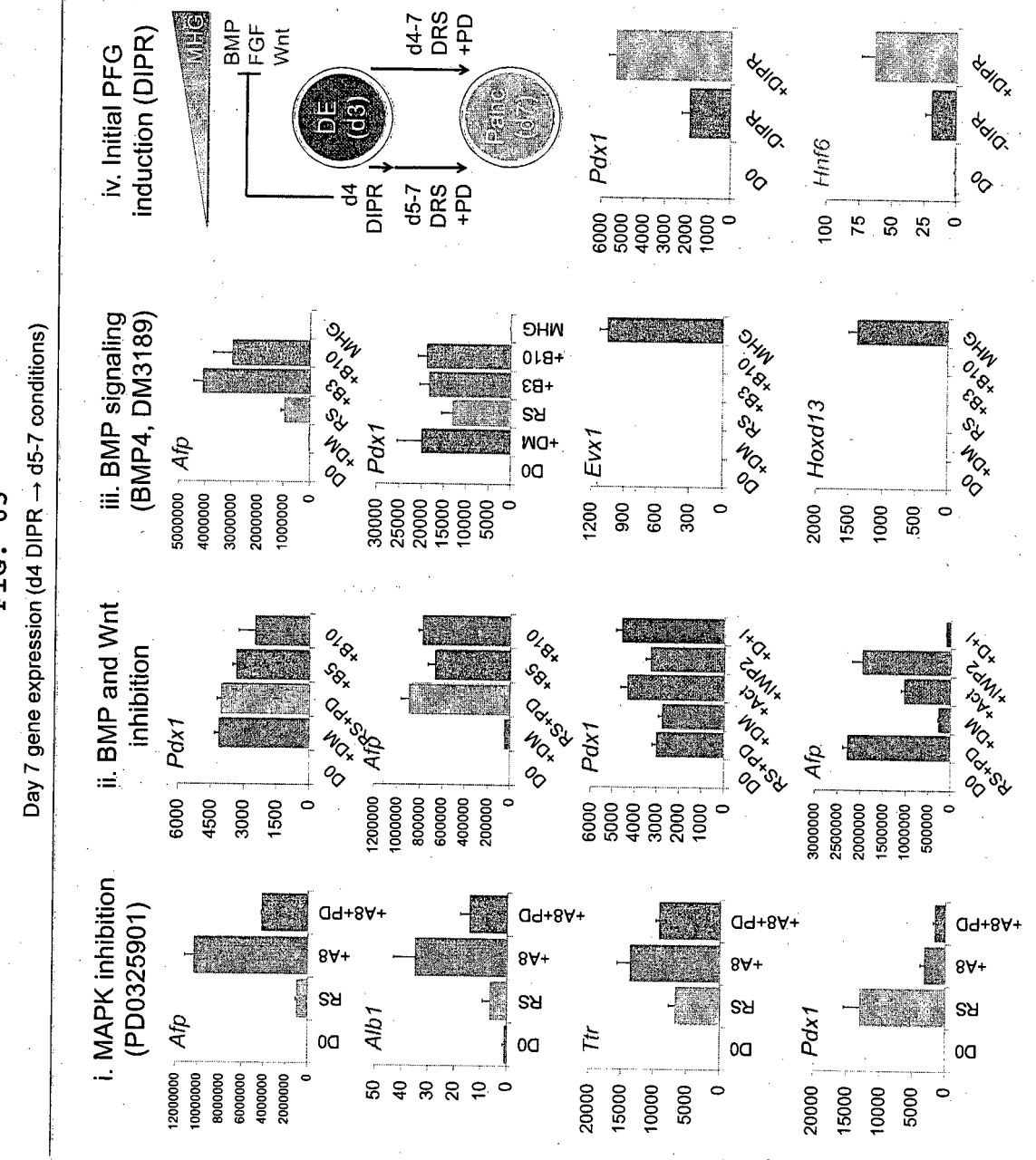


FIG. 64

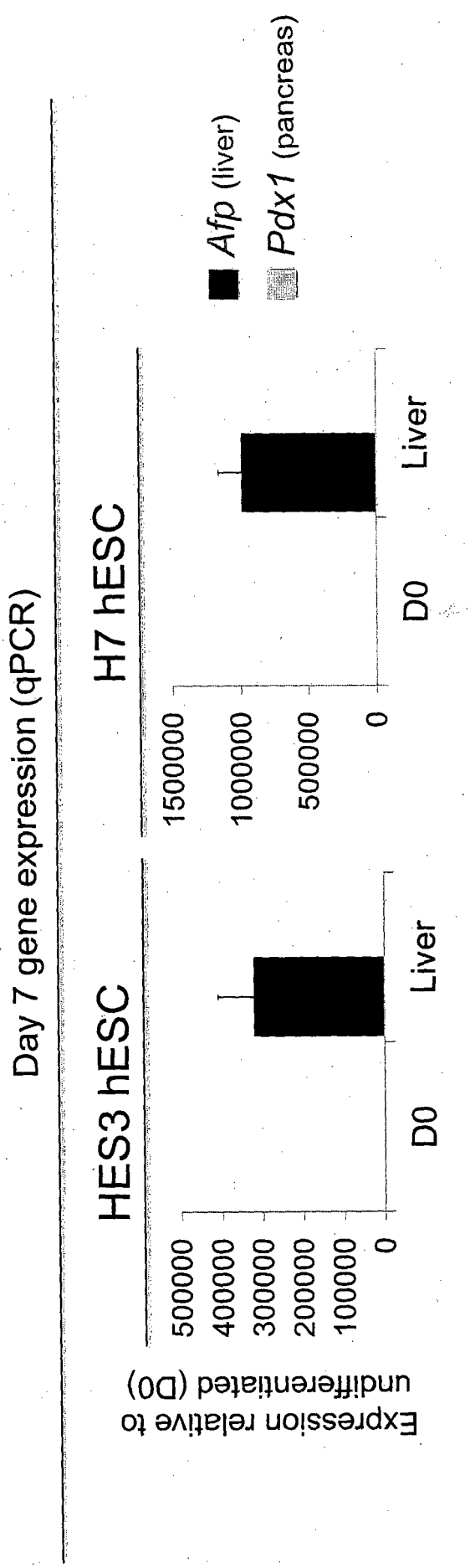

Day 7 gene expression (qPCR)
HES3 hESC
H7 hESC
Afp (liver)
Pdx1 (pancreas)
Expression relative to undifferentiated (D0)
500000
400000
300000
200000
100000
0
1500000
1000000
500000
0
D0
Liver
D0
Liver

