FIG. 1

A

List of small molecules compounds	
Small molecules	Target pathway
PD0325901 (PD03)	MAPK/ERK
BIO	Wnt/β catenin
ChIR99021 (CHIR)	Wnt/β catenin
RepSox (SOX)	Activin/TGFβ
A83-01 (A83)	Activin/TGFβ
IDE-1	Activin/TGFβ
Pifithrin-α (PFTα)	P53
Forskolin (FOR)	PKA
PD173074 (PD17)	FGF
Dorsomorphin (DOR)	BMP
PD153035 (PD15)	EGF

FIG. 1

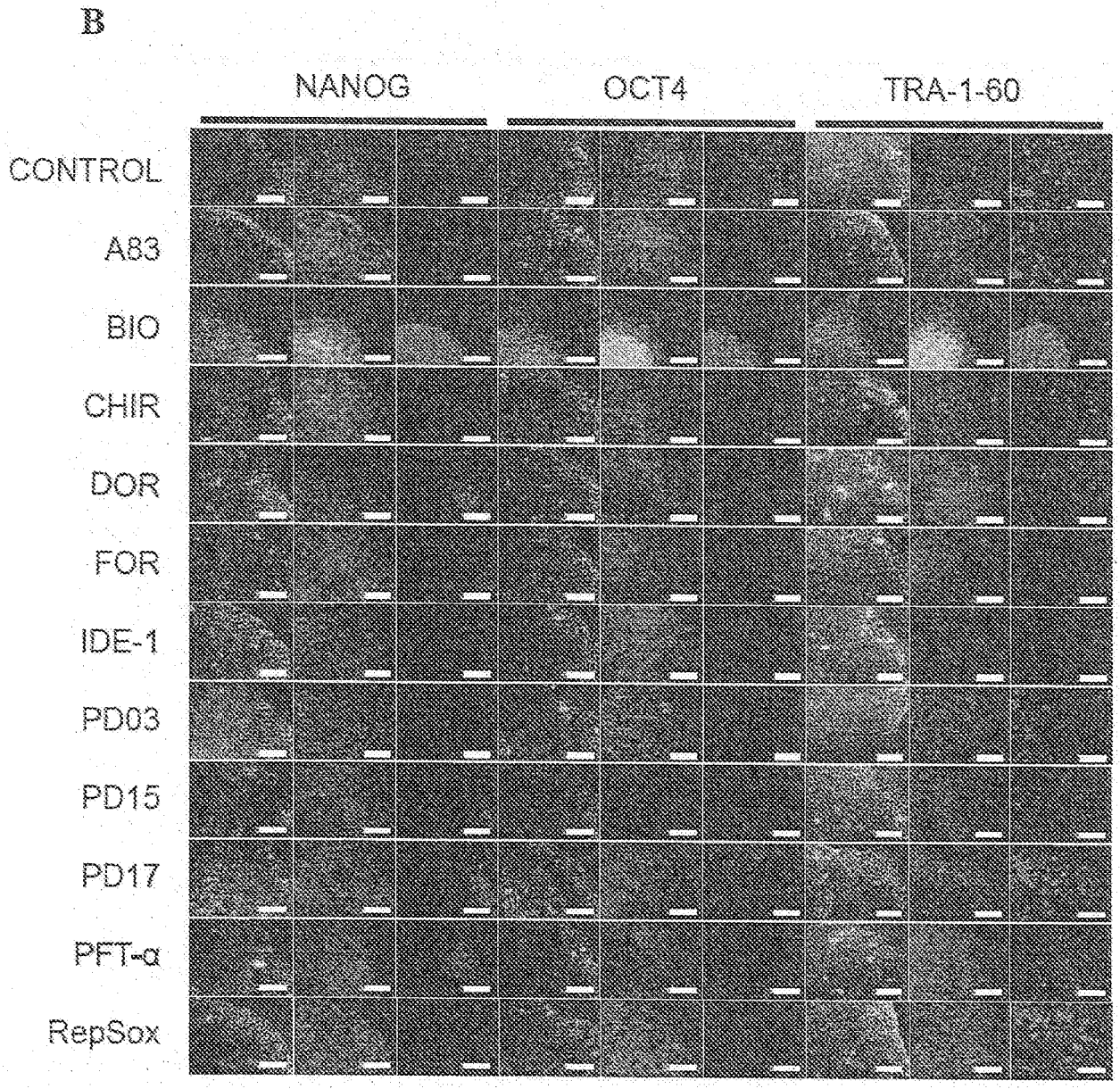
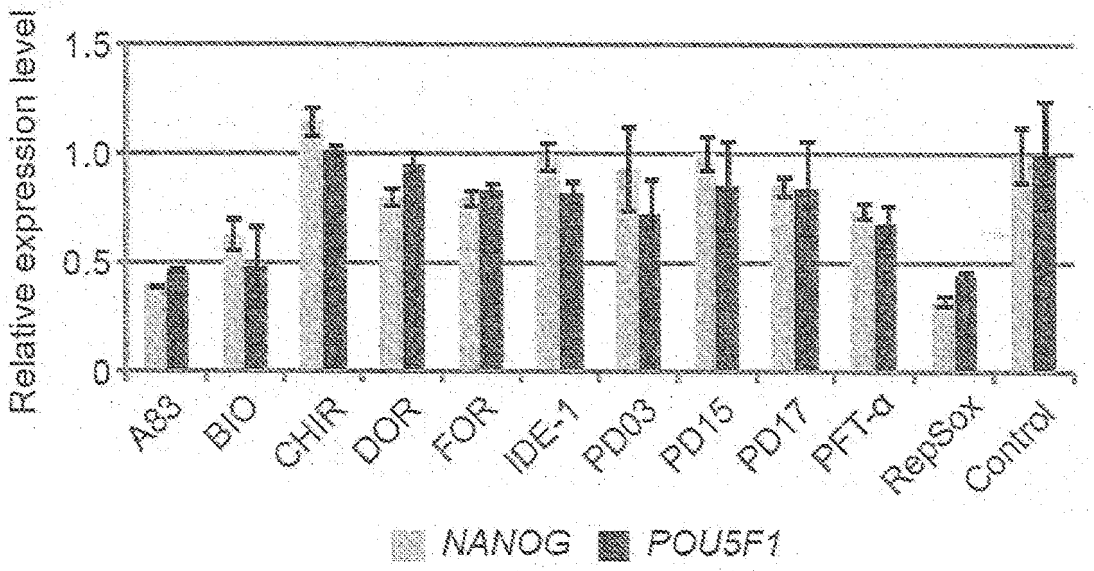

B
NANOG
OCT4
TRA-1-60
CONTROL
A83
BIO
CHIR
DOR
FOR
IDE-1
PD03
PD15
PD17
PFT-α
RepSox

FIG. 1

C



D

Combinations of small molecules used			
No.	Small molecules		
1	BIO/IDE-1/SOX	13	DOR/FOR/ PFTα
2	PD17/BIO/SOX	14	PD17/IDE-1/ PFTα
3	BIO/SOX/DOR	15	PD03/CHIR/FOR
4	PD03/IDE/SOX	16	PD03/CHIR/DOR
5	PD17/PD03/BIO	17	A83/CHIR/DOR
6	A83/FOR/DOR	18	PD15/DOR/CHIR
7	A83/PFTα/DOR	19	PD03/BIO/ PFTα
8	A83/BIO/DOR	20	PD17/BIO/DOR
9	PD15/ PFTα/FOR	21	PD17/FOR/DOR
10	PD15/FOR/IDE-1	22	PD03/BIO/DOR
11	BIO/FOR/DOR	23	PD17/BIO/IDE-1
12	DOR/FOR/IDE-1	24	PD17/CHIR/DOR

FIG. 1

E

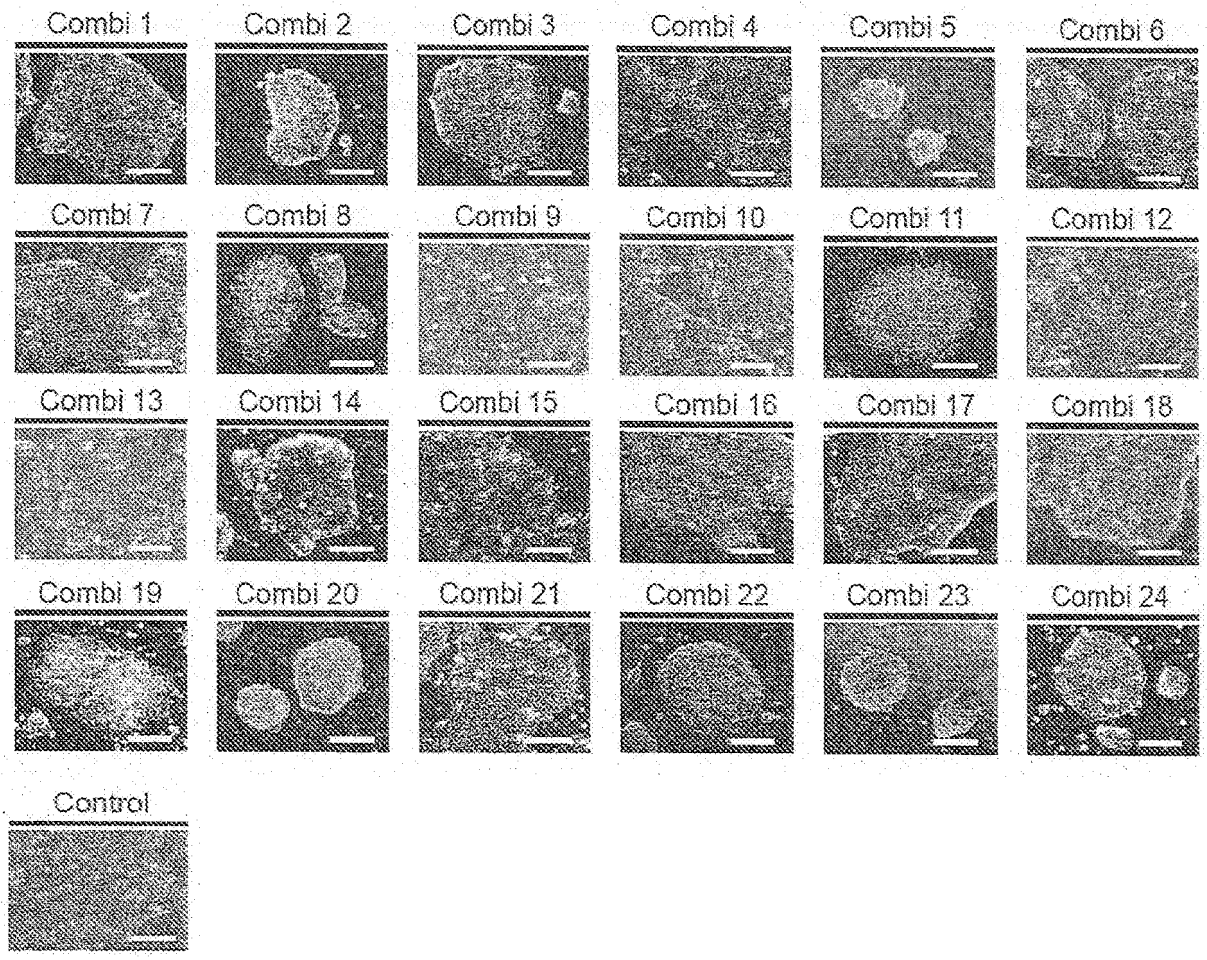

Combi 1
Combi 2
Combi 3
Combi 4
Combi 5
Combi 6
Combi 7
Combi 8
Combi 9
Combi 10
Combi 11
Combi 12
Combi 13
Combi 14
Combi 15
Combi 16
Combi 17
Combi 18
Combi 19
Combi 20
Combi 21
Combi 22
Combi 23
Combi 24
Control

Fig. 1

F

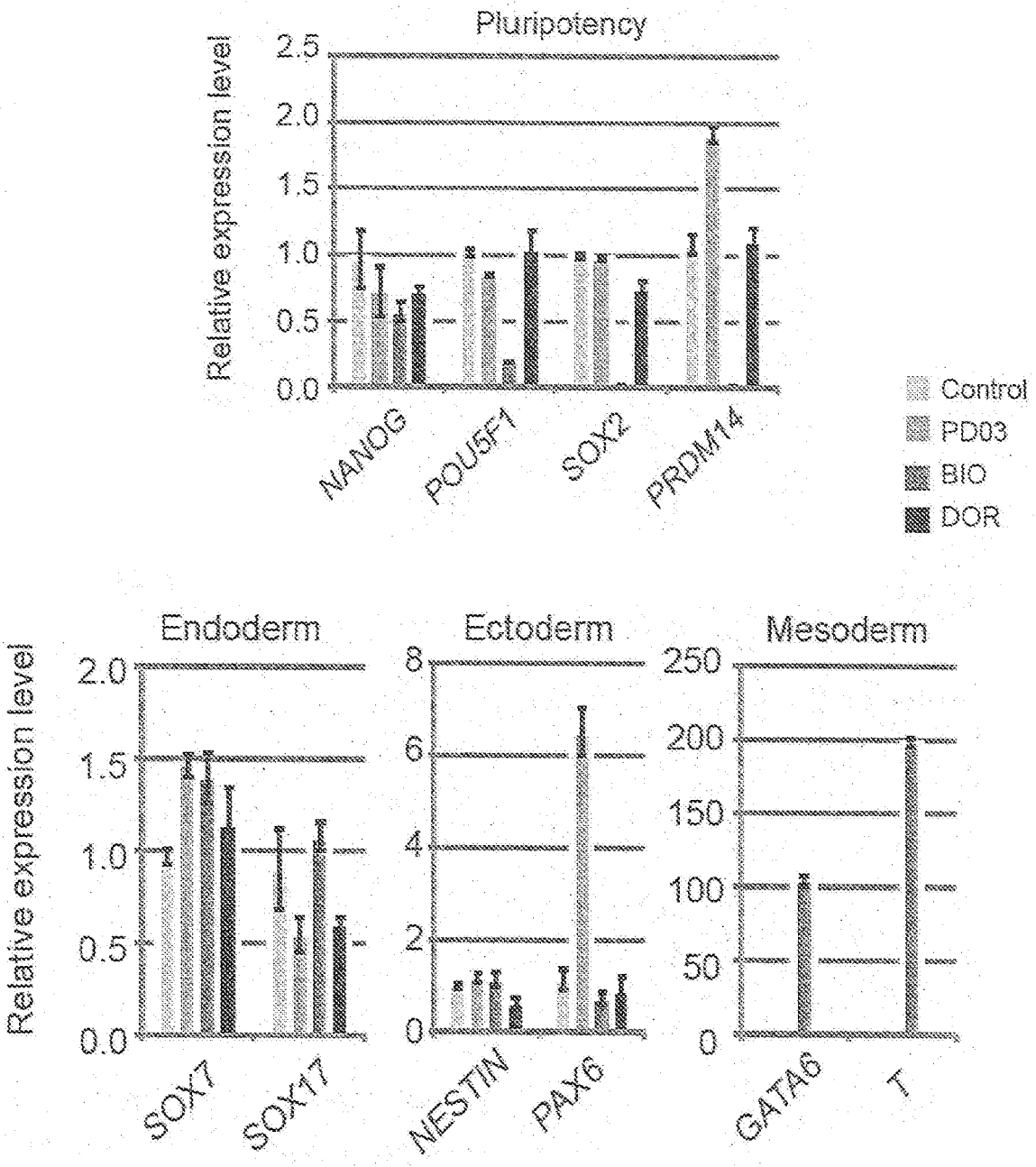

Pluripotency
Relative expression level
2.5
2.0
1.5
1.0
0.5
0.0
NANOG
POU5F1
SOX2
PRDM14
Control
PD03
BIO
DOR
Endoderm
Ectoderm
Mesoderm
2.0
1.5
1.0
0.5
0.0
8
6
4
2
0
250
200
150
100
50
0
SOX7
SOX17
NESTIN
PAX6
GATA6
T

Fig. 1

G

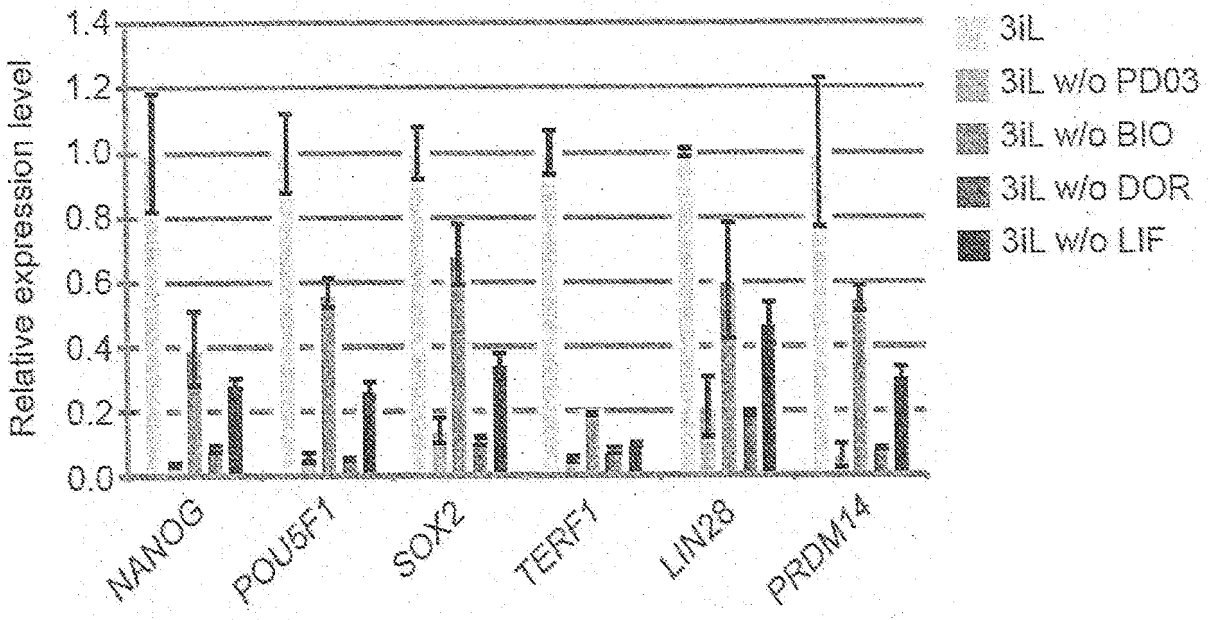

Relative expression level
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
NANOG
POU5F1
SOX2
TERF1
LIN28
PRDM14
3iL
3iL w/o PD03
3iL w/o BIO
3iL w/o DOR
3iL w/o LIF

FIG. 2

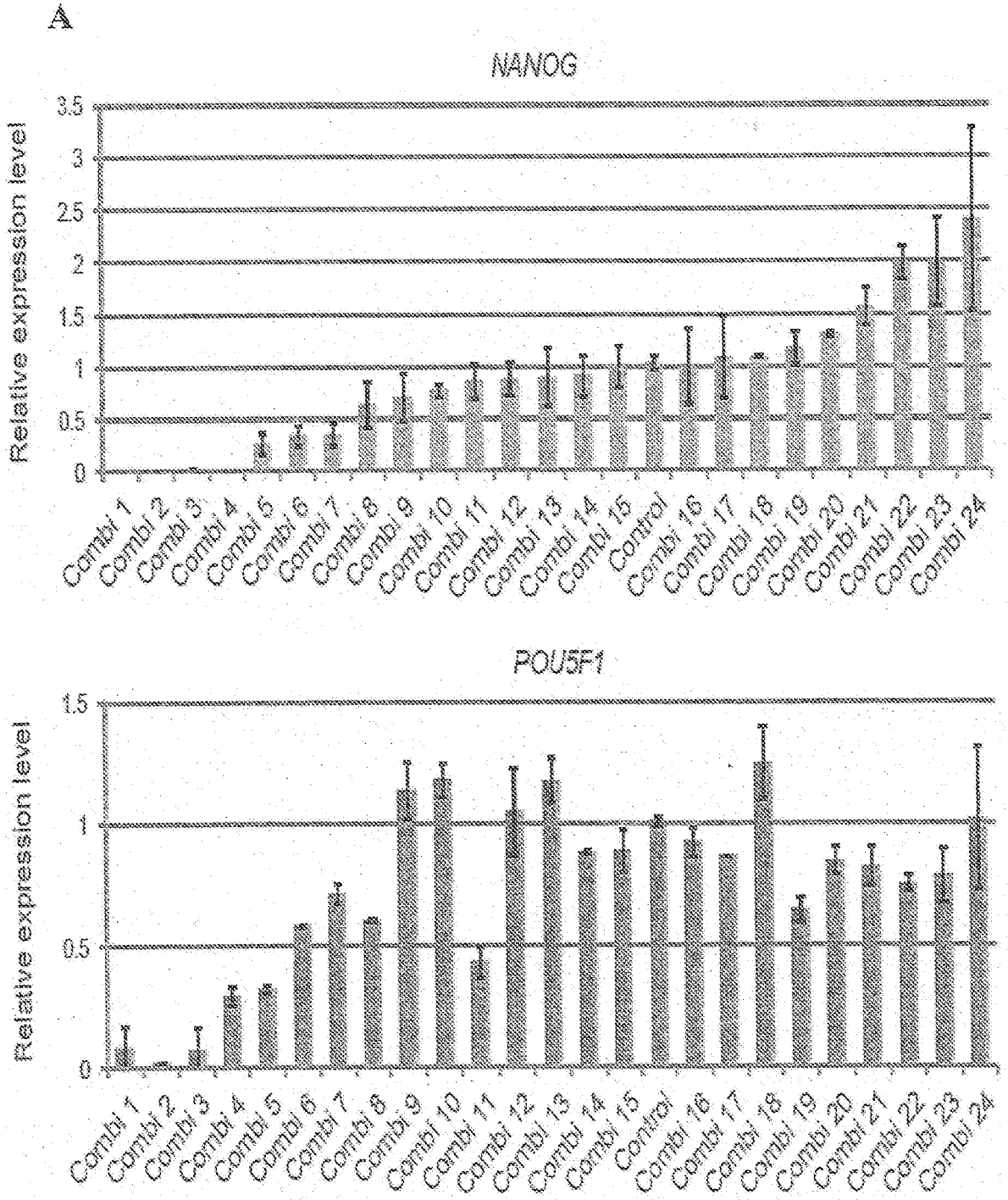
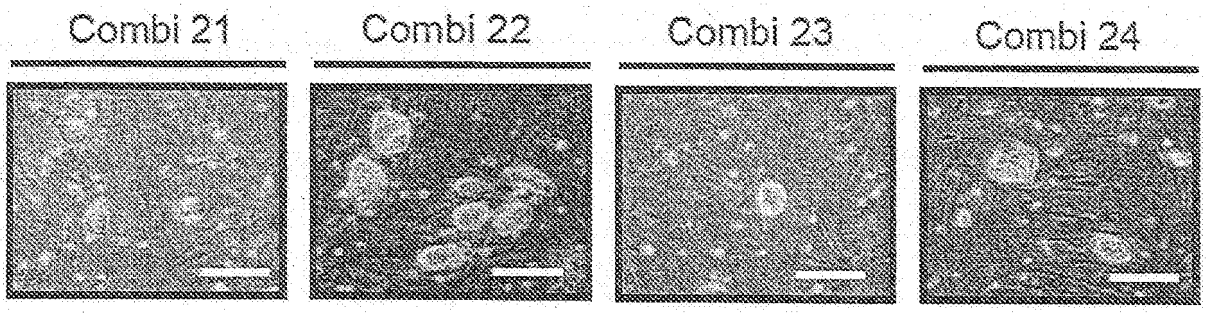

A
NANOG
Relative expression level
3.5
3
2.5
2
1.5
1
0.5
0
Combi 1
Combi 2
Combi 3
Combi 4
Combi 5
Combi 6
Combi 7
Combi 8
Combi 9
Combi 10
Combi 11
Combi 12
Combi 13
Combi 14
Combi 15
Control
Combi 16
Combi 17
Combi 18
Combi 19
Combi 20
Combi 21
Combi 22
Combi 23
Combi 24
POU5F1
Relative expression level
1.5
1
0.5
0
Combi 1
Combi 2
Combi 3
Combi 4
Combi 5
Combi 6
Combi 7
Combi 8
Combi 9
Combi 10
Combi 11
Combi 12
Combi 13
Combi 14
Combi 15
Control
Combi 16
Combi 17
Combi 18
Combi 19
Combi 20
Combi 21
Combi 22
Combi 23
Combi 24

FIG. 2

B


Combi 21
Combi 22
Combi 23
Combi 24

C

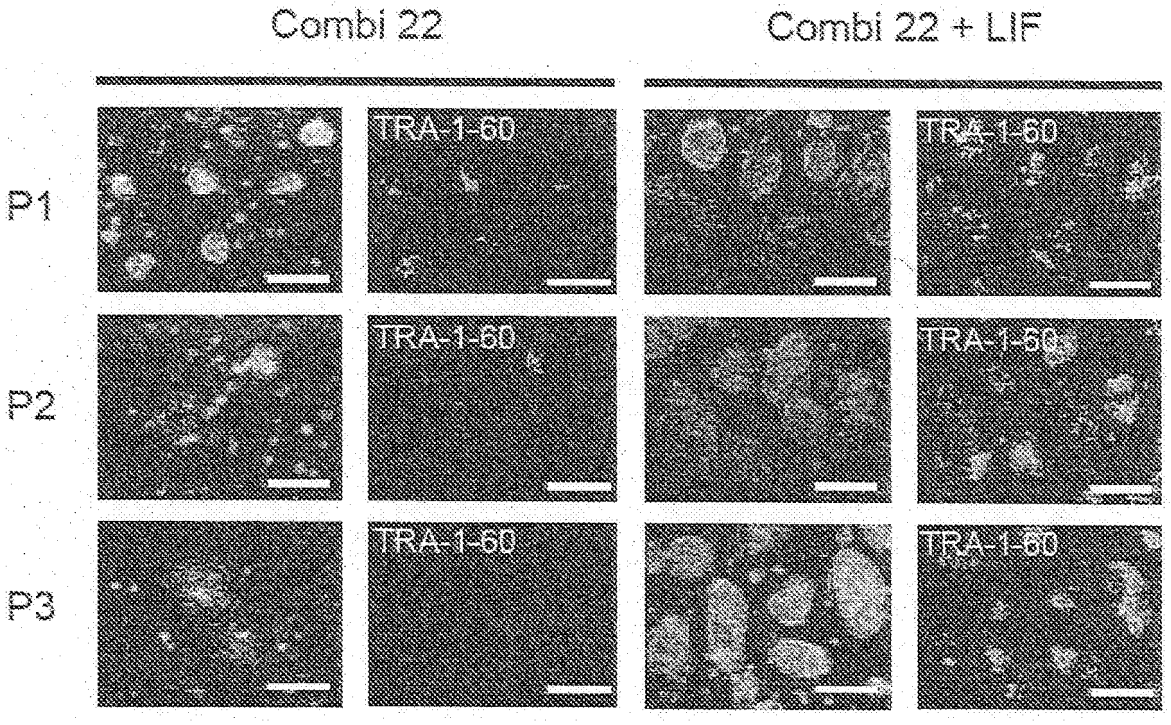
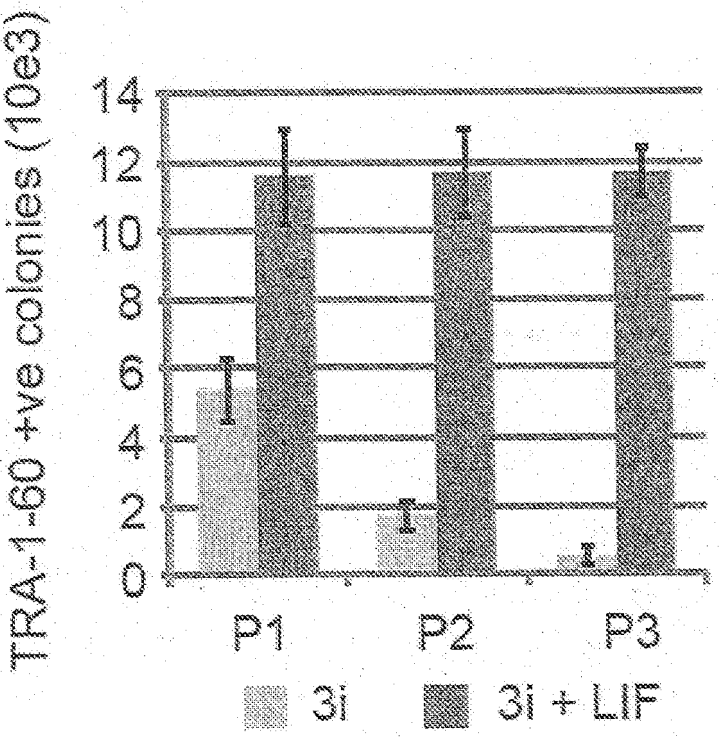

Combi 22
Combi 22 + LIF
P1
P2
P3
TRA-1-60
TRA-1-60
TRA-1-60
TRA-1-60
TRA-1-60
TRA-1-60

FIG. 2

D


TRA-1-60 +ve colonies (10e3)
14
12
10
8
6
4
2
0
P1
P2
P3
3i
3i + LIF

E

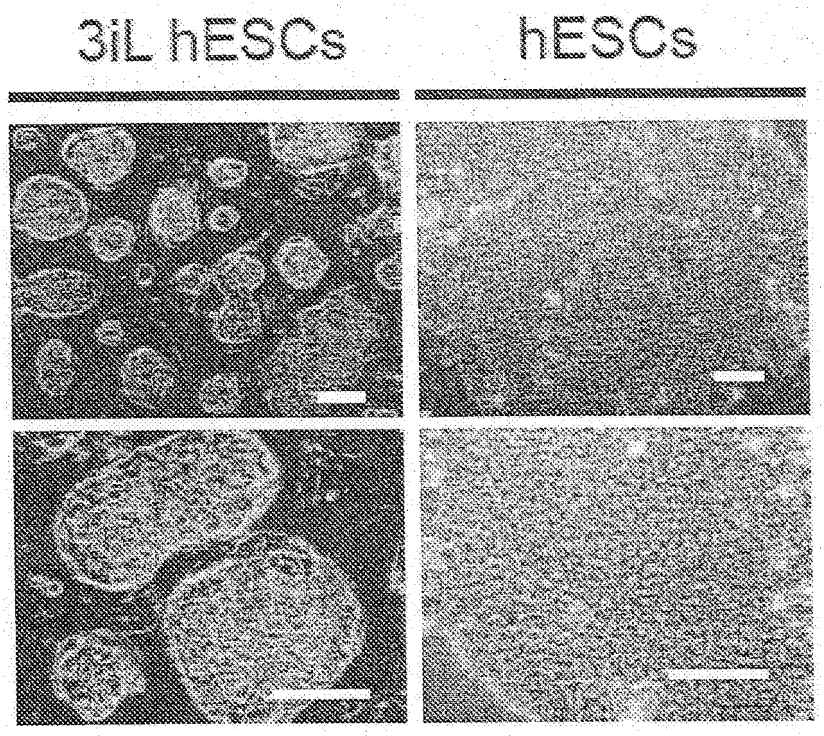

3iL hESCs
hESCs

FIG. 2

F

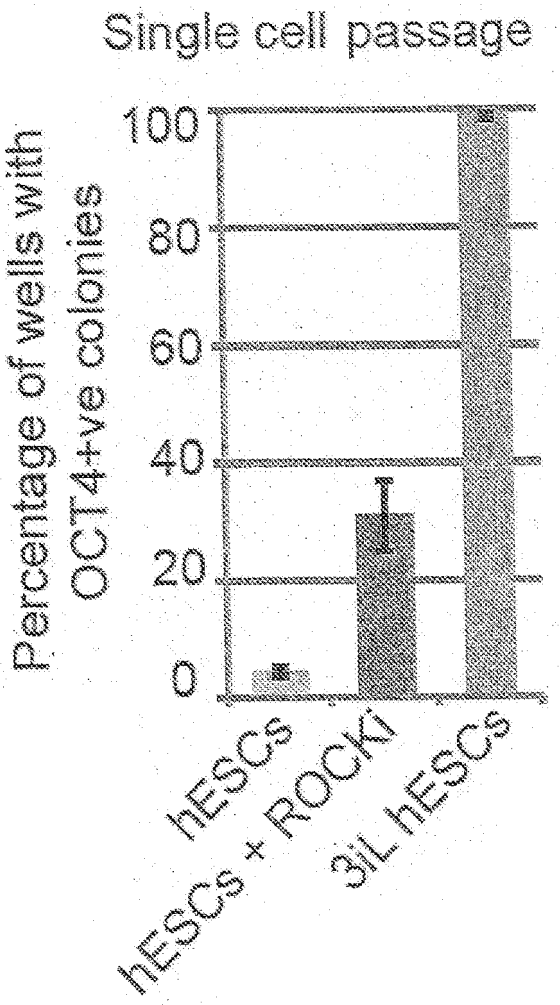
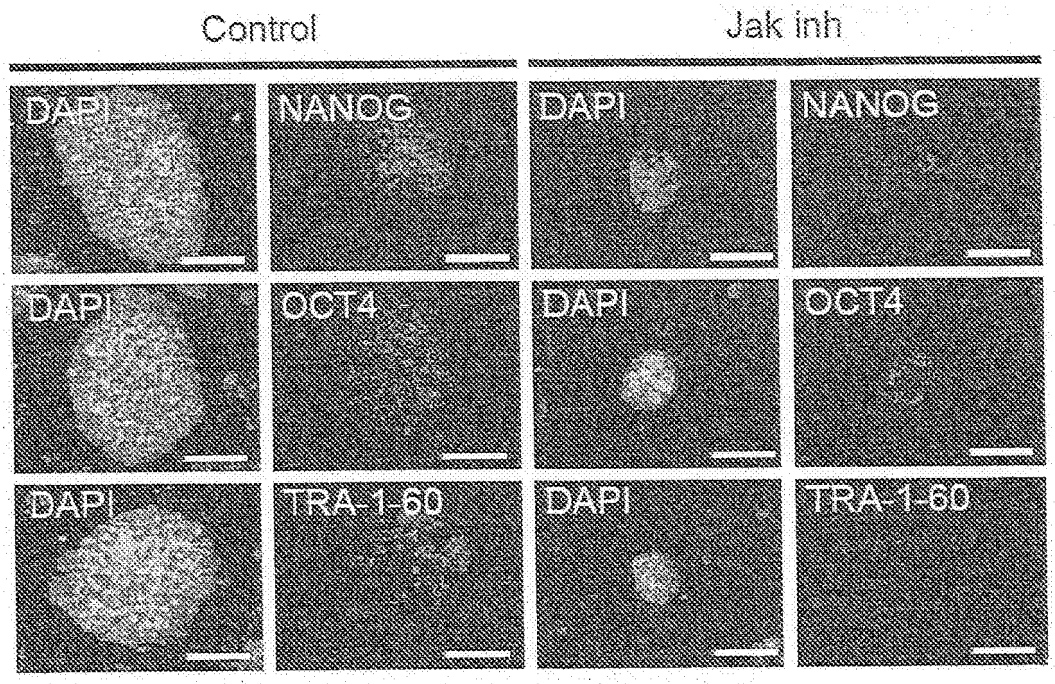

Single cell passage
Percentage of wells with OCT4+ve colonies
100
80
60
40
20
0
hESCs
hESCs + ROCKi
3iL hESCs

FIG. 3

A


Control
Jak inh
DAPI
NANOG
DAPI
NANOG
DAPI
OCT4
DAPI
OCT4
DAPI
TRA-1-60
DAPI
TRA-1-60

B

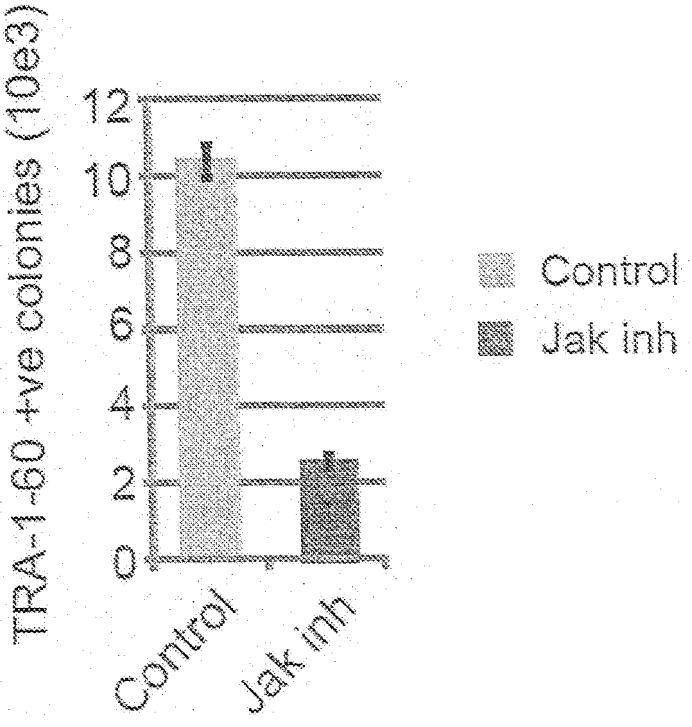
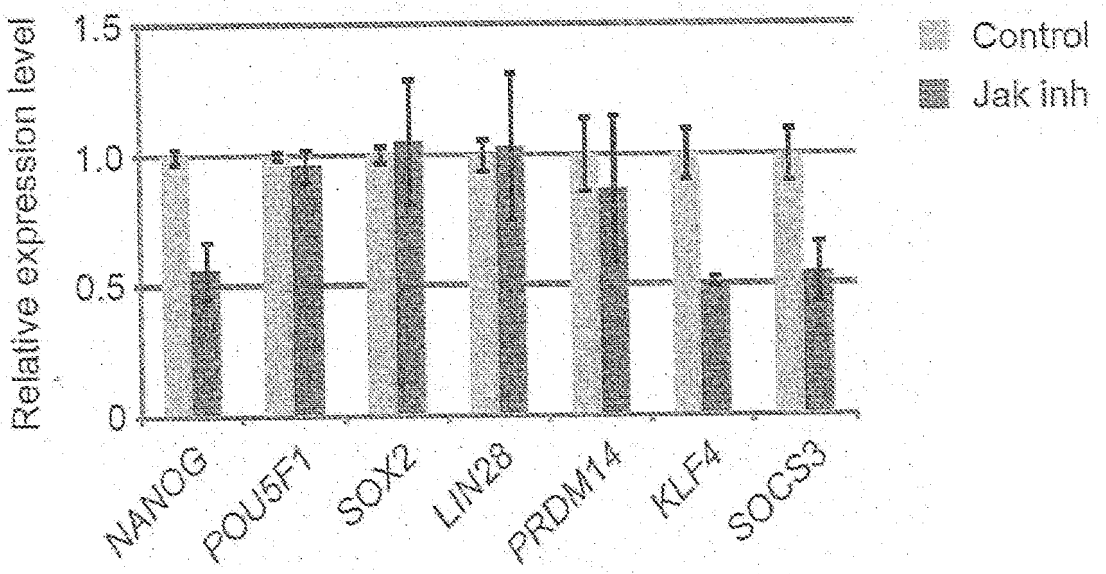

TRA-1-60 +ve colonies (10e3)
12
10
8
6
4
2
0
Control
Jak inh
Control
Jak inh

Fig. 3

C


Relative expression level
1.5
1.0
0.5
0
Control
Jak inh
NANOG
POU5F1
SOX2
LIN28
PRDM14
KLF4
SOCS3