FIG. 65

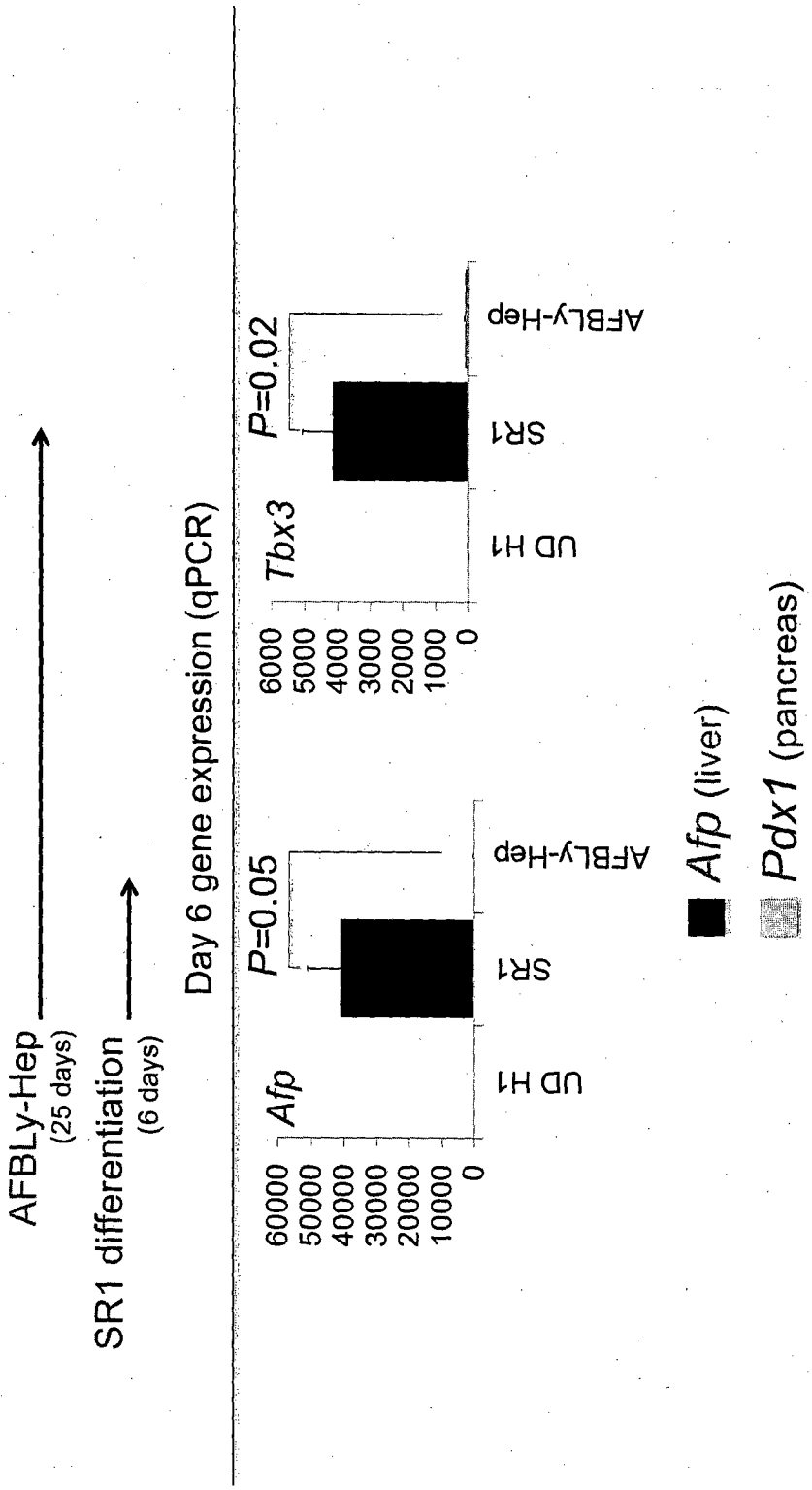

AFBLy-Hep
(25 days)
SR1 differentiation
(6 days)
Day 6 gene expression (qPCR)
Afp
P=0.05
60000
50000
40000
30000
20000
10000
0
UD H1
SR1
AFBLy-Hep
Tbx3
P=0.02
6000
5000
4000
3000
2000
1000
0
UD H1
SR1
AFBLy-Hep
Afp (liver)
Pdx1 (pancreas)