D

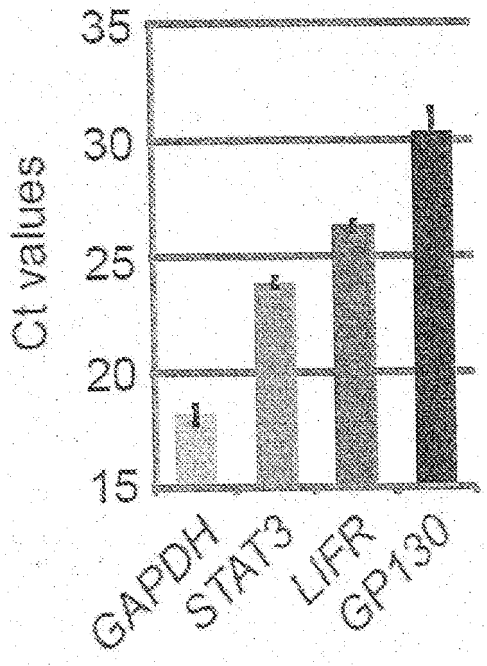
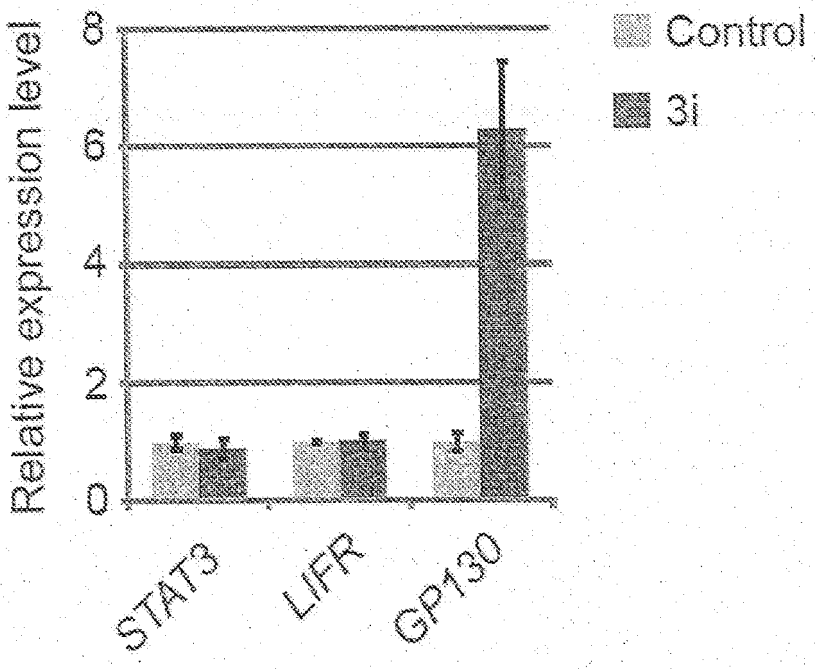

Ct values
35
30
25
20
15
GAPDH
STAT3
LIFR
GP130

Fig. 3

E


Relative expression level
0
2
4
6
8
STAT3
LIFR
GP130
Control
3i

F

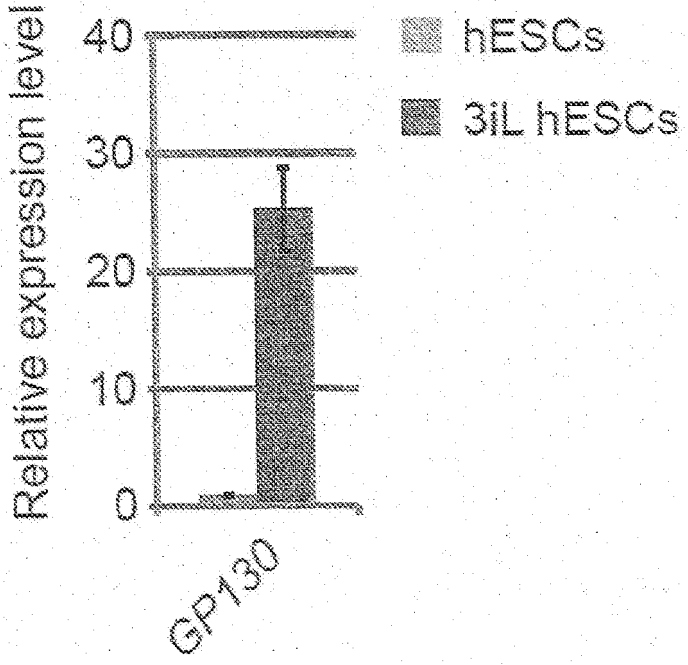
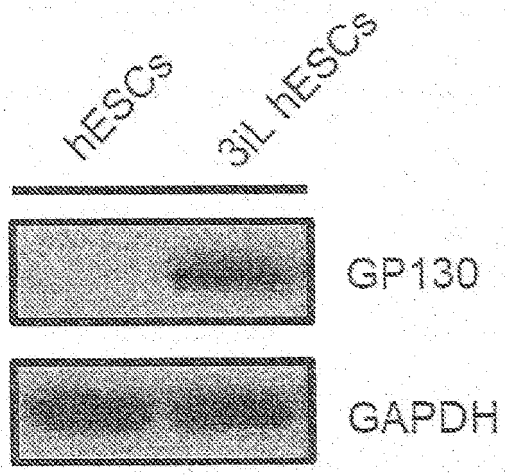

Relative expression level
0
10
20
30
40
GP130
hESCs
3iL hESCs

Fig. 3

G


hESCs
3iL hESCs
GP130
GAPDH

H

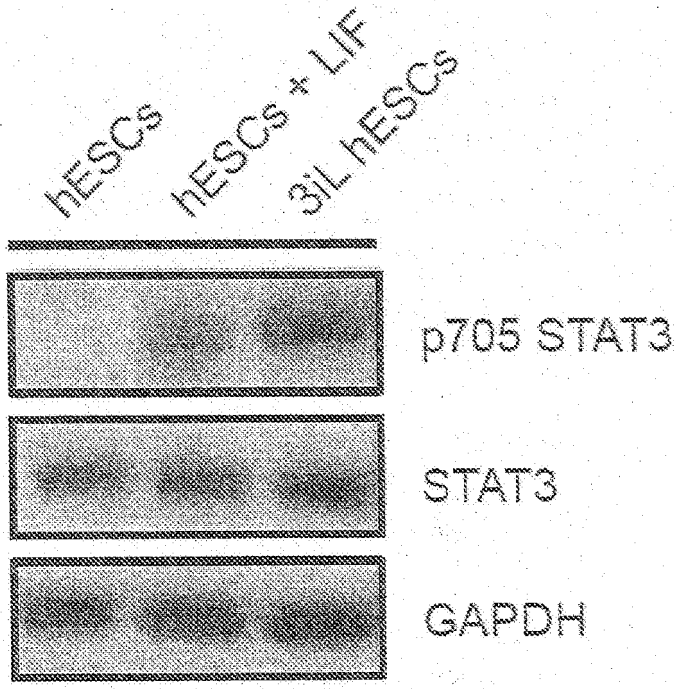

hESCs
hESCs + LIF
3iL hESCs
p705 STAT3
STAT3
GAPDH

Fig. 3

I

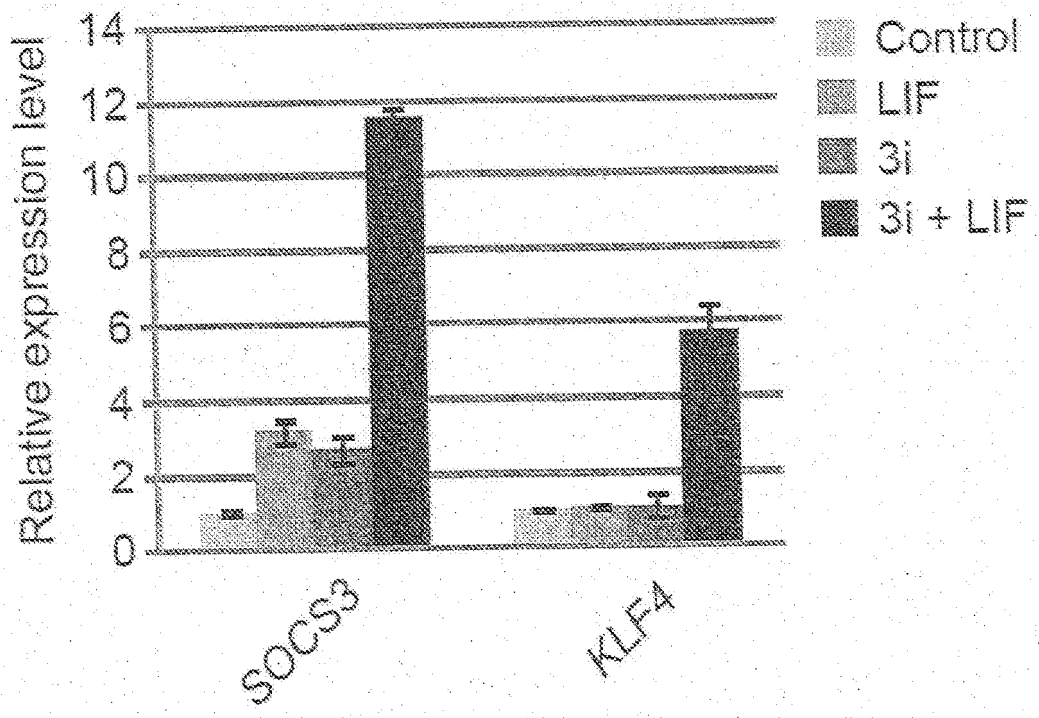

Relative expression level
14
12
10
8
6
4
2
0
SOCS3
KLF4
Control
LIF
3i
3i + LIF

Fig. 4

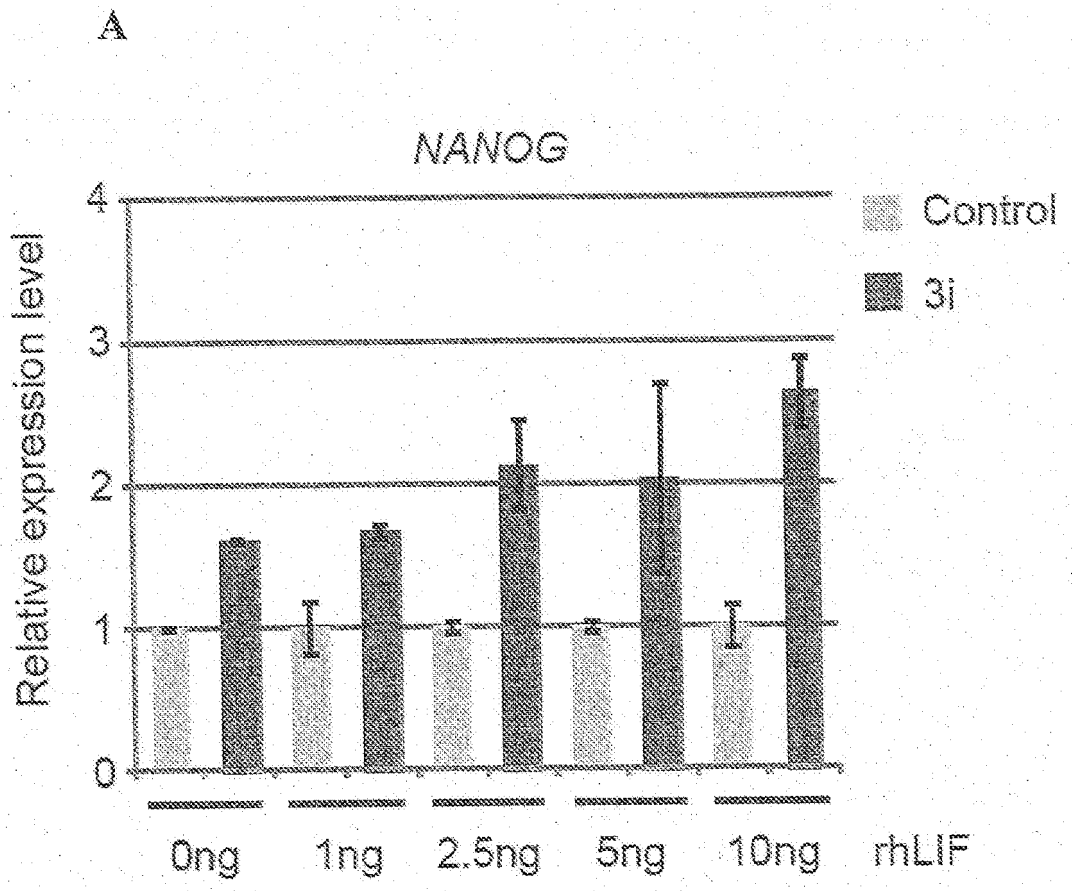

A
NANOG
Relative expression level
0
1
2
3
4
Control
3i
0ng
1ng
2.5ng
5ng
10ng
rhLIF

Fig. 4

B

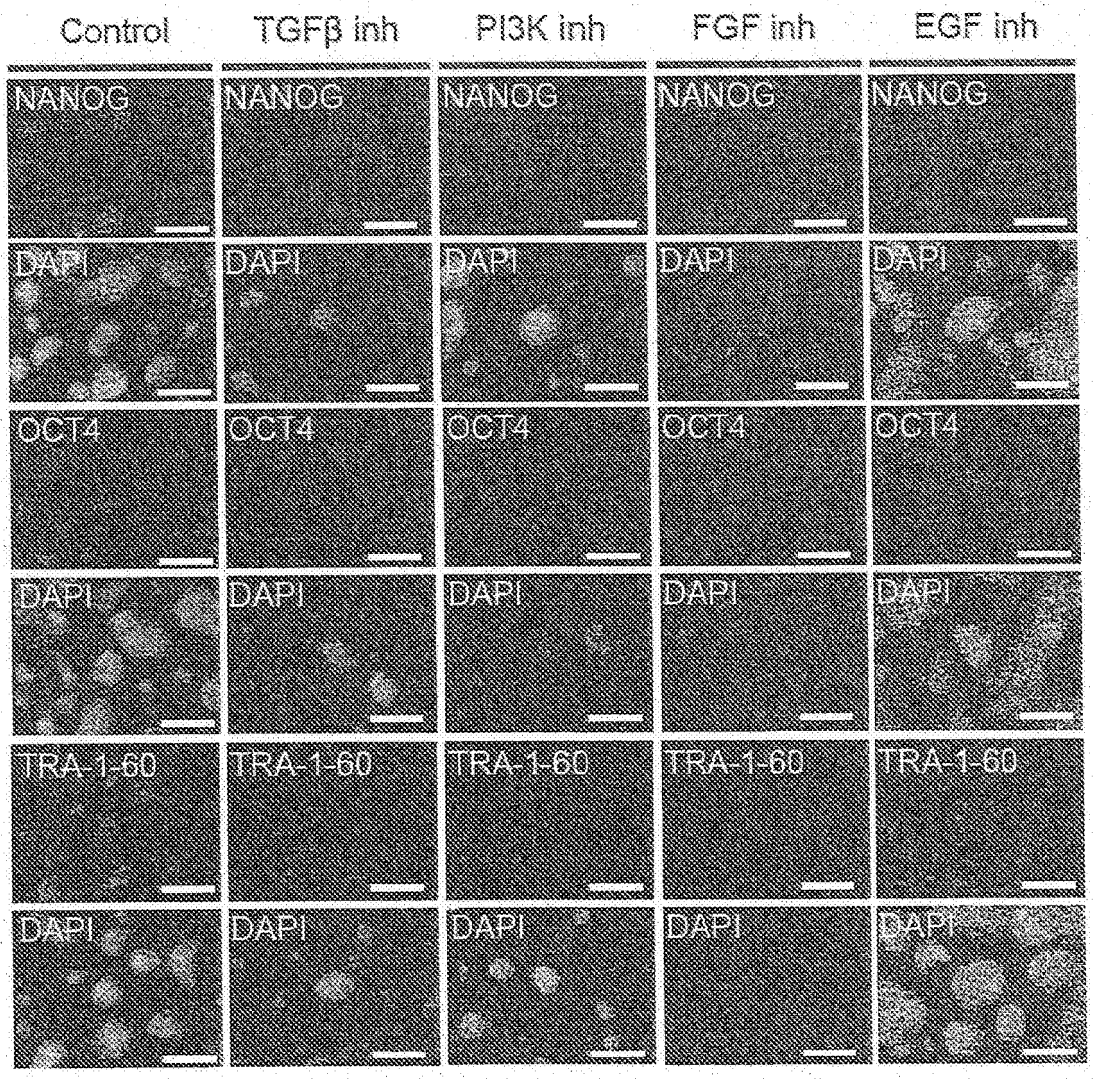

Control
TGFβ inh
PI3K inh
FGF inh
EGF inh
NANOG
DAPI
OCT4
TRA-1-60

Fig. 4

C

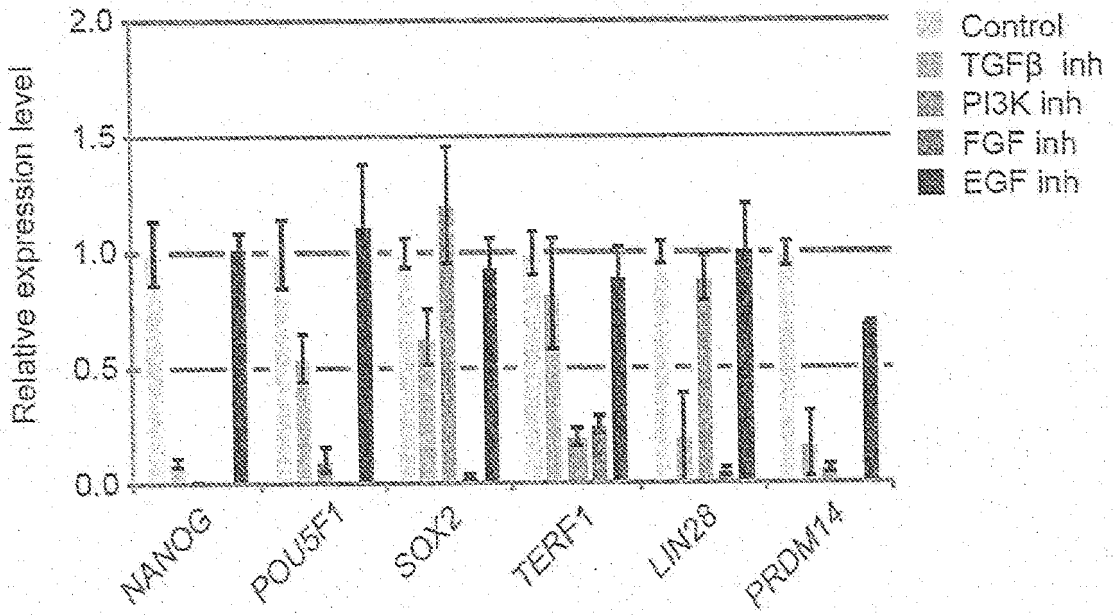

Relative expression level
2.0
1.5
1.0
0.5
0.0
NANOG
POU5F1
SOX2
TERF1
LIN28
PRDM14
Control
TGFβ inh
PI3K inh
FGF inh
EGF inh

Fig. 5

A

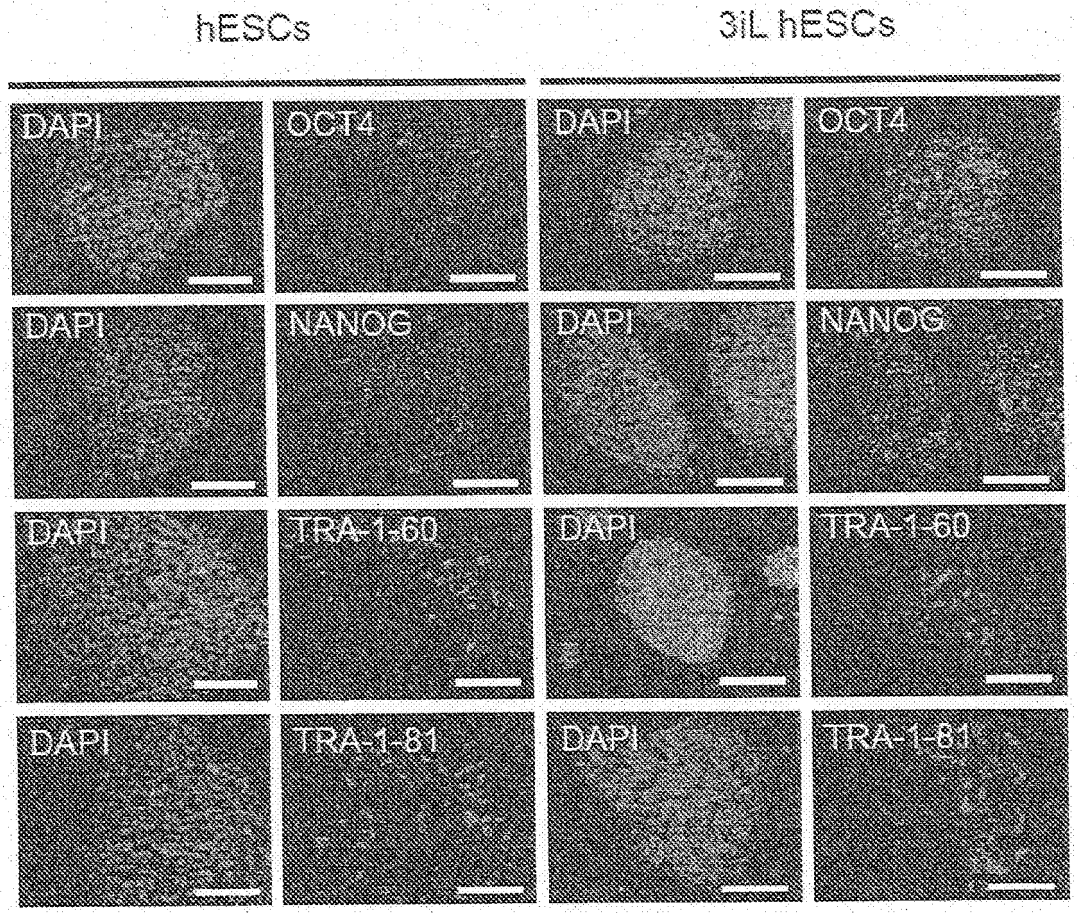
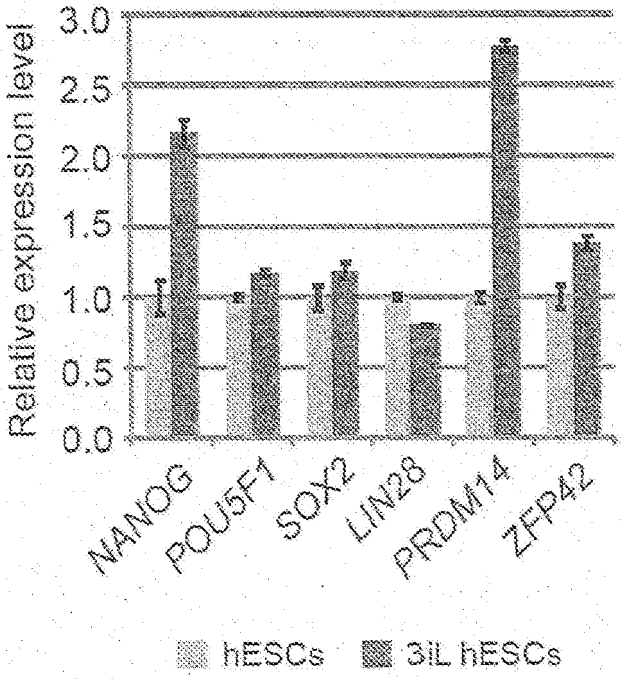

hESCs
3iL hESCs
DAPI
OCT4
DAPI
OCT4
DAPI
NANOG
DAPI
NANOG
DAPI
TRA-1-60
DAPI
TRA-1-60
DAPI
TRA-1-81
DAPI
TRA-1-81

Fig. 5

B


Relative expression level
3.0
2.5
2.0
1.5
1.0
0.5
0.0
NANOG
POU5F1
SOX2
LIN28
PRDM14
ZFP42
hESCs
3iL hESCs

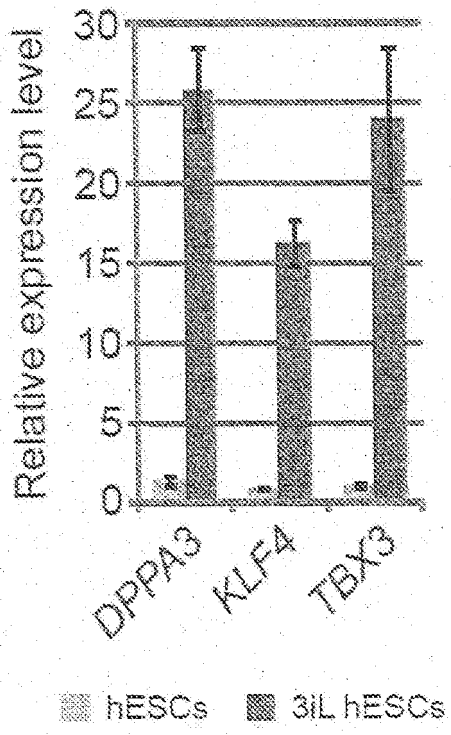

Relative expression level
30
25
20
15
10
5
0
DPPA3
KLF4
TBX3
hESCs
3iL hESCs

Fig. 5

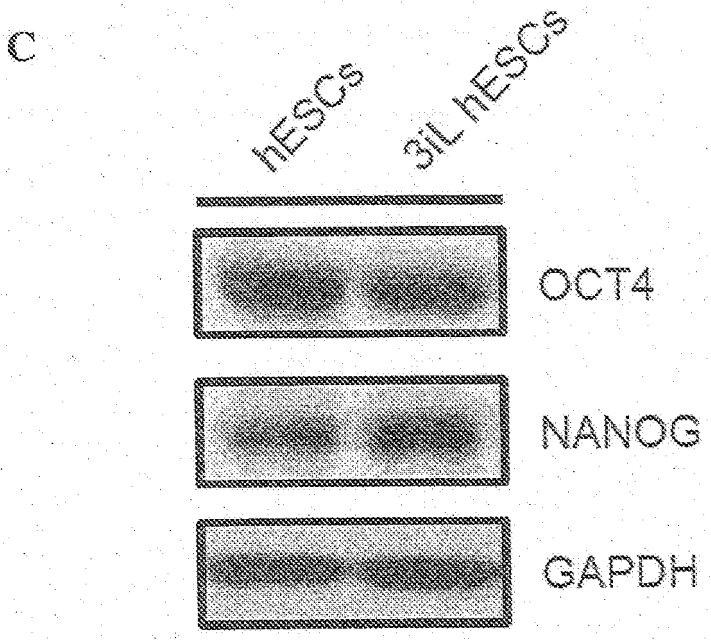


Fig. 5

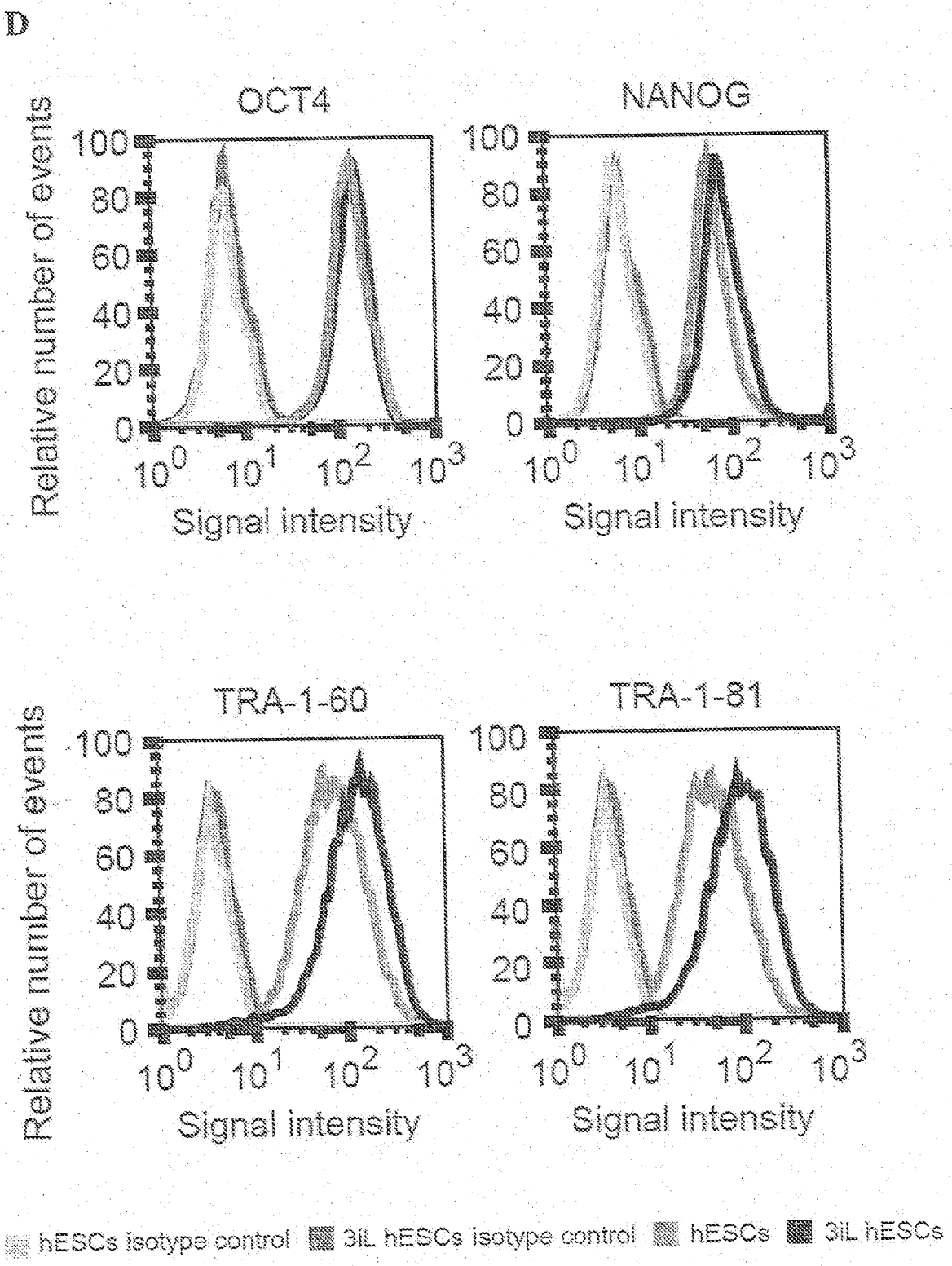

D
OCT4
NANOG
TRA-1-60
TRA-1-81
Relative number of events
Signal intensity
100
80
60
40
20
0
10^0
10^1
10^2
10^3
hESCs isotype control
3iL hESCs isotype control
hESCs
3iL hESCs

Fig. 5

E

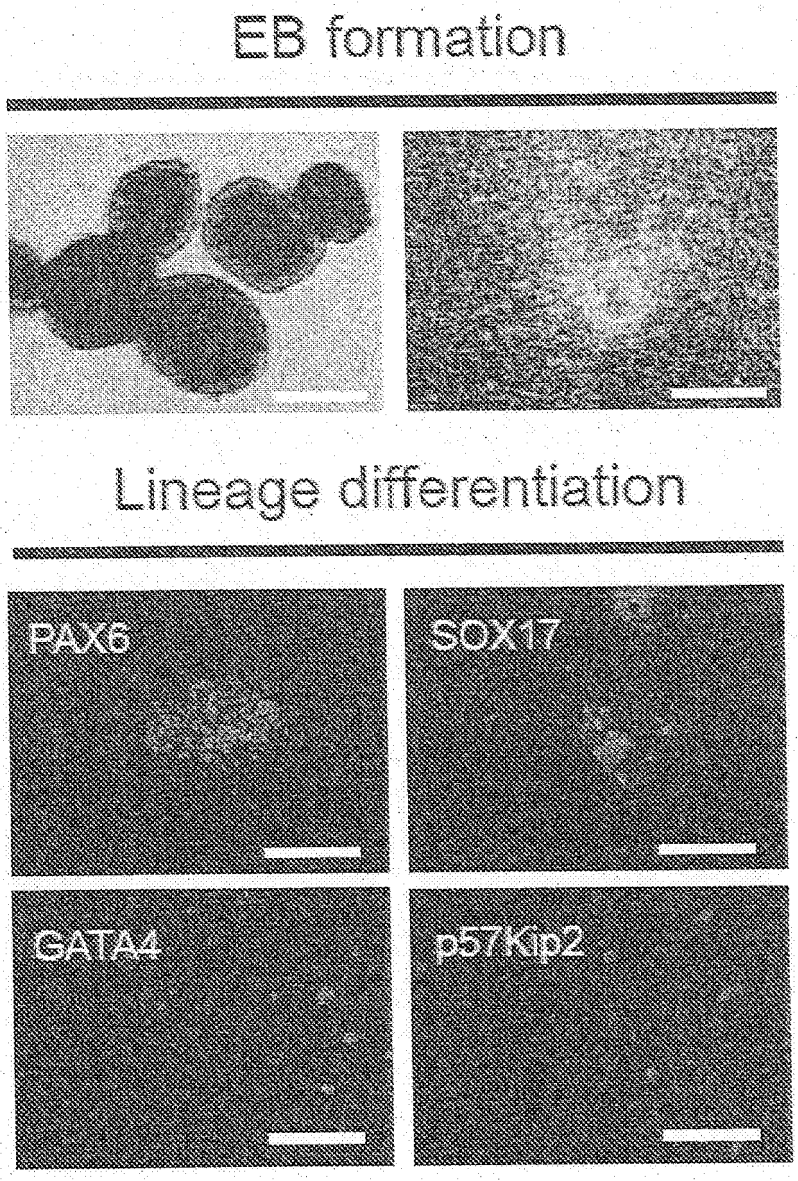

EB formation
Lineage differentiation
PAX6
SOX17
GATA4
p57Kip2

Fig. 5

F

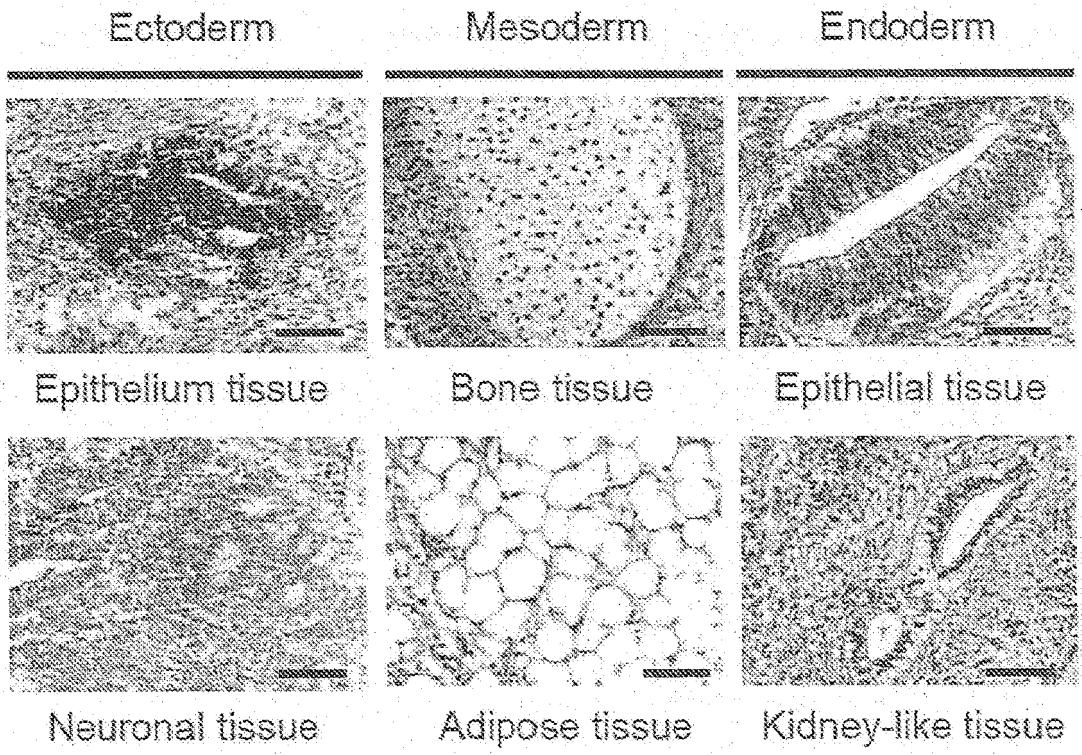
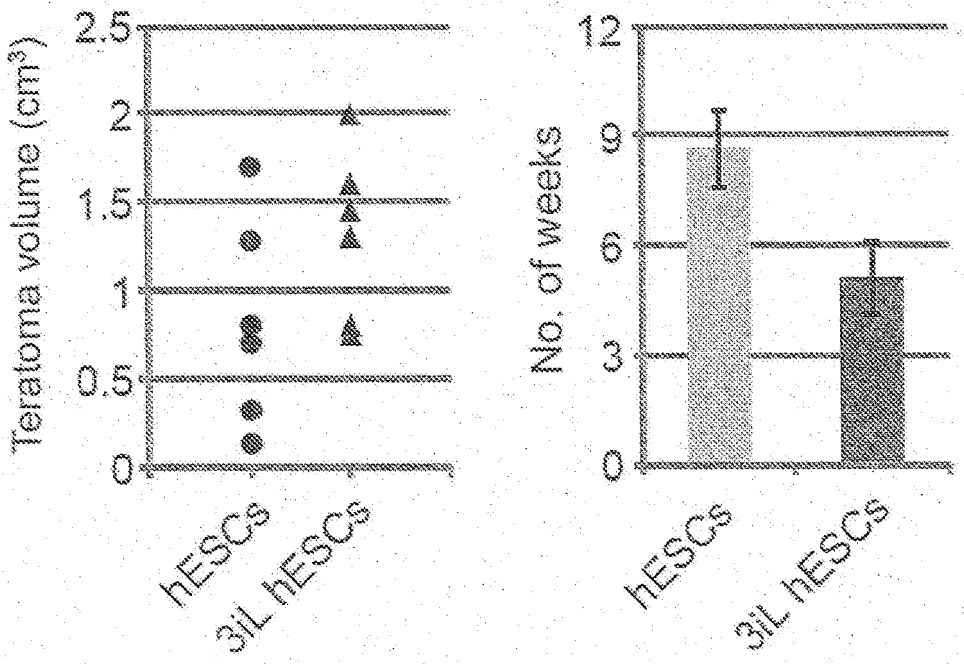

Ectoderm
Mesoderm
Endoderm
Epithelium tissue
Bone tissue
Epithelial tissue
Neuronal tissue
Adipose tissue
Kidney-like tissue

Fig. 5

G


Teratoma volume (cm3)
2.5
2
1.5
1
0.5
0
hESCs
3iL hESCs
No. of weeks
12
9
6
3
0
hESCs
3iL hESCs

H

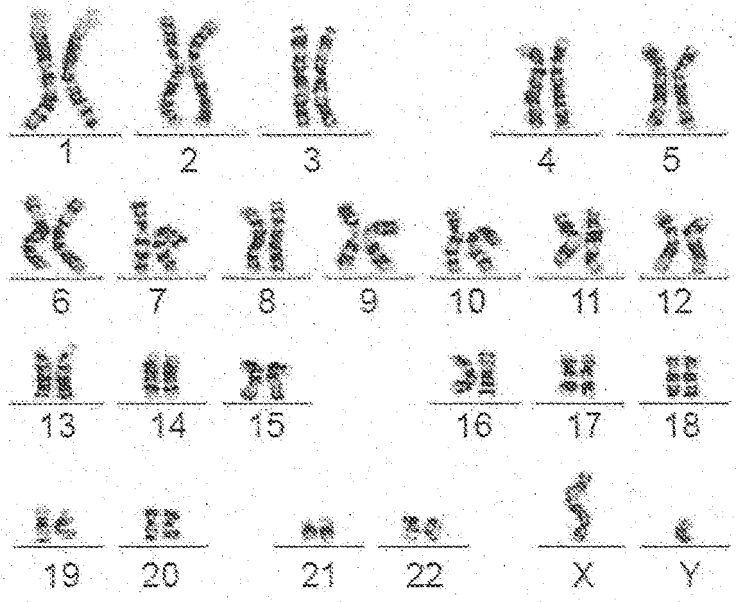
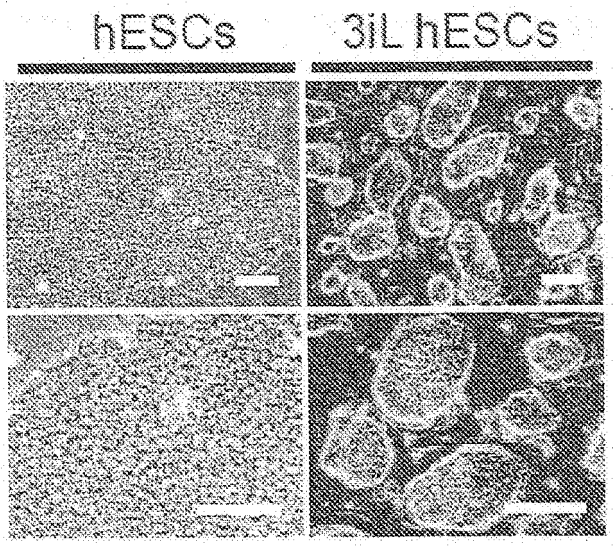