FIG. 66

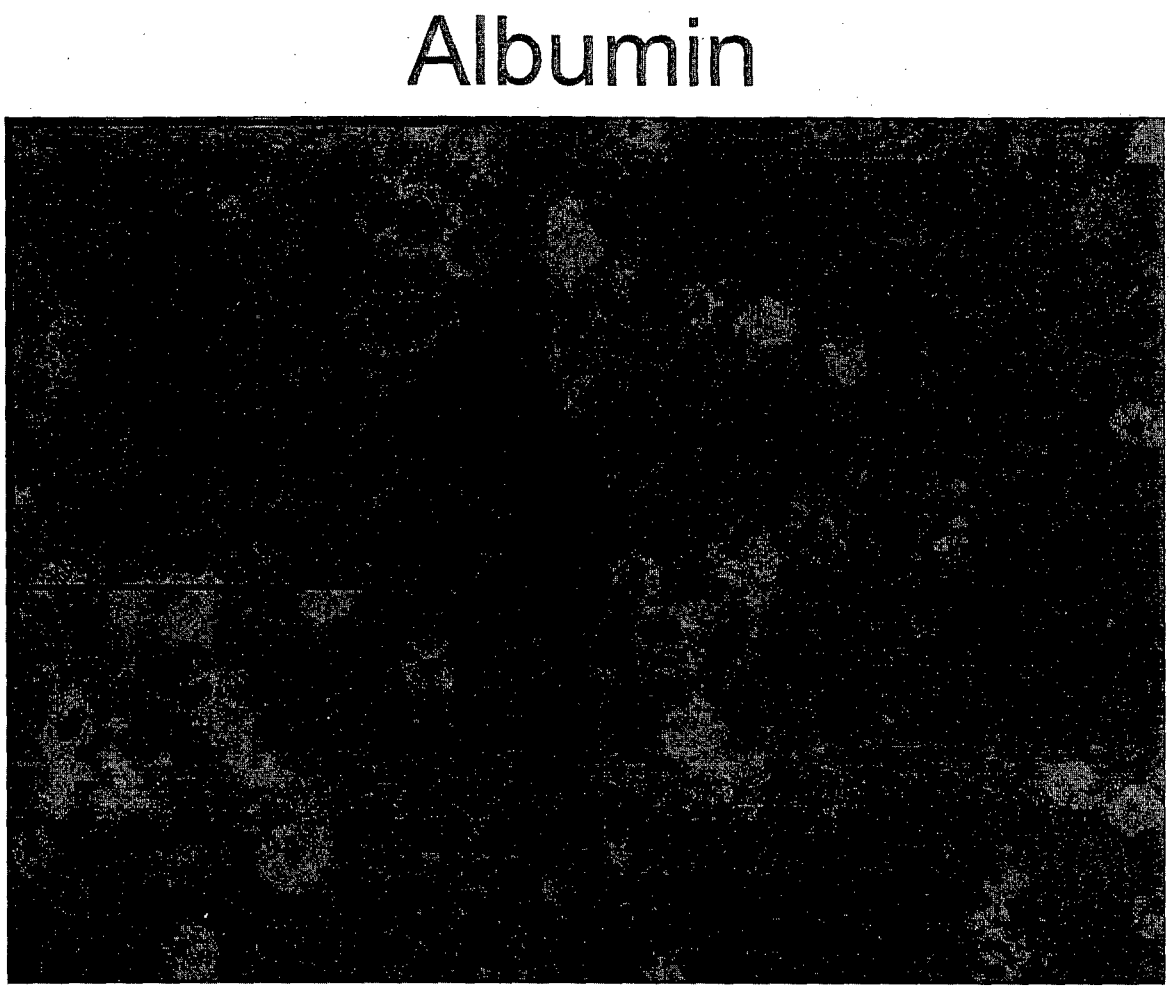


FIG. 67

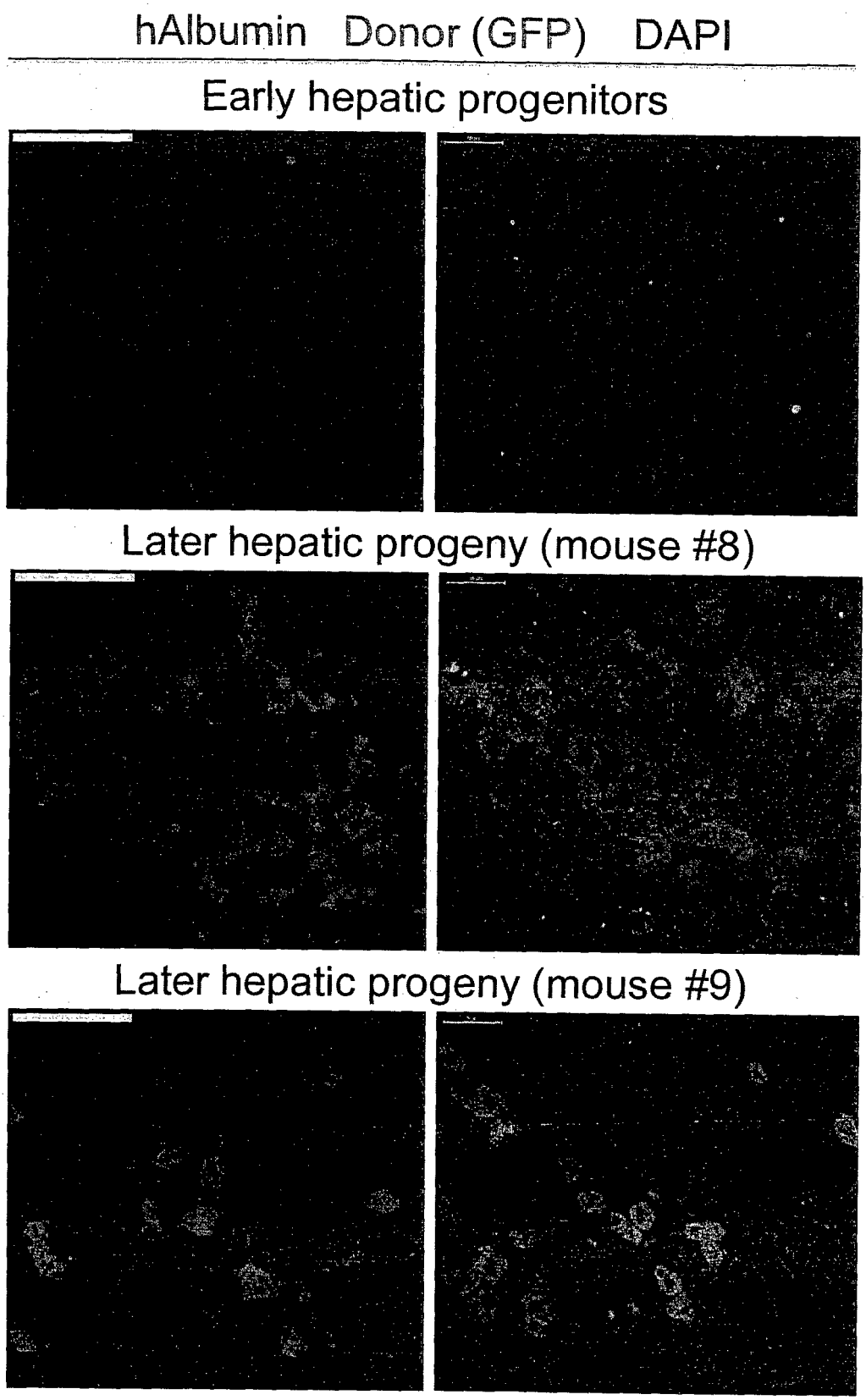

hAlbumin Donor (GFP) DAPI
Early hepatic progenitors
Later hepatic progeny (mouse #8)
Later hepatic progeny (mouse #9)

FIG. 68

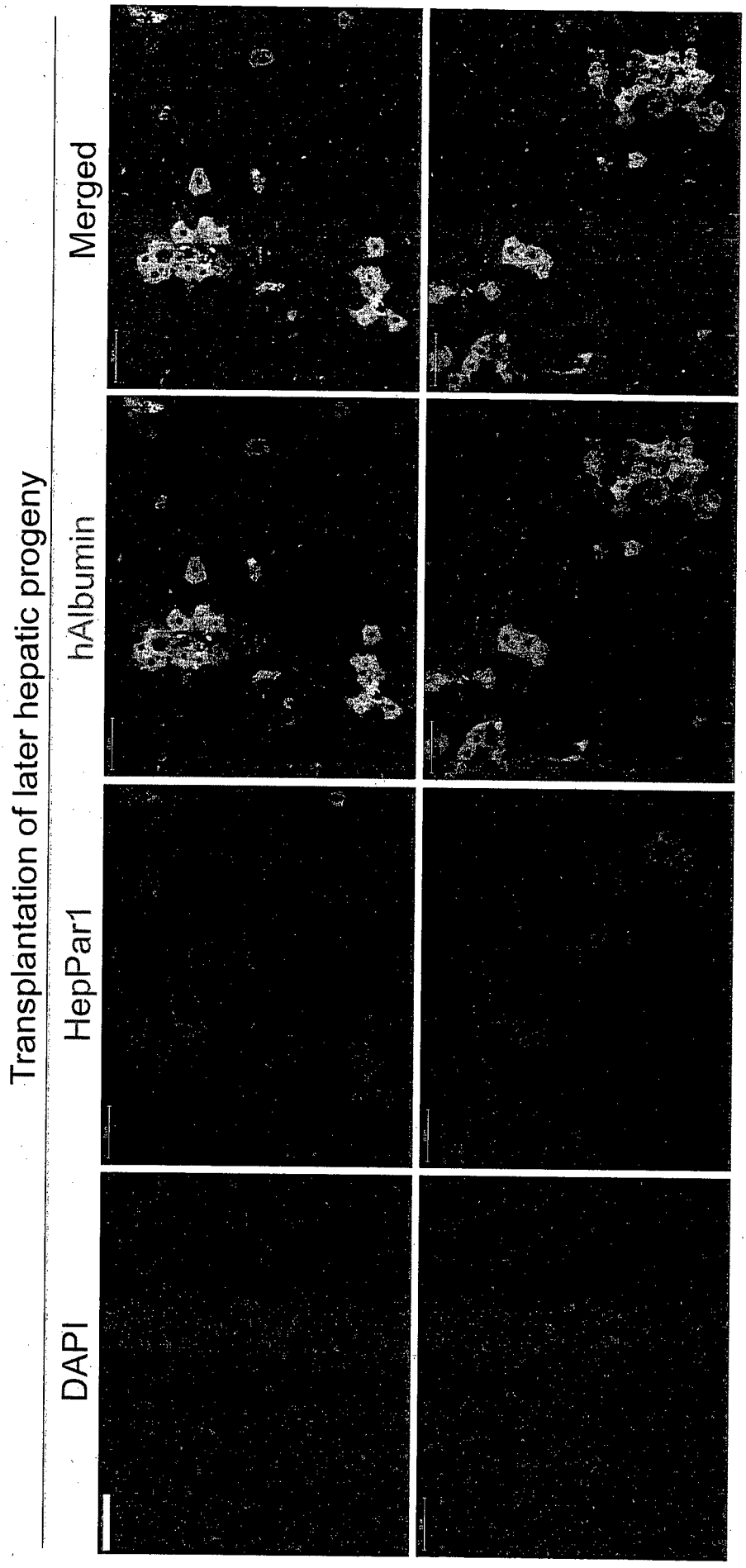

Transplantation of later hepatic progeny
DAPI
HepPar1
hAlbumin
Merged

FIG. 69


Early hepatic progenitors
DAPI
HepPar1
Afp
Merged
Later hepatic progeny
DAPI
HepPar1
Afp
Merged

FIG. 70

| hESC line | H7 chIP-seq & RNA-seq # of Total Passed Illumina Reads (# of Uniquely Aligned Reads) | | | | | | HES3 (# repli-cates) |
|---|---|---|---|---|---|---|---|
| | **Input** | **H3K4me3** | **H3K4me2** | **H3K27me3** | **H3K27ac** | **RNA-seq** | **Affymetrix microarray** |
| hESC | 46,953,326 (37,999,327) | 33,151,167 (23,968,294) | 32,407,688 (28,071,539) | 39,539,641 (28,812,536) | 43,680,960 (37,421,478) | 70,651,559 (54,097,899) | 3 |
| APS | 40,668,117 (32,778,502) | 33,621,173 (24,852,771) | 35,277,317 (30,405,520) | 36,807,047 (28,289,896) | 37,109,028 (31,761,617) | 90,049,689 (70,418,857) | 3 |
| DE | 48,121,636 (38,684,983) | 32,226,238 (24,024,660) | 35,819,126 (30,360,291) | 34,701,839 (27,362,400) | 42,379,490 (36,162,419) | 85,434,777 (68,458,887) | 3 |
| AFG | 42,425,219 (34,203,212) | 34,289,338 (25,343,250) | 36,016,254 (31,503,417) | 36,586,138 (28,987,197) | 41,460,711 (36,427,381) | 88,299,484 (71,416,623) | 3 |
| PFG | 39,138,045 (31,423,936) | 34,958,923 (26,369,516) | 32,781,185 (28,808,105) | 38,752,066 (31,017,154) | 37,947,007 (33,321,267) | 89,052,131 (73,263,188) | 3 |
| MHG | 43,654,736 (35,469,473) | 33,831,958 (25,238,641) | 30,663,675 (26,708,061) | 35,329,148 (28,295,115) | 37,394,612 (32,219,198) | 88,531,796 (68,860,031) | 3 |