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X
Y

Fig. 6

A hES3:


hESCs
3iL hESCs

B

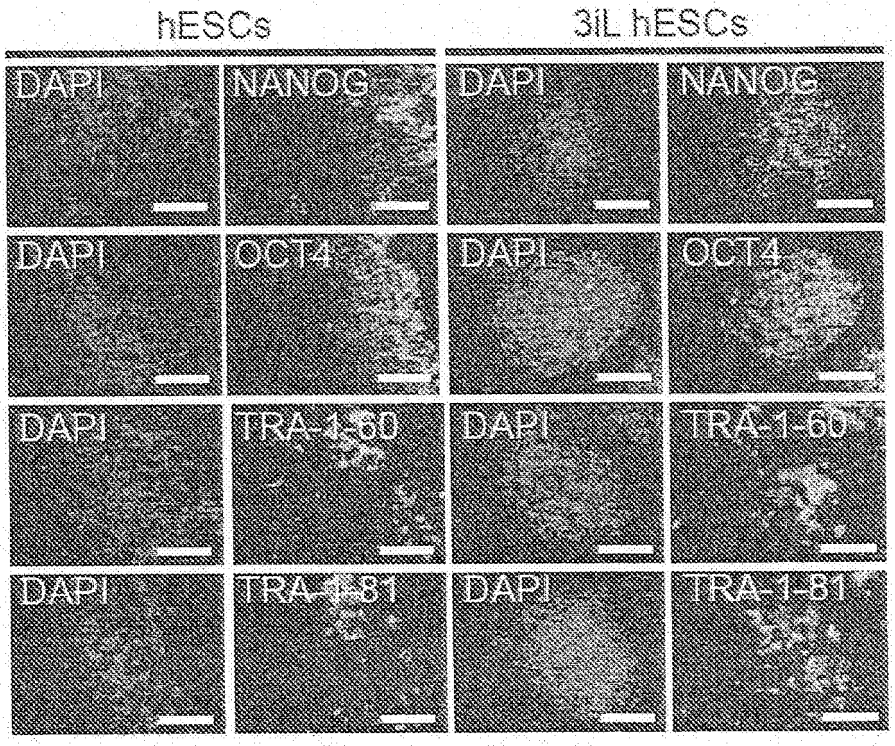

hESCs
3iL hESCs
DAPI
NANOG
DAPI
NANOG
DAPI
OCT4
DAPI
OCT4
DAPI
TRA-1-60
DAPI
TRA-1-60
DAPI
TRA-1-81
DAPI
TRA-1-81

Fig. 6

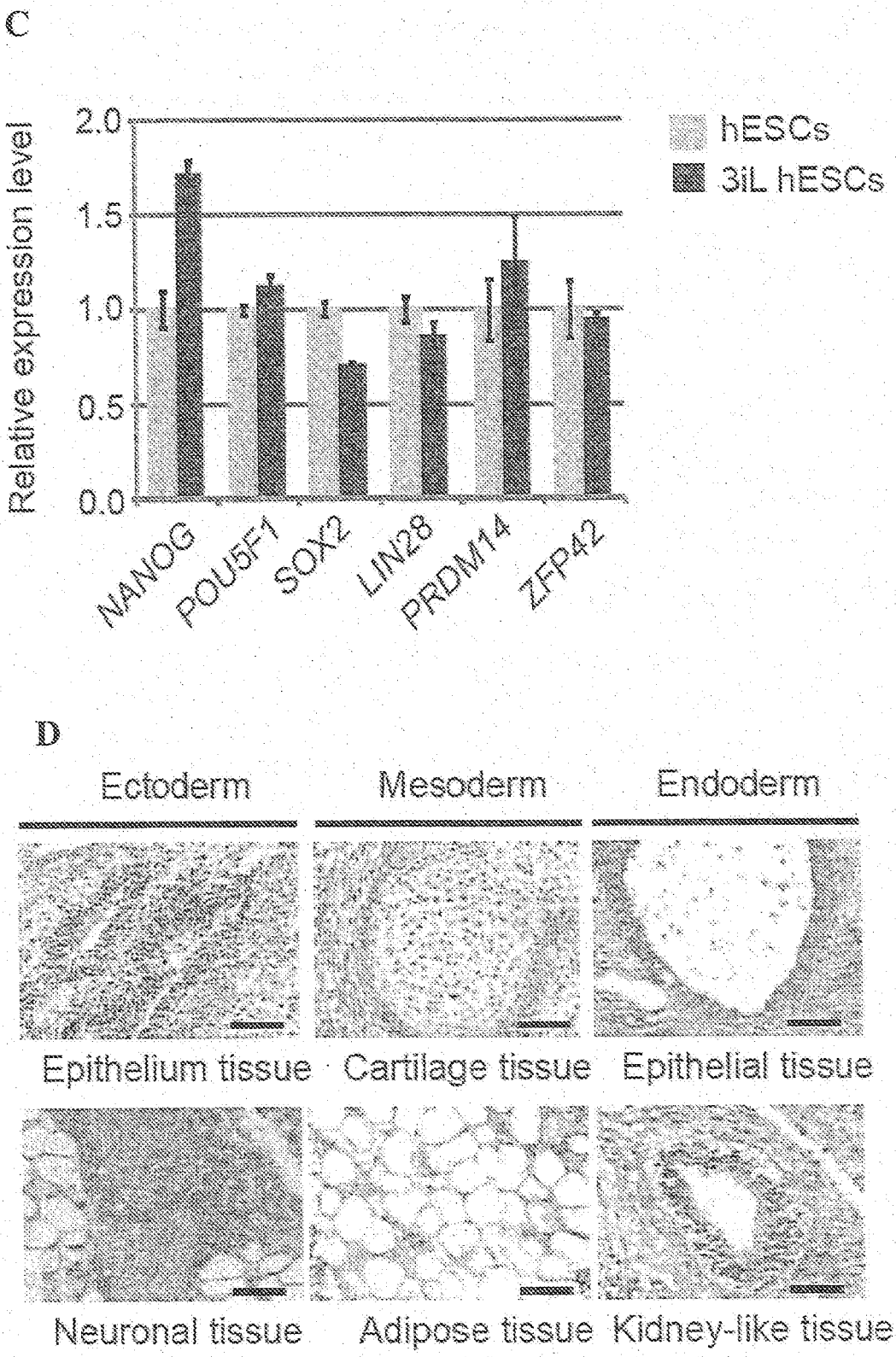

C
Relative expression level
2.0
1.5
1.0
0.5
0.0
hESCs
3iL hESCs
NANOG
POU5F1
SOX2
LIN28
PRDM14
ZFP42
D
Ectoderm
Mesoderm
Endoderm
Epithelium tissue
Cartilage tissue
Epithelial tissue
Neuronal tissue
Adipose tissue
Kidney-like tissue

Fig. 6

E

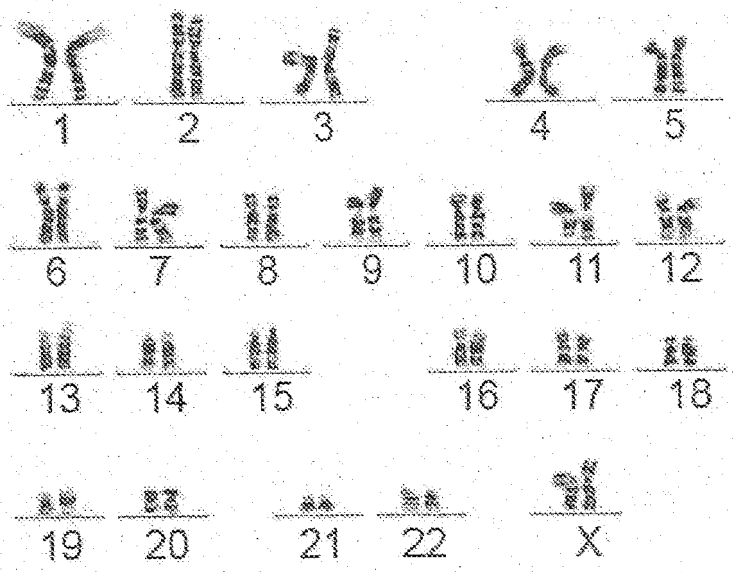


Fig. 6

F

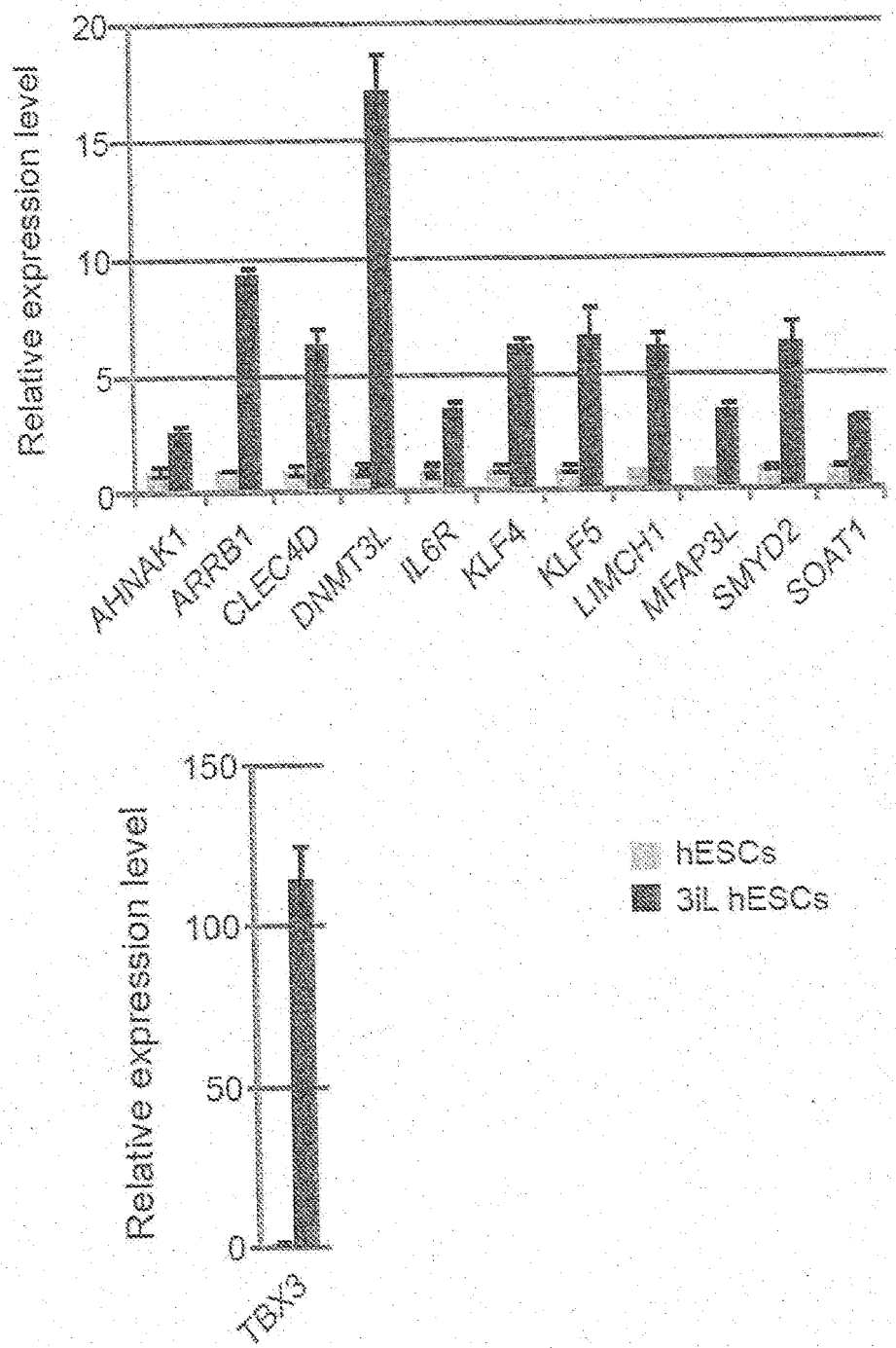
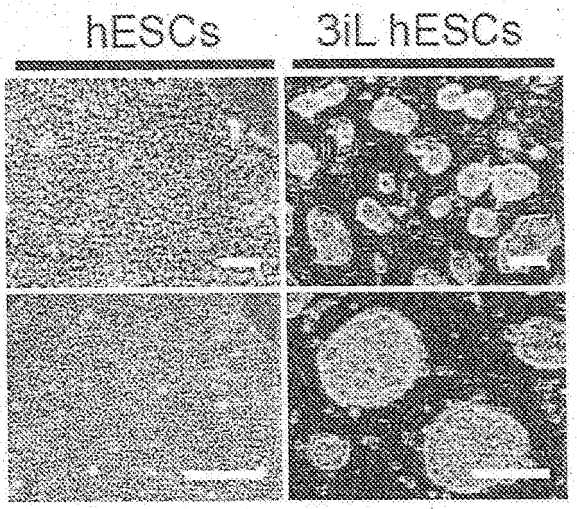

Relative expression level
20
15
10
5
0
AHNAK1
ARRB1
CLEC4D
DNMT3L
IL6R
KLF4
KLF5
LIMCH1
MFAP3L
SMYD2
SOAT1
Relative expression level
150
100
50
0
TBX3
hESCs
3iL hESCs

Fig. 6

G


hES2:
hESCs
3iL hESCs

H

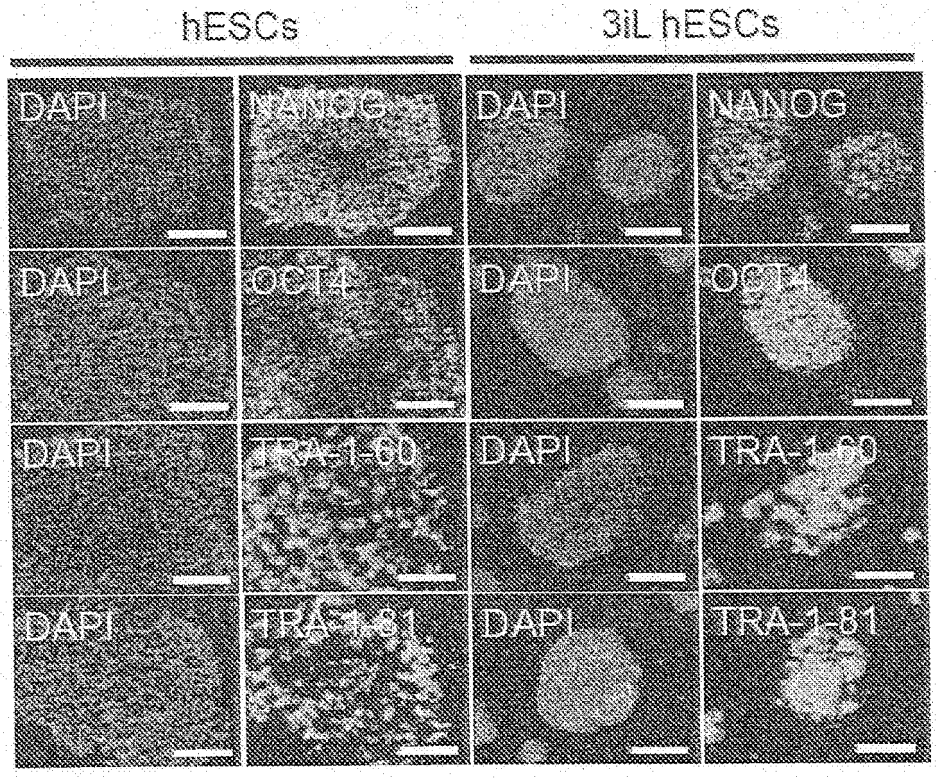
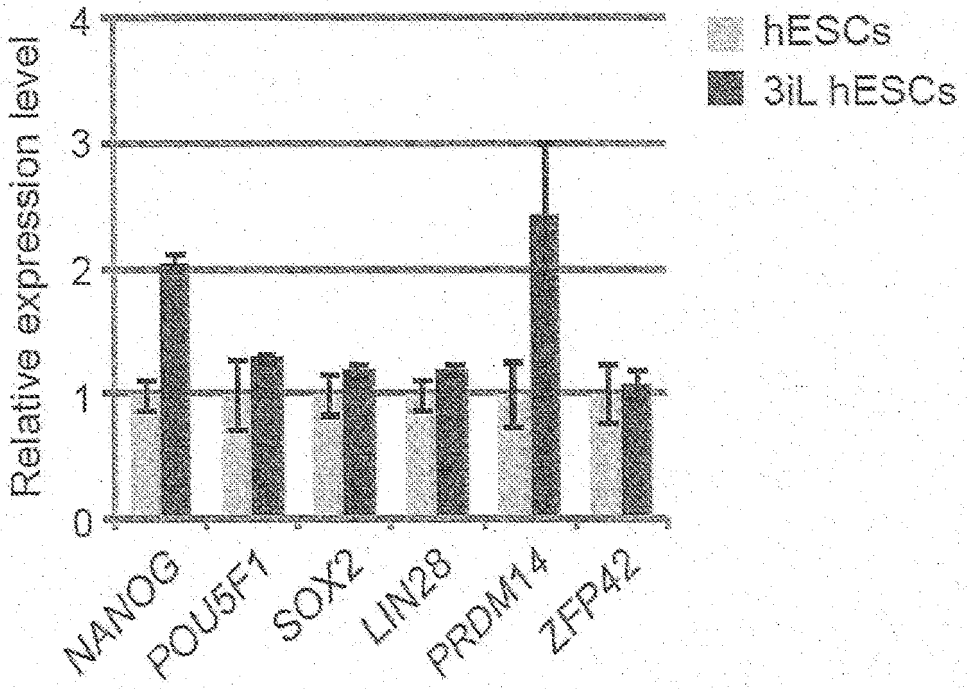

hESCs
3iL hESCs
DAPI
NANOG
DAPI
NANOG
DAPI
OCT4
DAPI
OCT4
DAPI
TRA-1-60
DAPI
TRA-1-60
DAPI
TRA-1-81
DAPI
TRA-1-81

Fig. 6

I


Relative expression level
0
1
2
3
4
hESCs
3iL hESCs
NANOG
POU5F1
SOX2
LIN28
PRDM14
ZFP42