FIG. 71

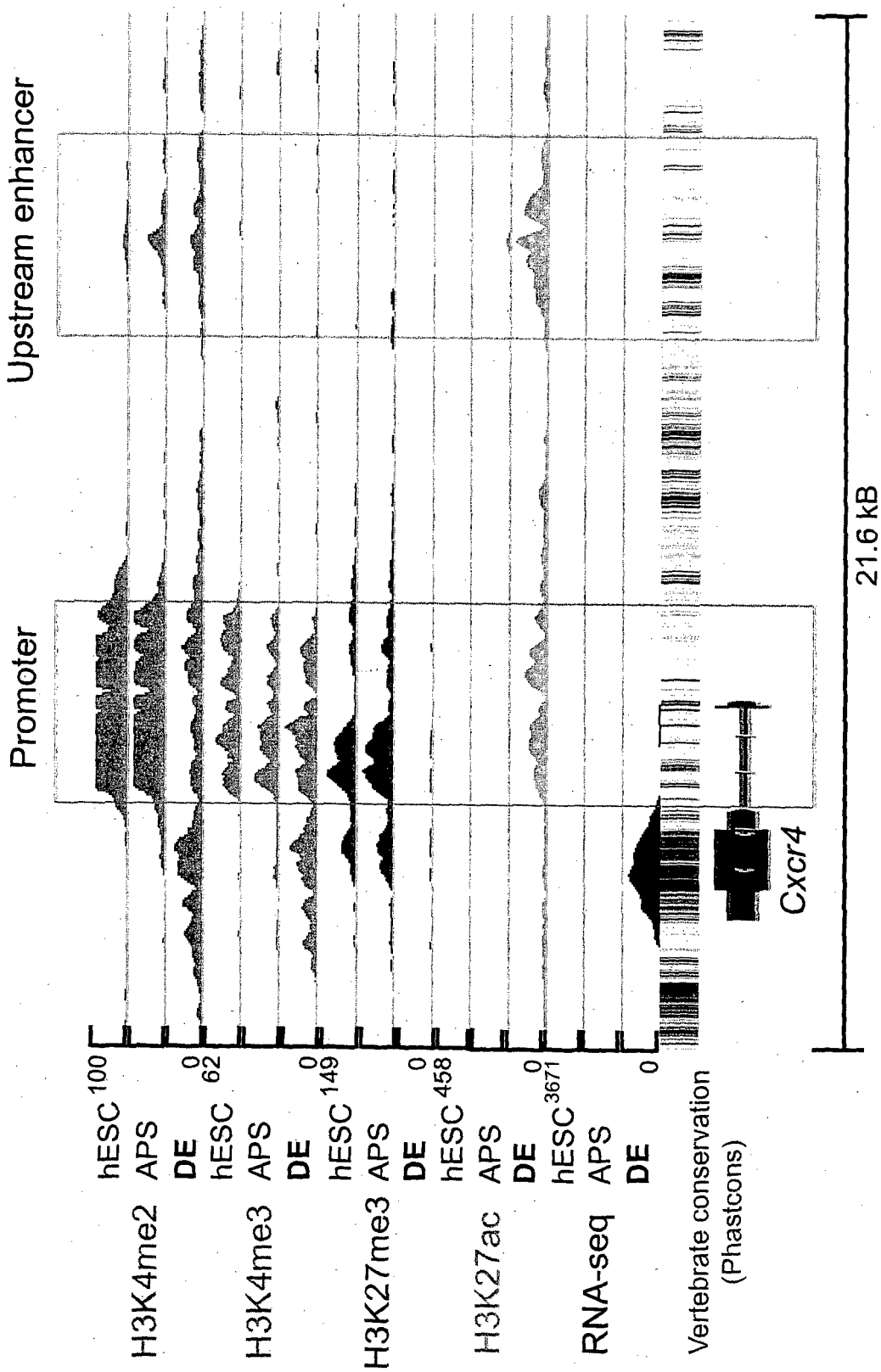

Promoter
Upstream enhancer
21.6 kB
Cxcr4
H3K4me2
H3K4me3
H3K27me3
H3K27ac
RNA-seq
hESC
APS
DE
Vertebrate conservation
(Phastcons)
100
62
149
458
3671
0

FIG. 72

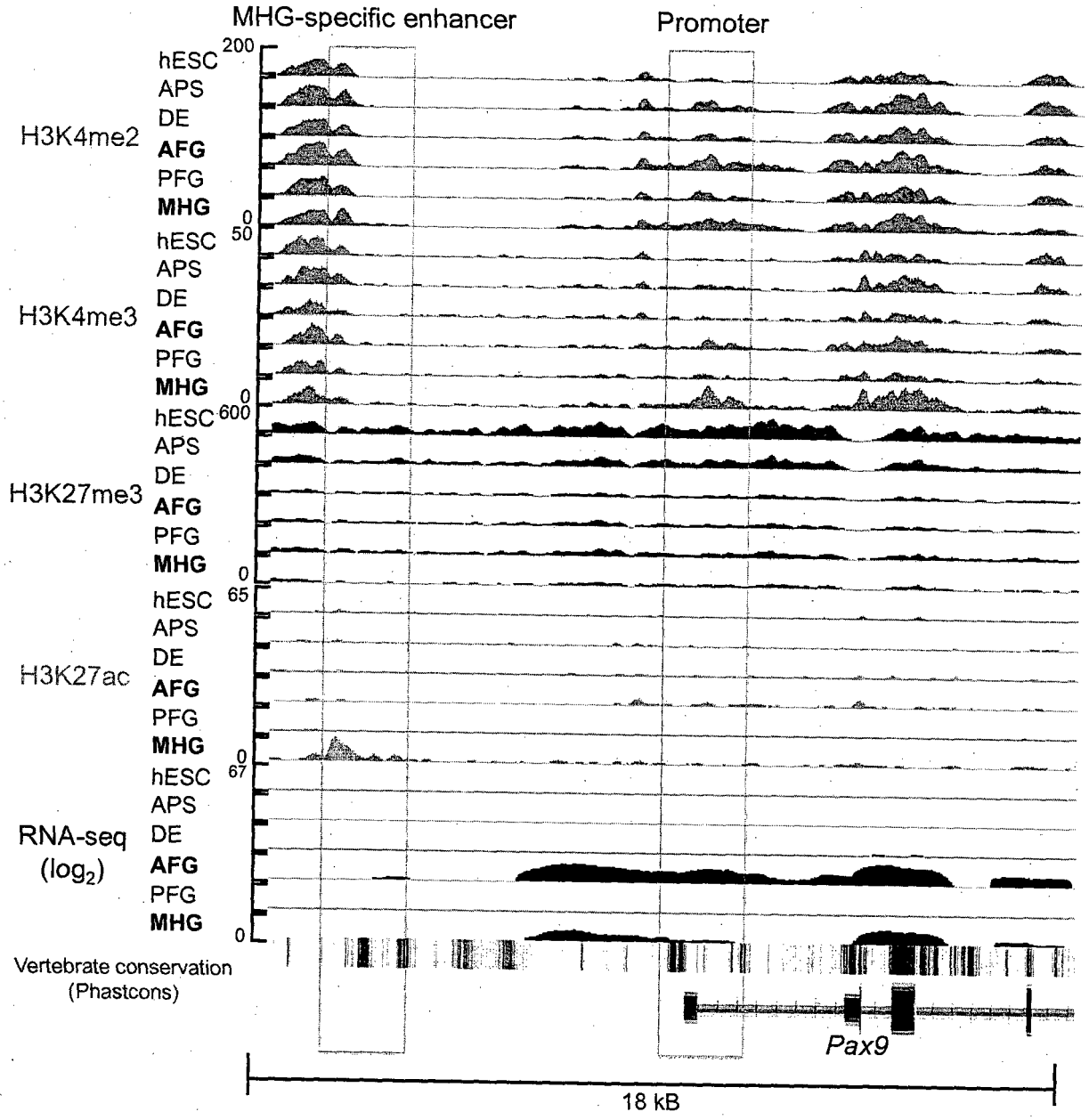

MHG-specific enhancer
Promoter
H3K4me2
H3K4me3
H3K27me3
H3K27ac
RNA-seq (log2)
hESC
APS
DE
AFG
PFG
MHG
200
0
50
600
65
67
Vertebrate conservation (Phastcons)
Pax9
18 kB

FIG. 73

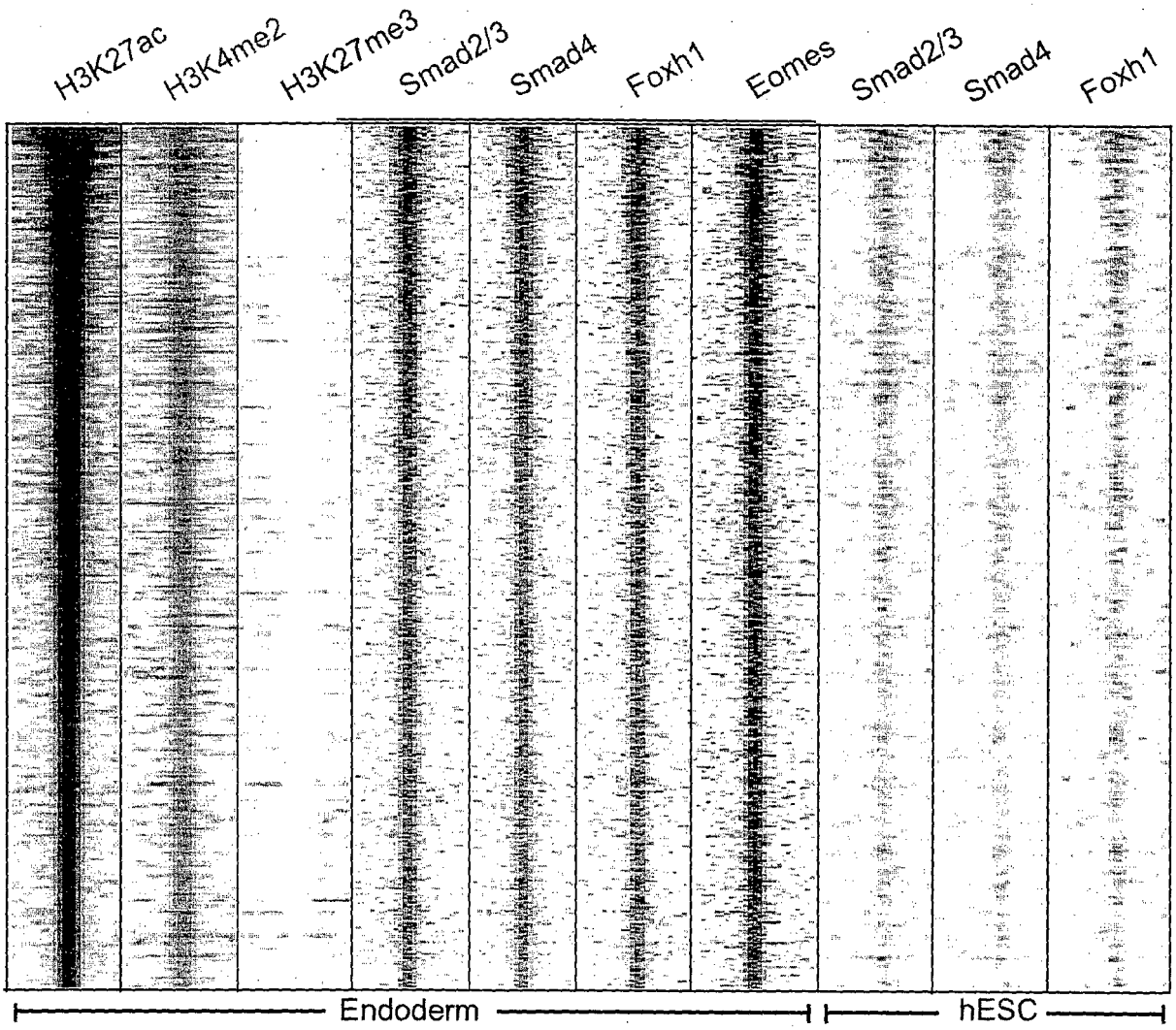

H3K27ac
H3K4me2
H3K27me3
Smad2/3
Smad4
Foxh1
Eomes
Smad2/3
Smad4
Foxh1
Endoderm
hESC