J

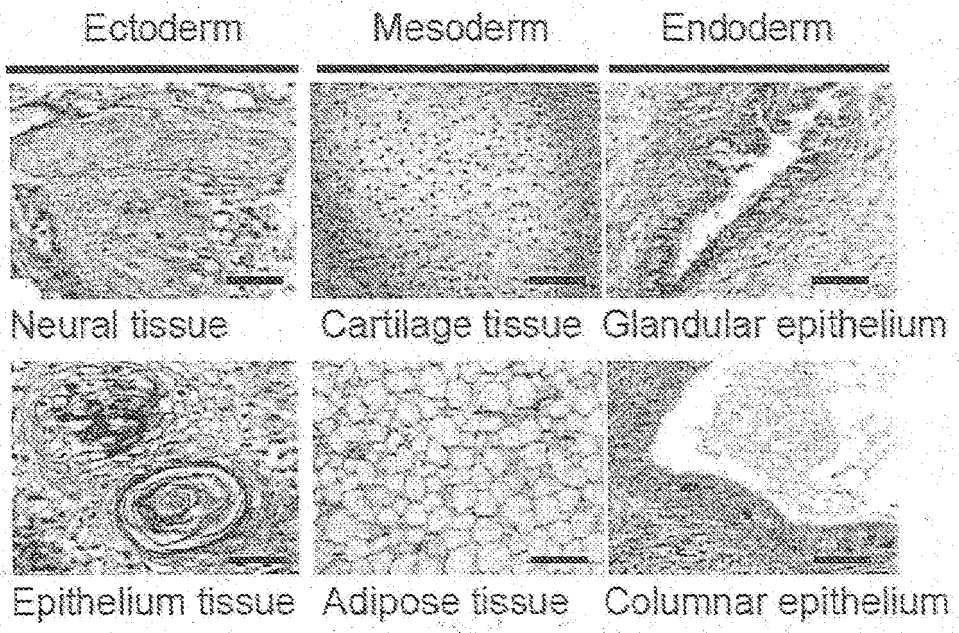

Ectoderm
Mesoderm
Endoderm
Neural tissue
Cartilage tissue
Glandular epithelium
Epithelium tissue
Adipose tissue
Columnar epithelium

Fig. 6

K

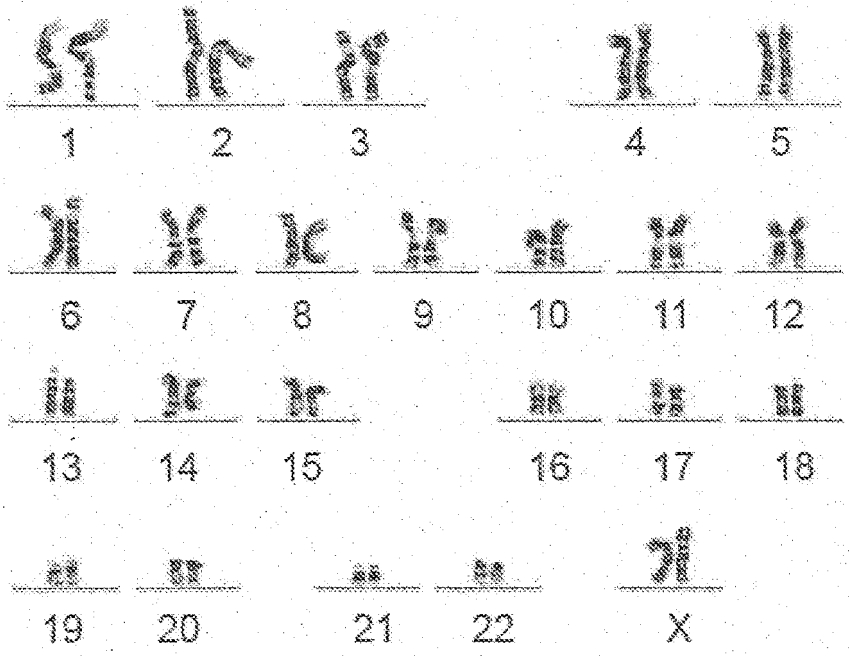
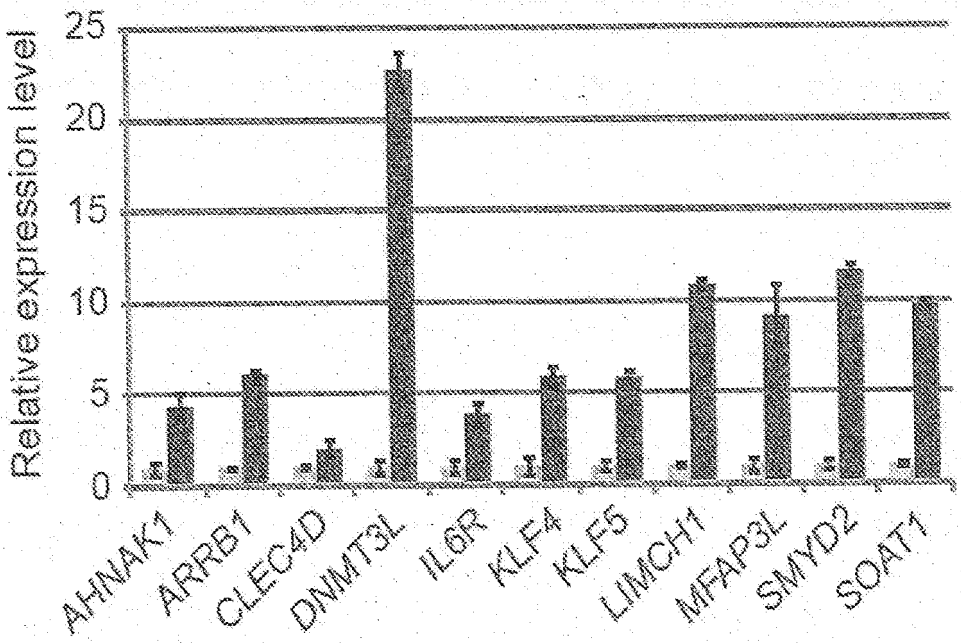


Fig. 6

L


Relative expression level
25
20
15
10
5
0
AHNAK1
ARRB1
CLEC4D
DNMT3L
IL6R
KLF4
KLF5
LIMCH1
MFAP3L
SMYD2
SOAT1

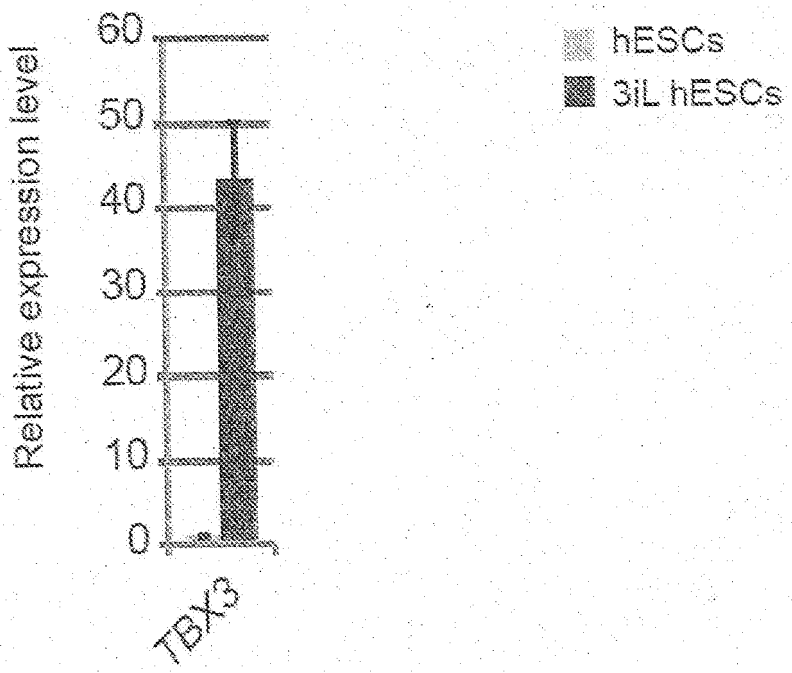
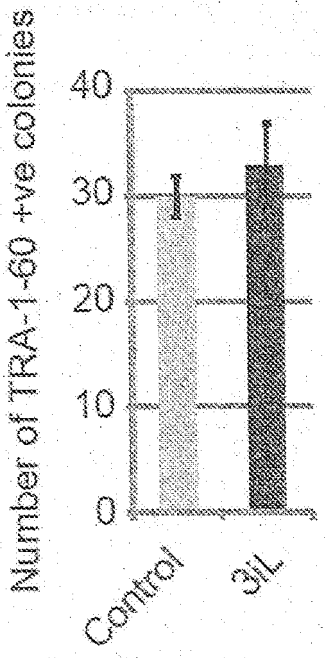

Relative expression level
60
50
40
30
20
10
0
TBX3
hESCs
3iL hESCs

Fig. 6

M


Number of TRA-1-60 +ve colonies
40
30
20
10
0
Control
3iL

N

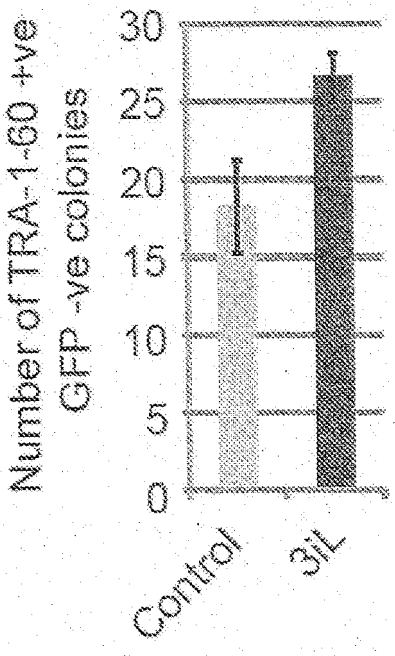
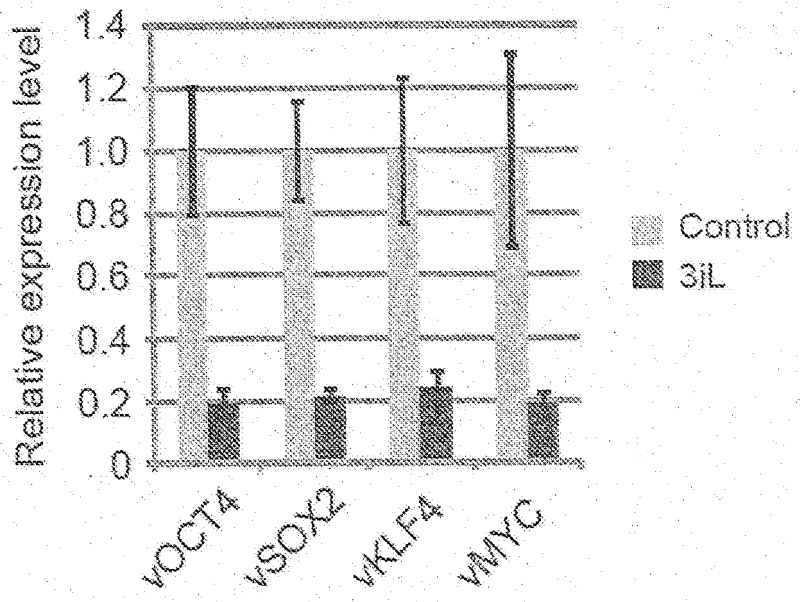

Number of TRA-1-60 +ve GFP -ve colonies
30
25
20
15
10
5
0
Control
3iL

Fig. 6

O


Relative expression level
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
vOCT4
vSOX2
vKLF4
vMYC
Control
3iL

P

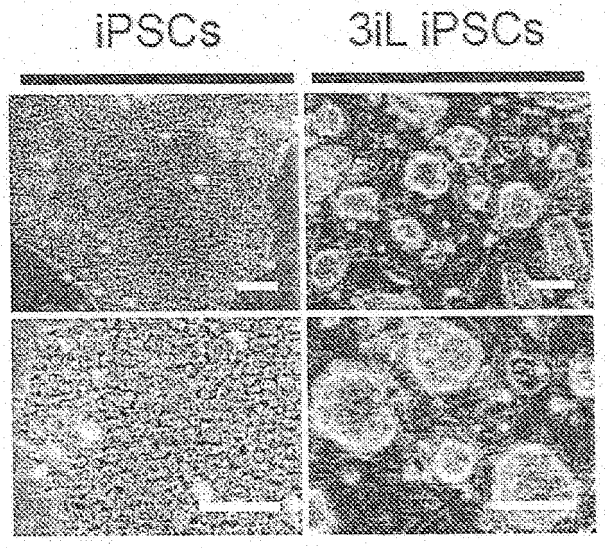

iPSCs
3iL iPSCs

Fig. 6

Q

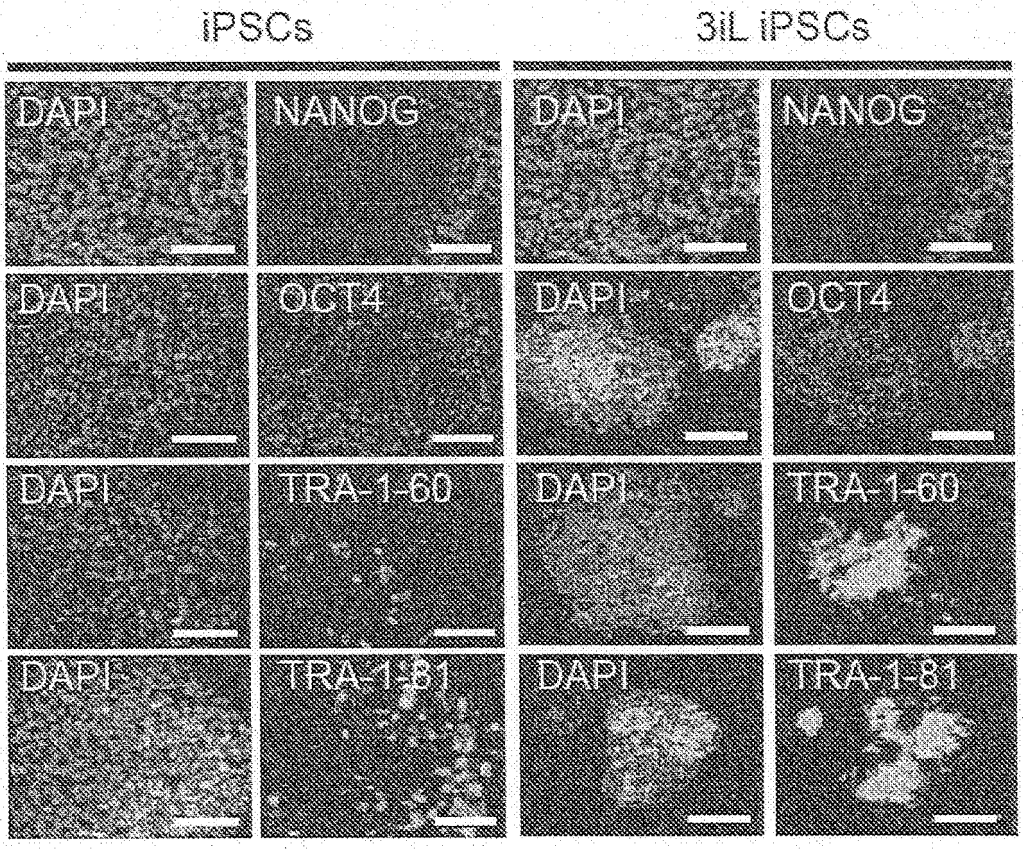
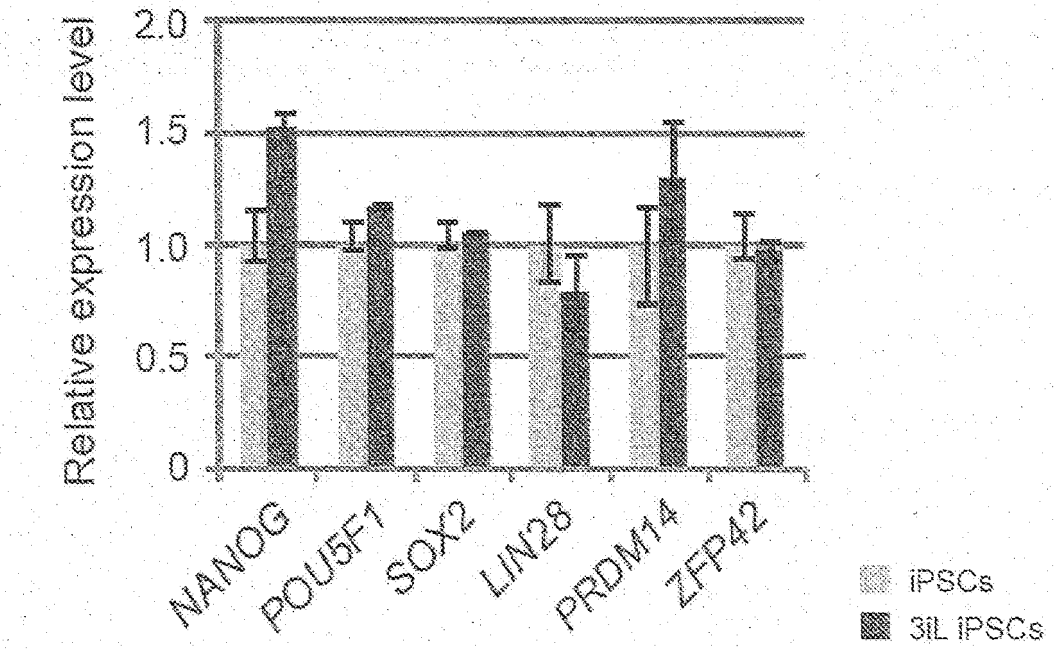

iPSCs
3iL iPSCs
DAPI
NANOG
DAPI
NANOG
DAPI
OCT4
DAPI
OCT4
DAPI
TRA-1-60
DAPI
TRA-1-60
DAPI
TRA-1-81
DAPI
TRA-1-81

Fig. 6

R


Relative expression level
2.0
1.5
1.0
0.5
0
NANOG
POU5F1
SOX2
LIN28
PRDM14
ZFP42
iPSCs
3iL iPSCs

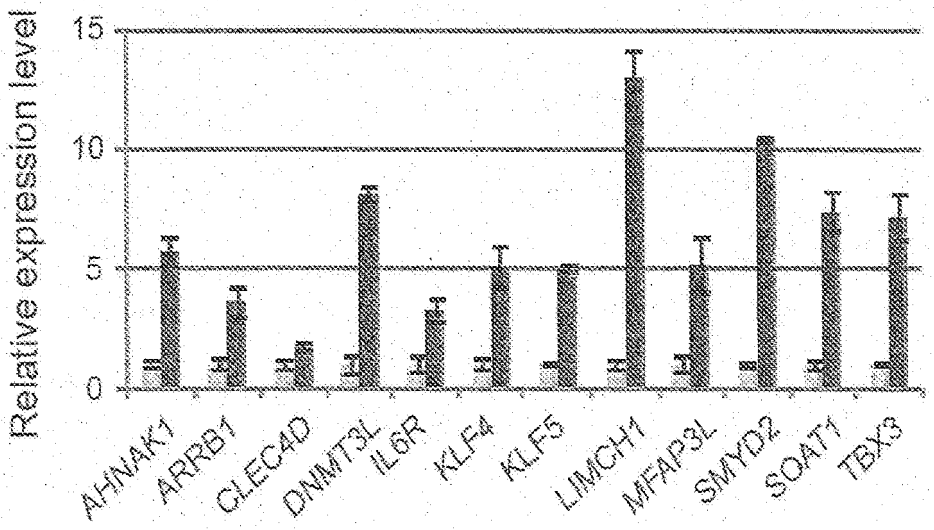

Relative expression level
15
10
5
0
AHNAK1
ARRB1
CLEC4D
DNMT3L
IL6R
KLF4
KLF5
LIMCH1
MFAP3L
SMYD2
SOAT1
TBX3