FIG. 74

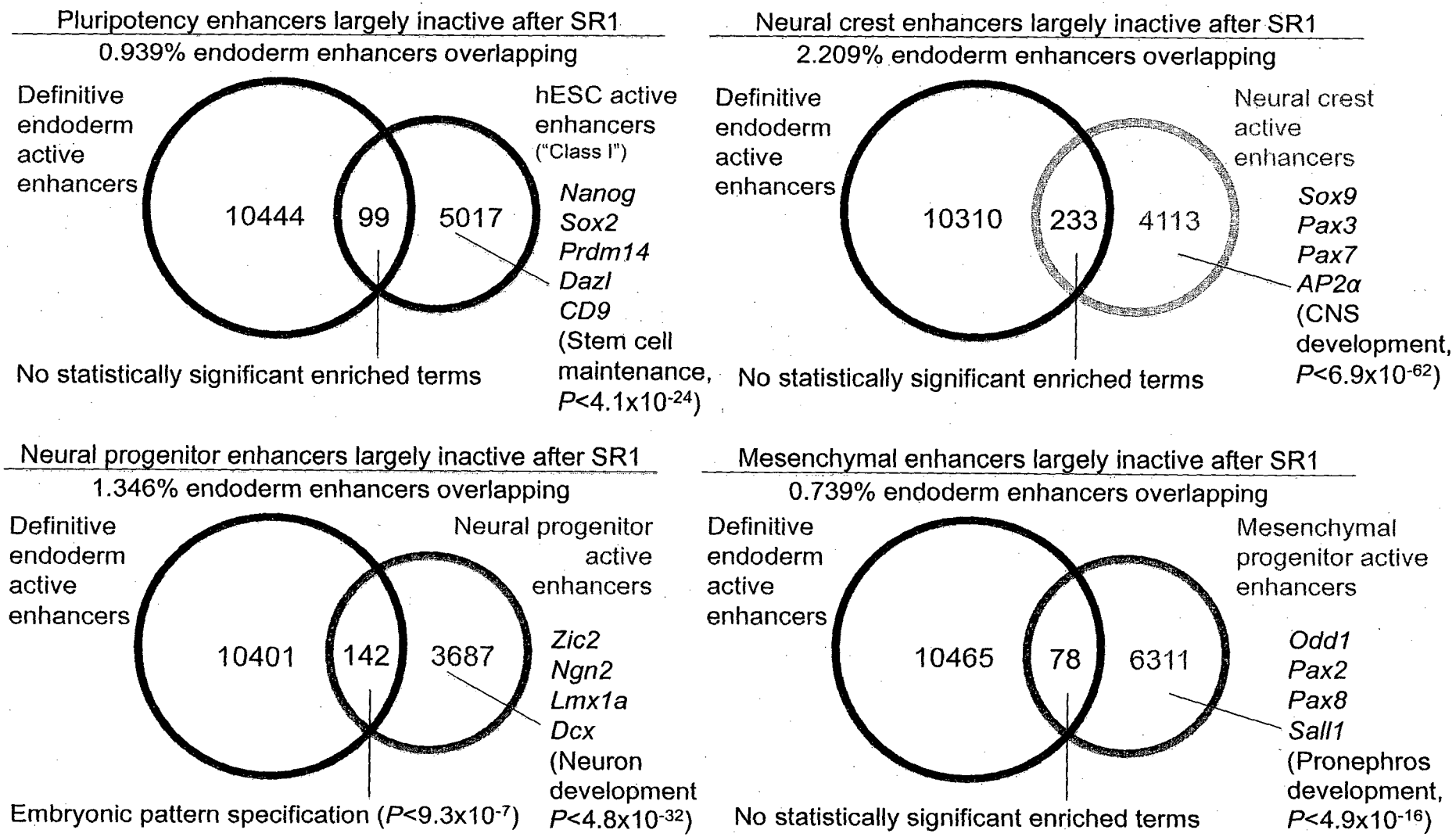
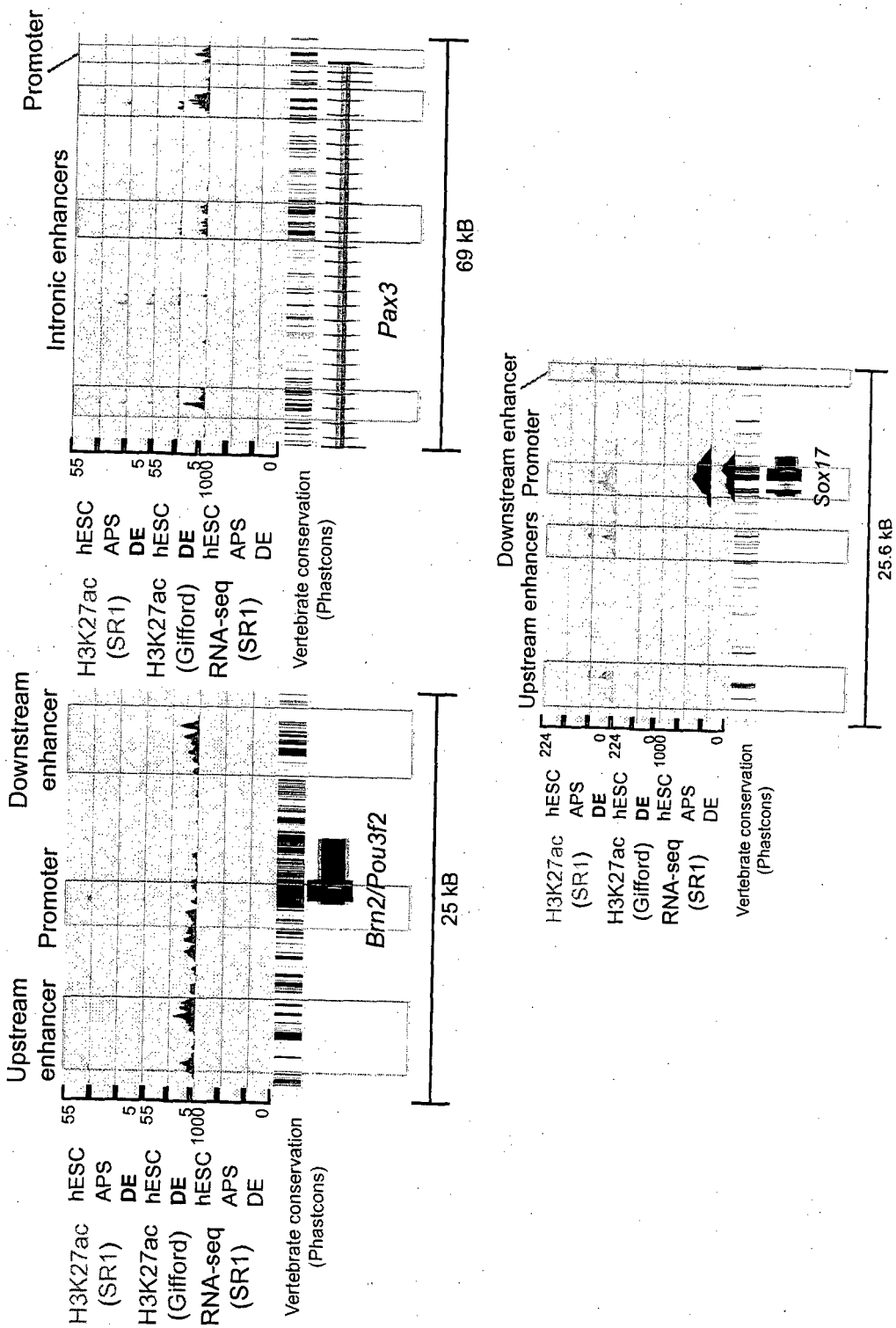

Pluripotency enhancers largely inactive after SR1
0.939% endoderm enhancers overlapping
Definitive endoderm active enhancers
10444
99
5017
hESC active enhancers ("Class I")
Nanog
Sox2
Prdm14
Dazl
CD9
(Stem cell maintenance, P<4.1x10-24)
No statistically significant enriched terms
Neural crest enhancers largely inactive after SR1
2.209% endoderm enhancers overlapping
Definitive endoderm active enhancers
10310
233
4113
Neural crest active enhancers
Sox9
Pax3
Pax7
AP2α
(CNS development, P<6.9x10-62)
No statistically significant enriched terms
Neural progenitor enhancers largely inactive after SR1
1.346% endoderm enhancers overlapping
Definitive endoderm active enhancers
10401
142
3687
Neural progenitor active enhancers
Zic2
Ngn2
Lmx1a
Dcx
(Neuron development P<4.8x10-32)
Embryonic pattern specification (P<9.3x10-7)
Mesenchymal enhancers largely inactive after SR1
0.739% endoderm enhancers overlapping
Definitive endoderm active enhancers
10465
78
6311
Mesenchymal progenitor active enhancers
Odd1
Pax2
Pax8
Sall1
(Pronephros development, P<4.9x10-16)
No statistically significant enriched terms

FIG. 75


Upstream enhancer
Promoter
Downstream enhancer
H3K27ac (SR1)
H3K27ac (Gifford)
RNA-seq (SR1)
hESC
APS
DE
hESC
DE
hESC
APS
DE
55
5
55
5
1000
0
Vertebrate conservation (Phastcons)
Brn2/Pou3f2
25 kB
Intronic enhancers
Promoter
Pax3
69 kB
Upstream enhancers
Promoter
Downstream enhancer
Sox17
25.6 kB
224
0
224
0
1000
0

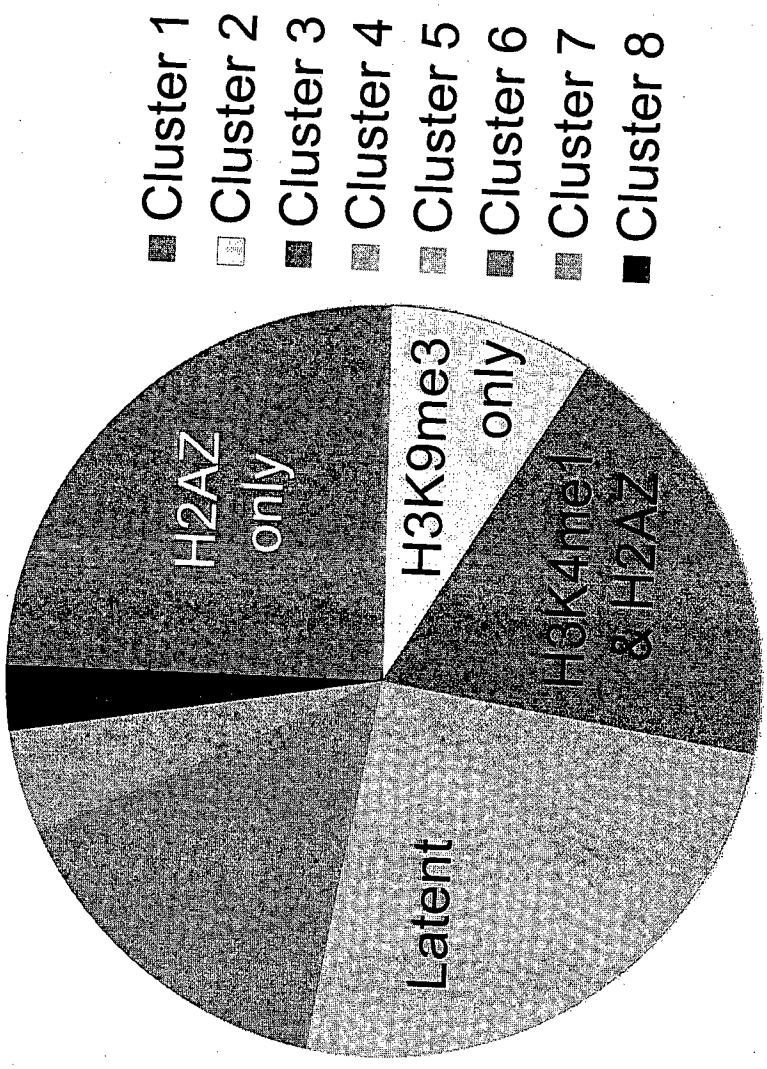
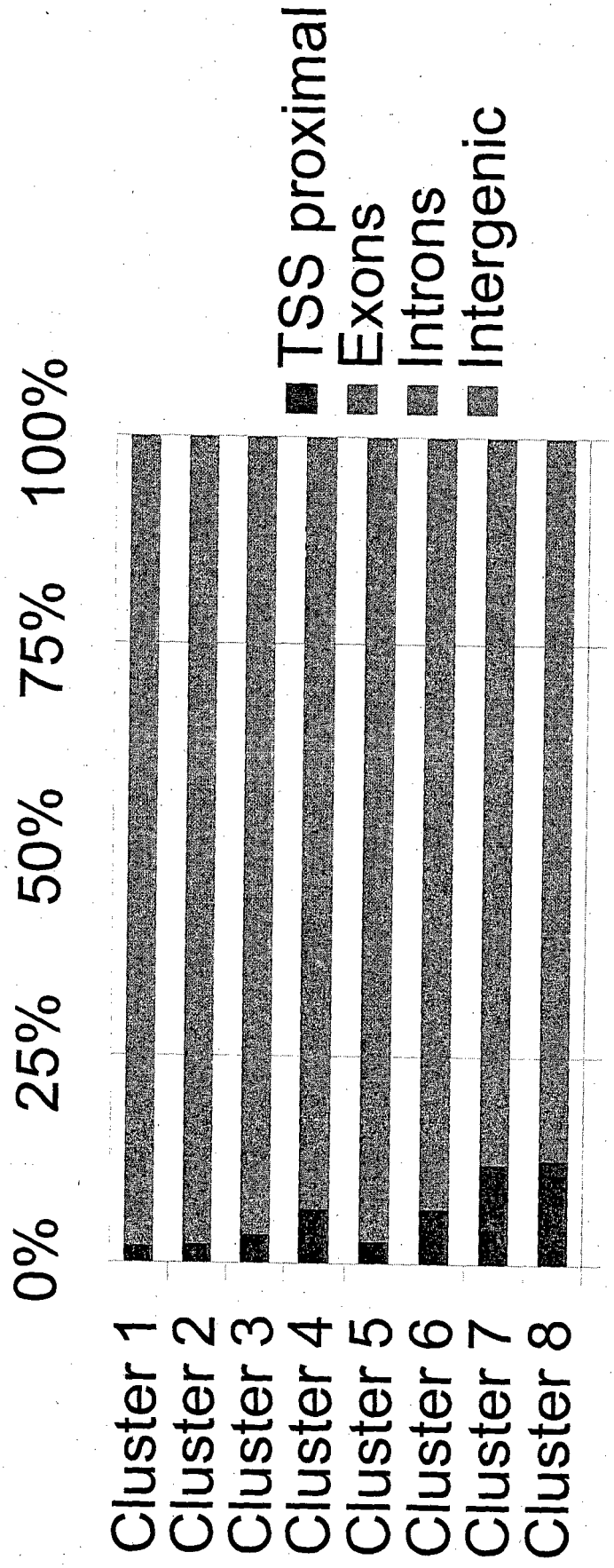

Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6
Cluster 7
Cluster 8
H2AZ only
H3K9me3 only
H3K4me1 & H2AZ
Latent

FIG. 76

FIG. 77


TSS proximal
Exons
Introns
Intergenic
0%
25%
50%
75%
100%
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6
Cluster 7
Cluster 8

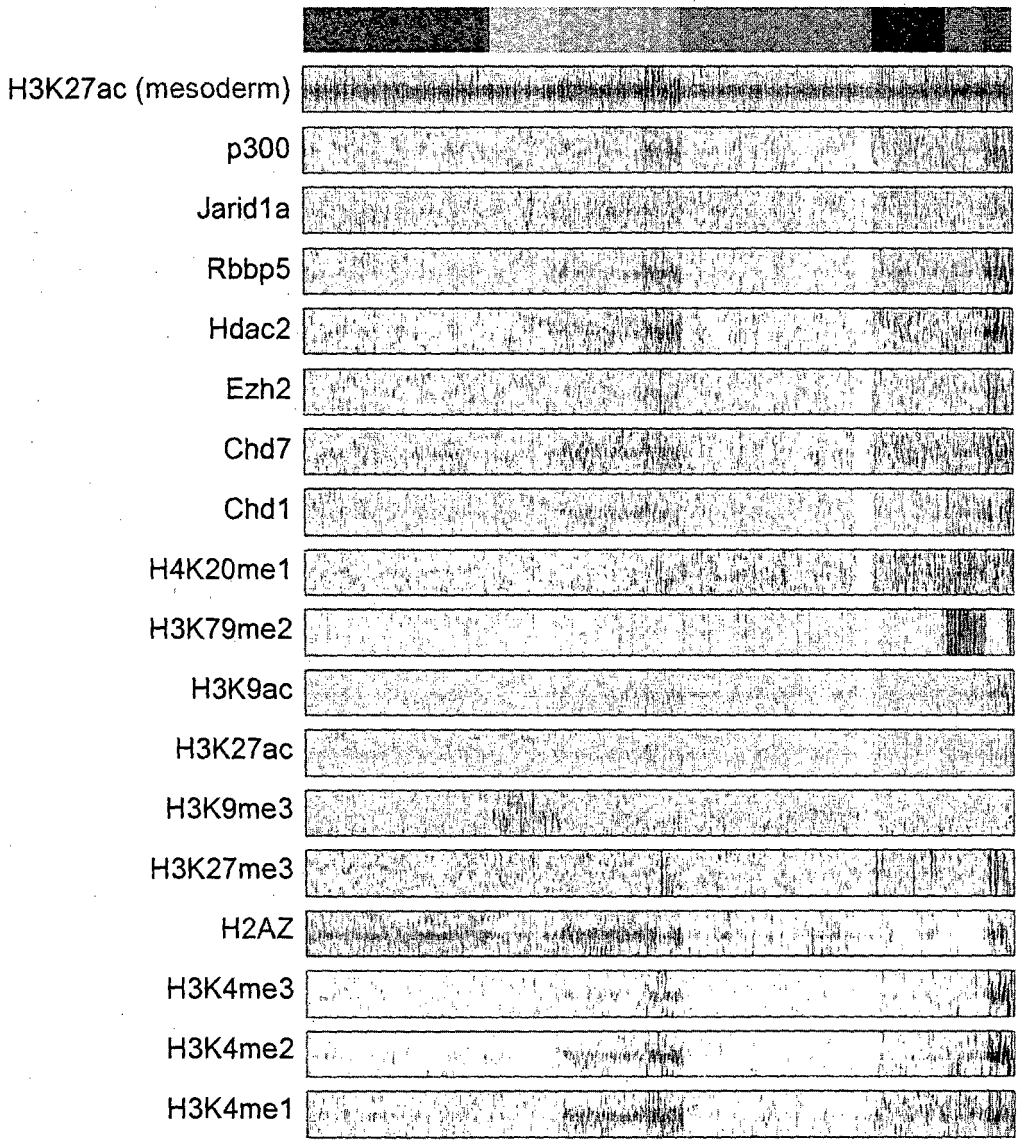

H3K27ac (mesoderm)
p300
Jarid1a
Rbbp5
Hdac2
Ezh2
Chd7
Chd1
H4K20me1
H3K79me2
H3K9ac
H3K27ac
H3K9me3
H3K27me3
H2AZ
H3K4me3
H3K4me2
H3K4me1