Fig. 7

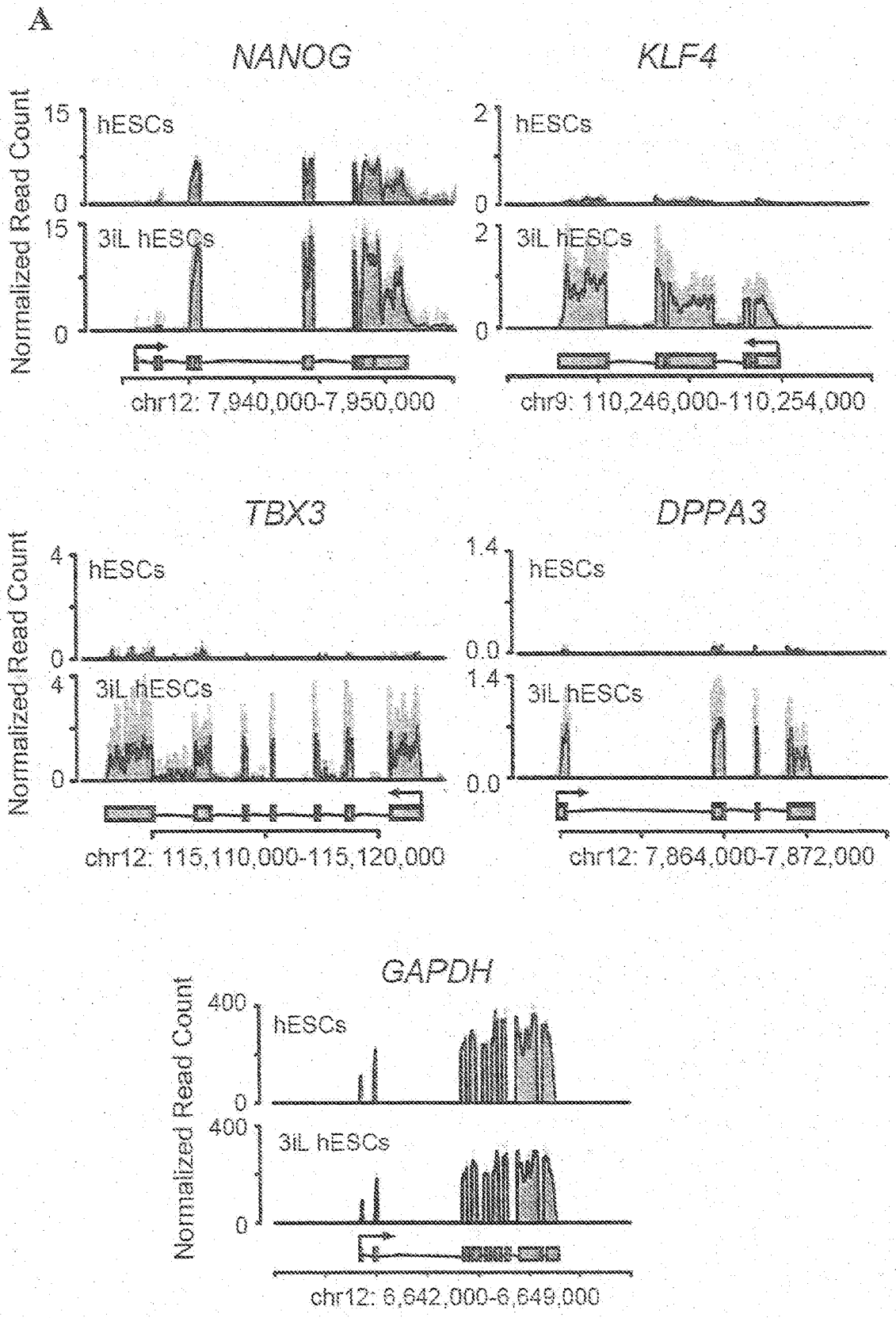

A
NANOG
KLF4
Normalized Read Count
15
hESCs
0
15
3iL hESCs
0
chr12: 7,940,000-7,950,000
2
hESCs
0
2
3iL hESCs
0
chr9: 110,246,000-110,254,000
TBX3
DPPA3
4
hESCs
0
4
3iL hESCs
0
chr12: 115,110,000-115,120,000
1.4
hESCs
0.0
1.4
3iL hESCs
0.0
chr12: 7,864,000-7,872,000
GAPDH
400
hESCs
0
400
3iL hESCs
0
chr12: 6,642,000-6,649,000

Fig. 7

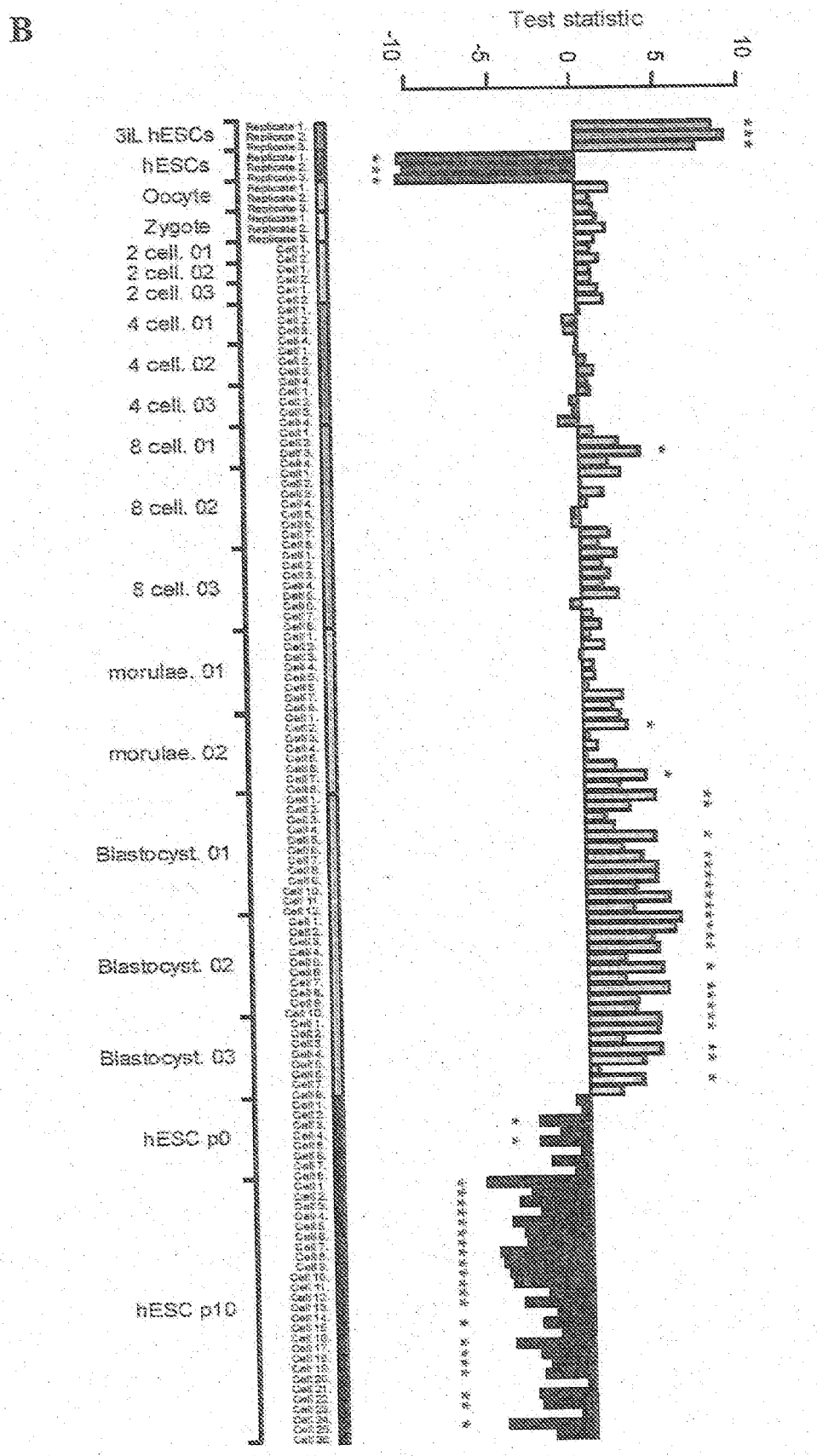

B
Test statistic
-10
-5
0
5
10
3iL hESCs
hESCs
Oocyte
Zygote
2 cell. 01
2 cell. 02
2 cell. 03
4 cell. 01
4 cell. 02
4 cell. 03
8 cell. 01
8 cell. 02
8 cell. 03
morulae. 01
morulae. 02
Blastocyst. 01
Blastocyst. 02
Blastocyst. 03
hESC p0
hESC p10

Fig. 7

C

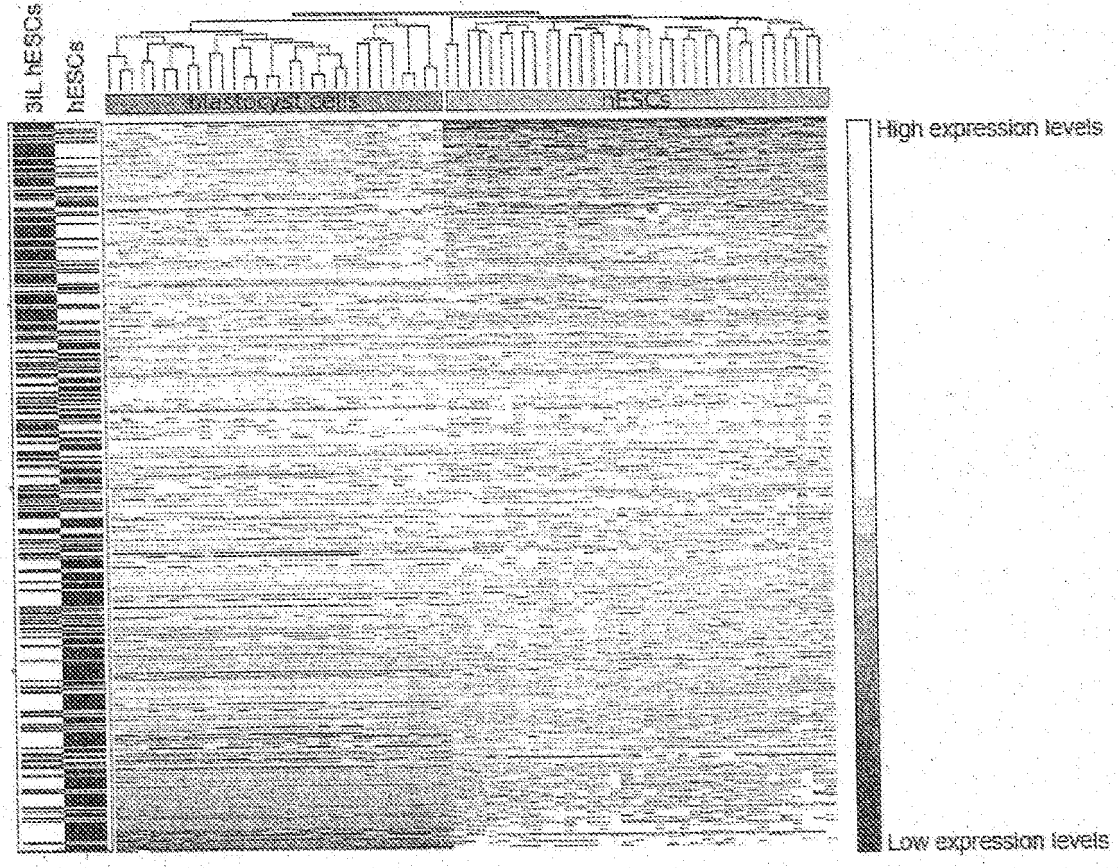

3iL hESCs
hESCs
blastocyst cells
hESCs
High expression levels
Low expression levels

Fig. 7

D

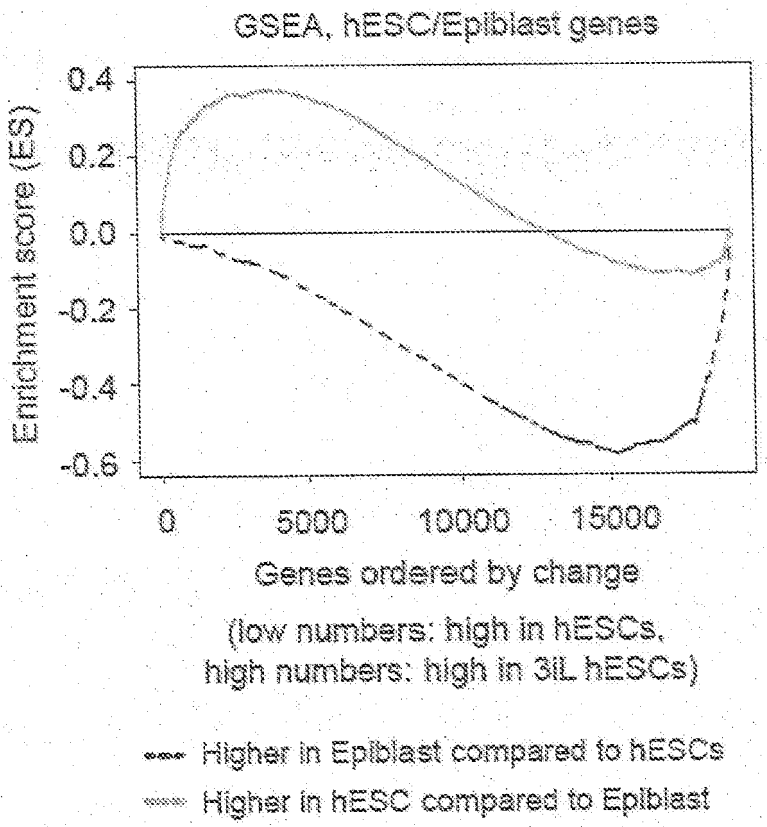

GSEA, hESC/Epiblast genes
Enrichment score (ES)
0.4
0.2
0.0
-0.2
-0.4
-0.6
0
5000
10000
15000
Genes ordered by change
(low numbers: high in hESCs,
high numbers: high in 3iL hESCs)
Higher in Epiblast compared to hESCs
Higher in hESC compared to Epiblast

Fig. 7

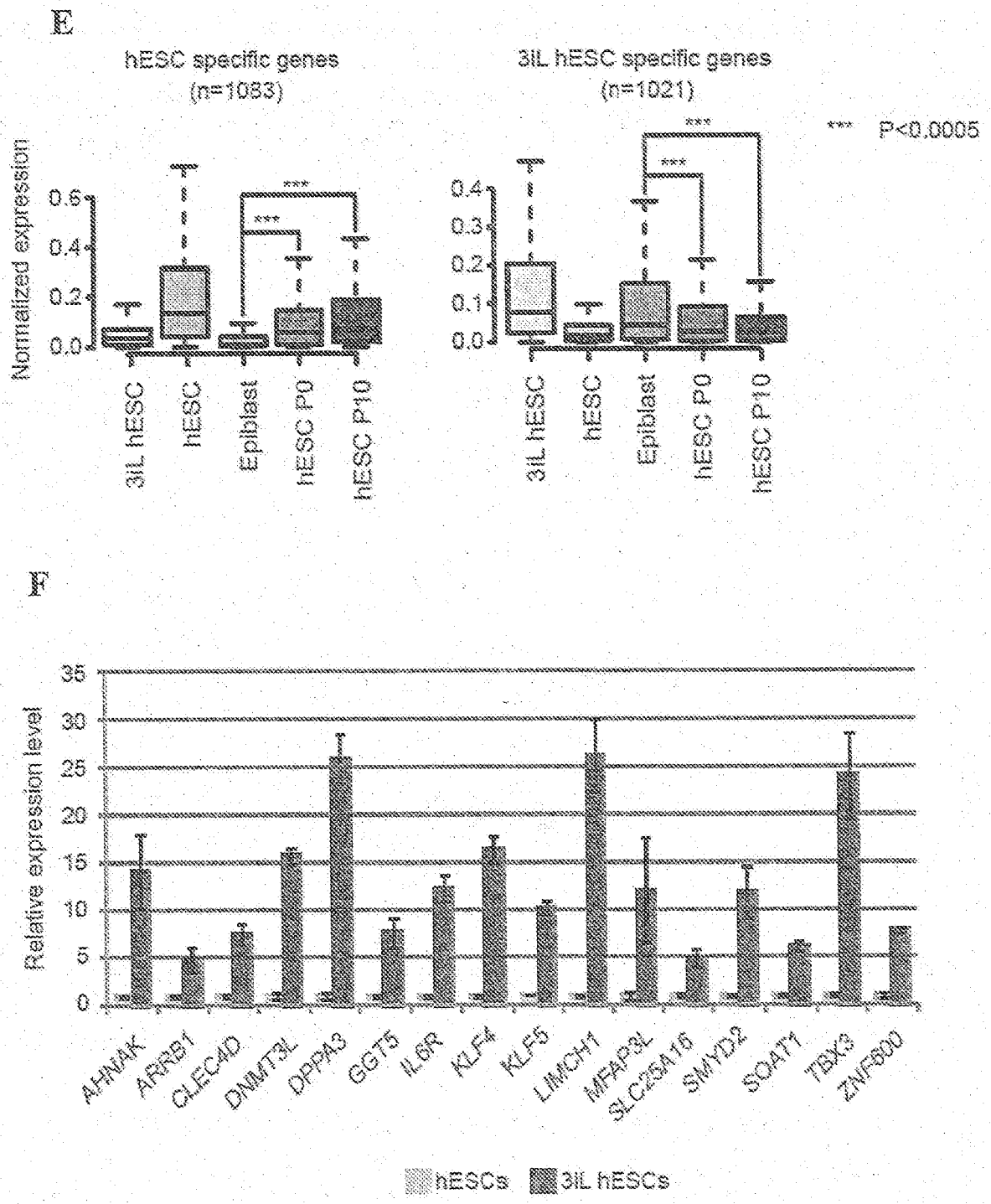
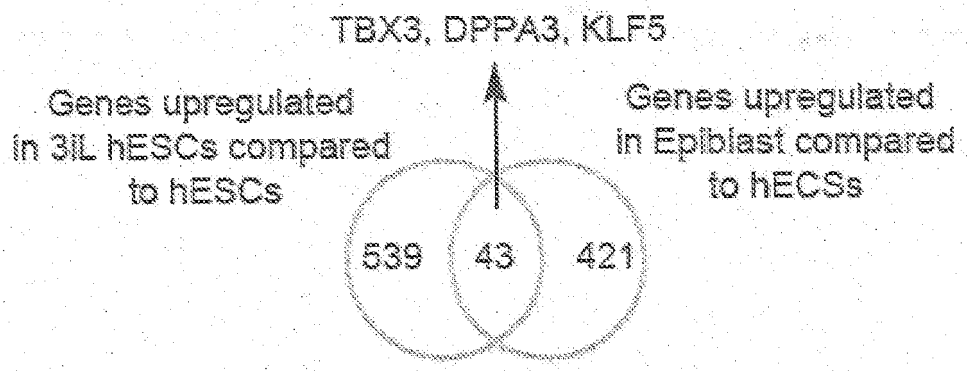

E
hESC specific genes
(n=1083)
3iL hESC specific genes
(n=1021)
*** P<0.0005
Normalized expression
0.6
0.4
0.2
0.0
0.4
0.3
0.2
0.1
0.0
***
***
***
3iL hESC
hESC
Epiblast
hESC P0
hESC P10
F
Relative expression level
35
30
25
20
15
10
5
0
AHNAK
ARRB1
CLEC4D
DNMT3L
DPPA3
GGT5
IL6R
KLF4
KLF5
LIMCH1
MFAP3L
SLC2SA16
SMYD2
SOAT1
TBX3
ZNF800
hESCs
3iL hESCs

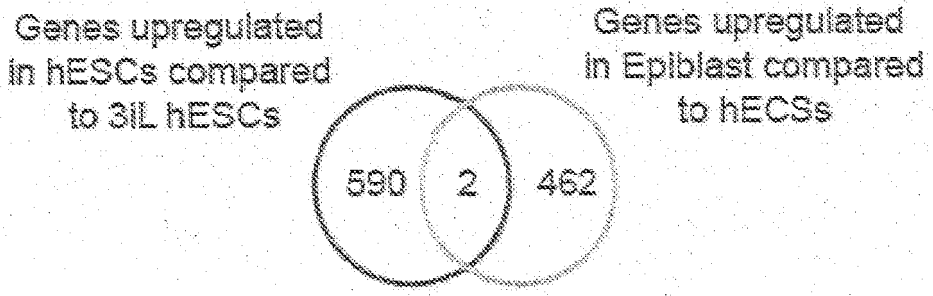
Fig. 8

A



p-value = 3.675e-12
(odds ratio = 3.824, higher than expected)