FIG. 78

FIG. 79

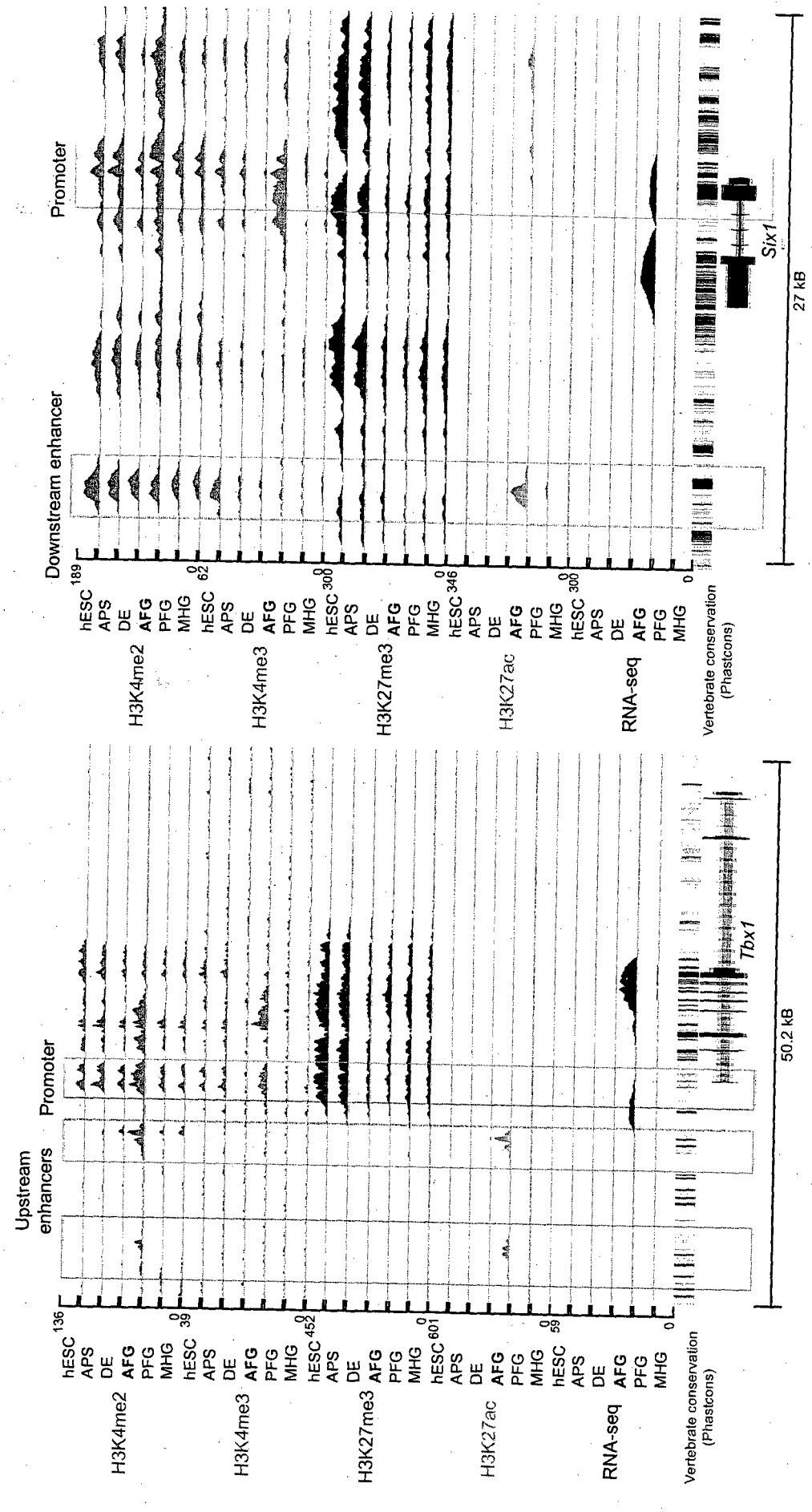
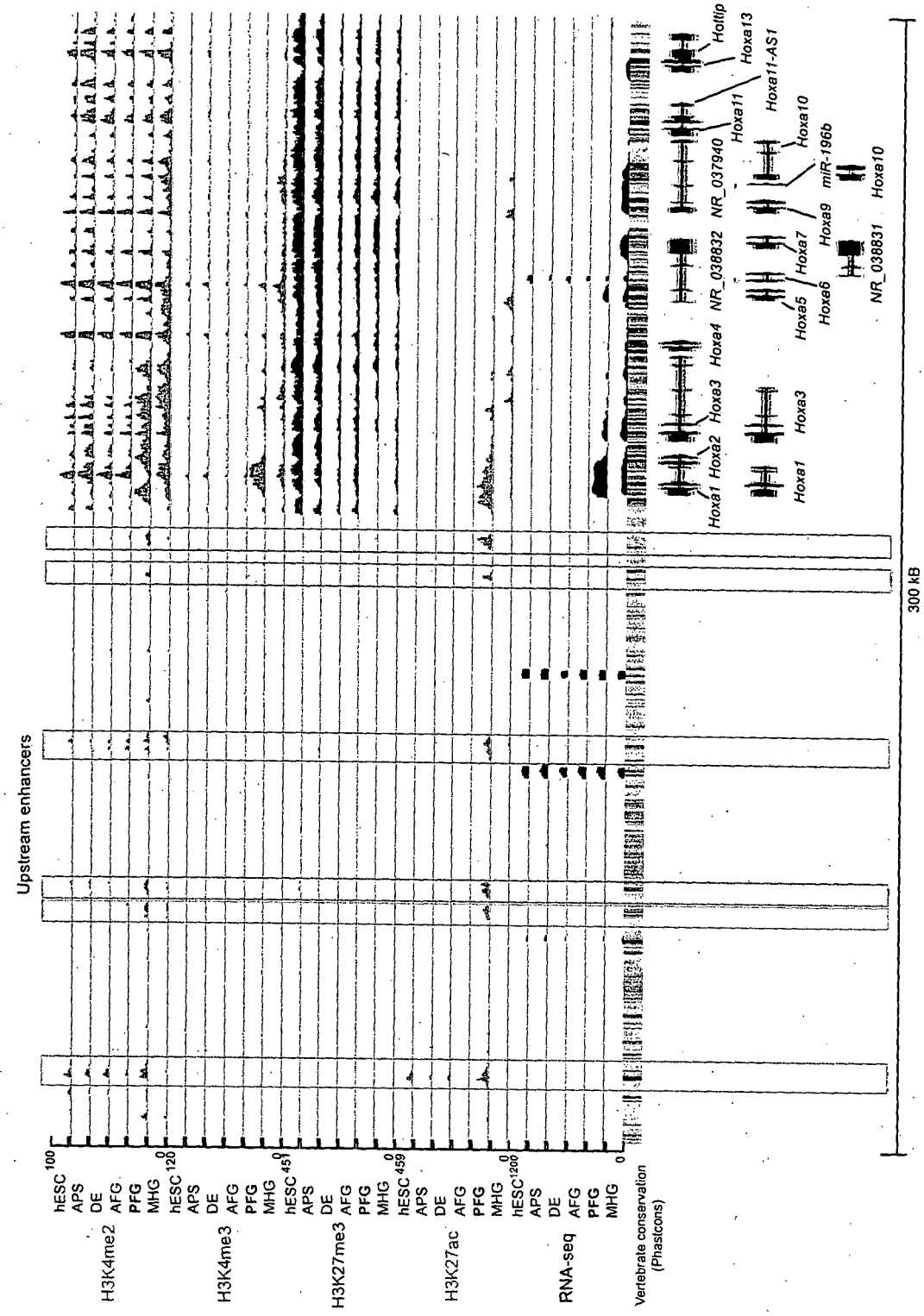

Upstream enhancers
Promoter
Downstream enhancer
Promoter
H3K4me2
H3K4me3
H3K27me3
H3K27ac
RNA-seq
hESC
APS
DE
AFG
PFG
MHG
Vertebrate conservation (Phastcons)
Tbx1
Six1
50.2 kB
27 kB

FIG. 80


Upstream enhancers
H3K4me2
H3K4me3
H3K27me3
H3K27ac
RNA-seq
hESC
APS
DE
AFG
PFG
MHG
Vertebrate conservation (Phastcons)
Hoxa1
Hoxa2
Hoxa3
Hoxa4
NR_038832
NR_037940
Hoxa11
Hottip
Hoxa13
Hoxa11-AS1
Hoxa10
miR-196b
Hoxa9
Hoxa7
Hoxa6
Hoxa5
NR_038831
300 kB