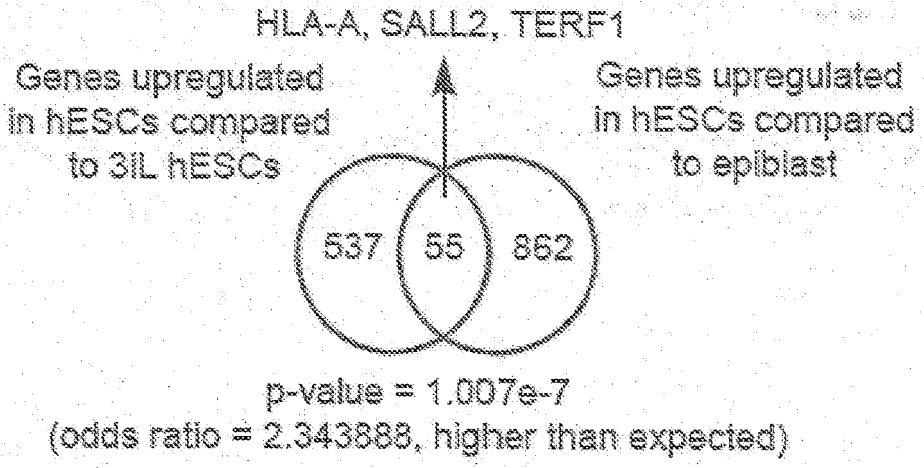
B



p-value = 0.0003056
(odds ratio = 0.148 lower than expected)

Fig. 8

C


HLA-A, SALL2, TERF1
Genes upregulated in hESCs compared to 3iL hESCs
Genes upregulated in hESCs compared to epiblast
537
55
862
p-value = 1.007e-7
(odds ratio = 2.343888, higher than expected)

D

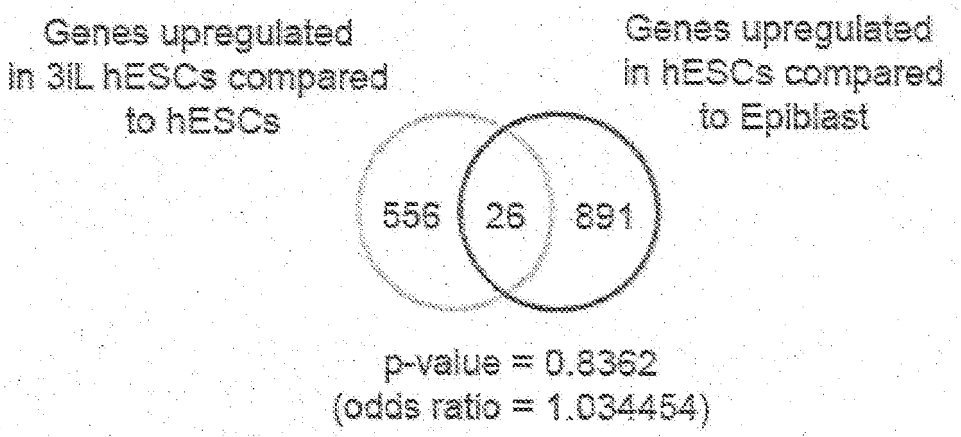
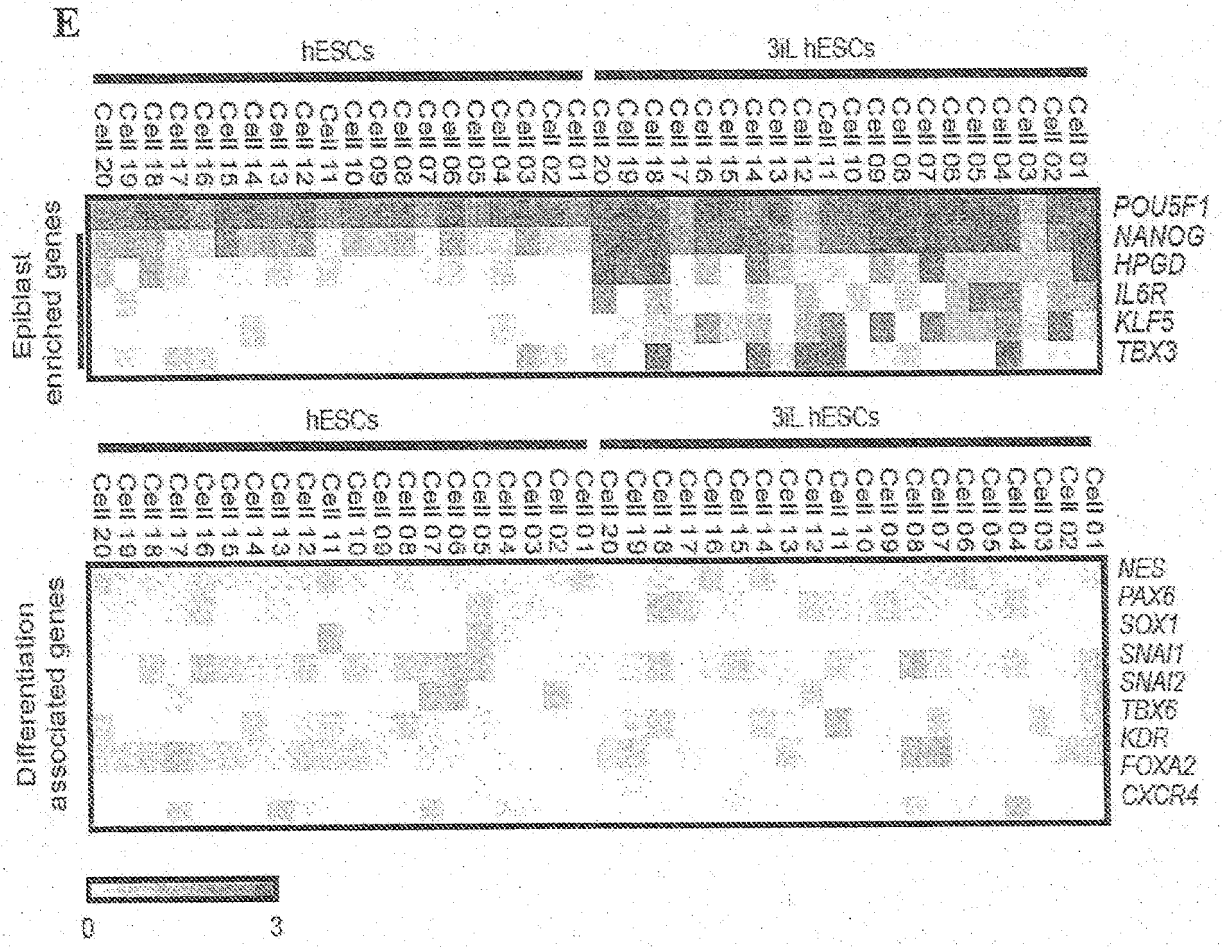

Genes upregulated in 3iL hESCs compared to hESCs
Genes upregulated in hESCs compared to Epiblast
556
26
891
p-value = 0.8362
(odds ratio = 1.034454)

Fig. 8


E
hESCs
3iL hESCs
Cell 20
Cell 19
Cell 18
Cell 17
Cell 16
Cell 15
Cell 14
Cell 13
Cell 12
Cell 11
Cell 10
Cell 09
Cell 08
Cell 07
Cell 06
Cell 05
Cell 04
Cell 03
Cell 02
Cell 01
Epiblast enriched genes
POU5F1
NANOG
HPGD
IL6R
KLF5
TBX3
hESCs
3iL hESCs
Differentiation associated genes
NES
PAX6
SOX1
SNAI1
SNAI2
TBX6
KDR
FOXA2
CXCR4
0
3

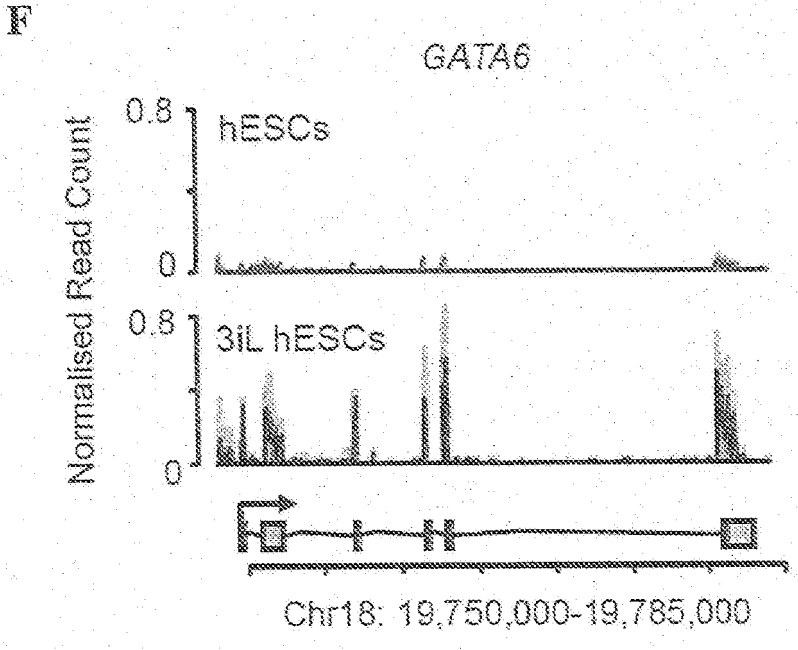

F
GATA6
Normalised Read Count
0.8
hESCs
0
0.8
3iL hESCs
0
Chr18: 19,750,000-19,785,000

Fig. 8

G

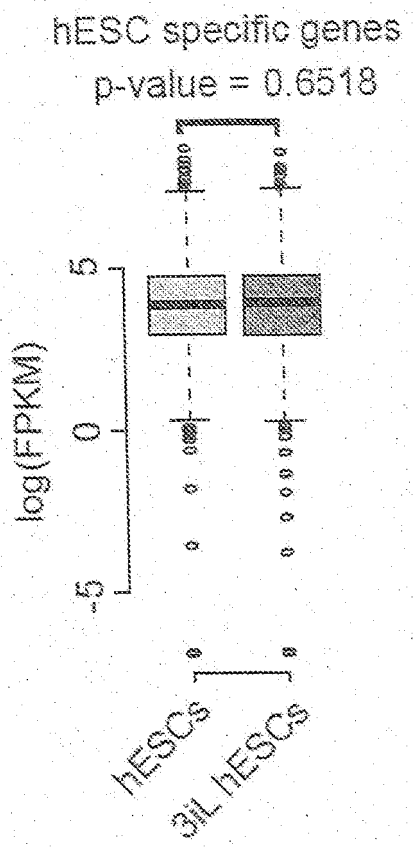

hESC specific genes
p-value = 0.6518
log(FPKM)
5
0
-5
hESCs
3iL hESCs

Fig. 8

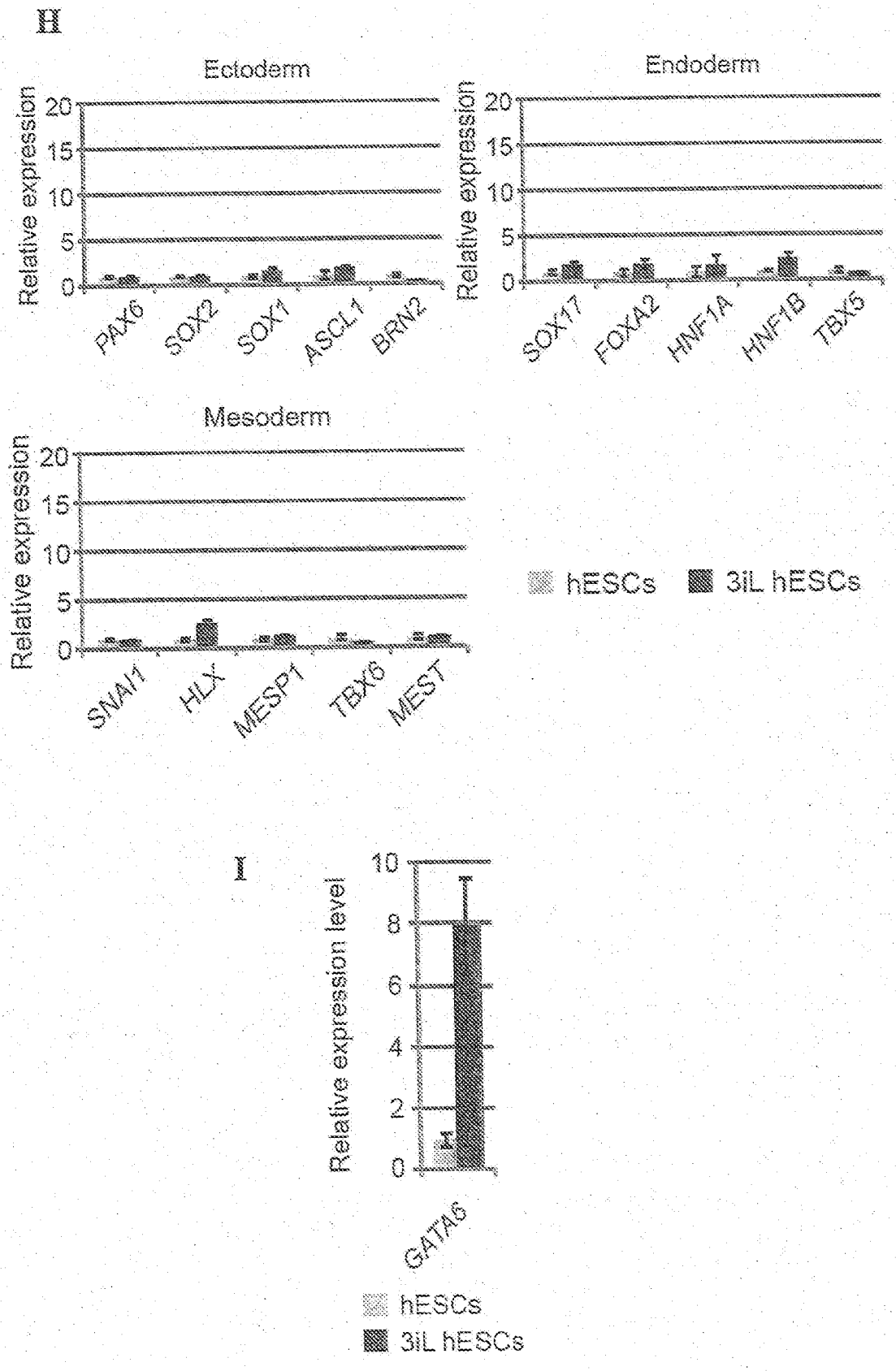
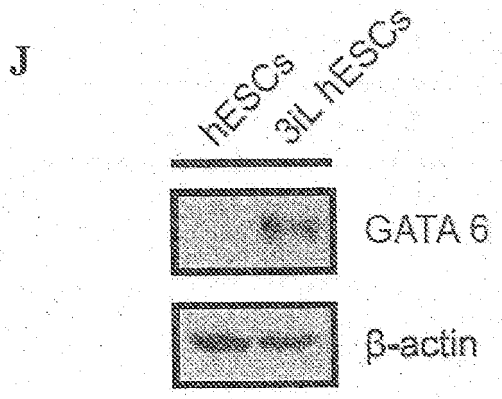

H
Ectoderm
Relative expression
20
15
10
5
0
PAX6
SOX2
SOX1
ASCL1
BRN2
Endoderm
SOX17
FOXA2
HNF1A
HNF1B
TBX5
Mesoderm
SNAI1
HLX
MESP1
TBX6
MEST
hESCs
3iL hESCs
I
Relative expression level
10
8
6
4
2
0
H1
GATA6
hESCs
3iL hESCs

Fig. 8


J
hESCs
3iL hESCs
GATA 6
β-actin

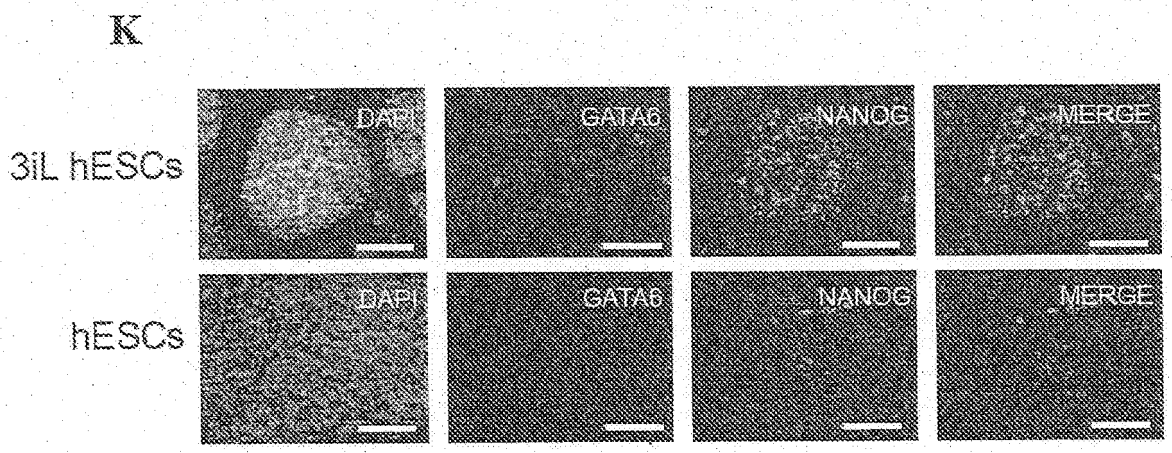

K
3iL hESCs
hESCs
DAPI
GATA6
NANOG
MERGE
DAPI
GATA6
NANOG
MERGE

Fig. 8

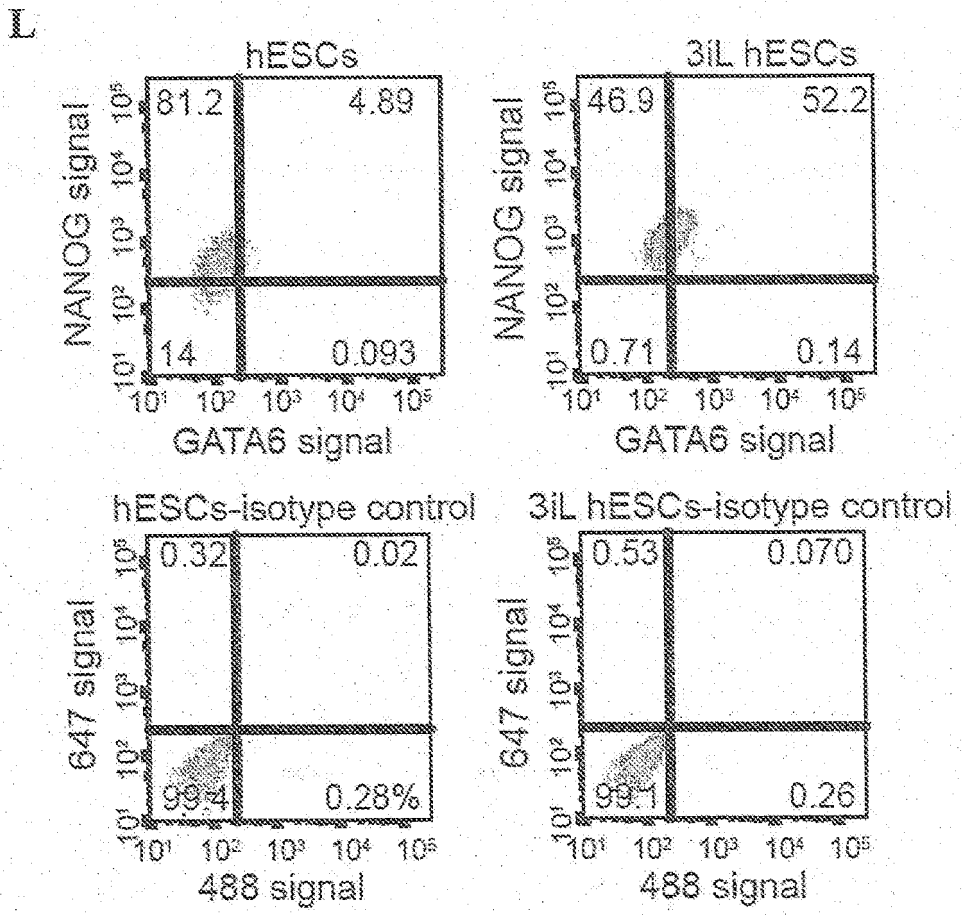

L
hESCs
81.2
4.89
14
0.093
NANOG signal
GATA6 signal
3iL hESCs
46.9
52.2
0.71
0.14
NANOG signal
GATA6 signal
hESCs-isotype control
0.32
0.02
99.4
0.28%
647 signal
488 signal
3iL hESCs-isotype control
0.53
0.070
99.1
0.26
647 signal
488 signal

Fig. 8

M

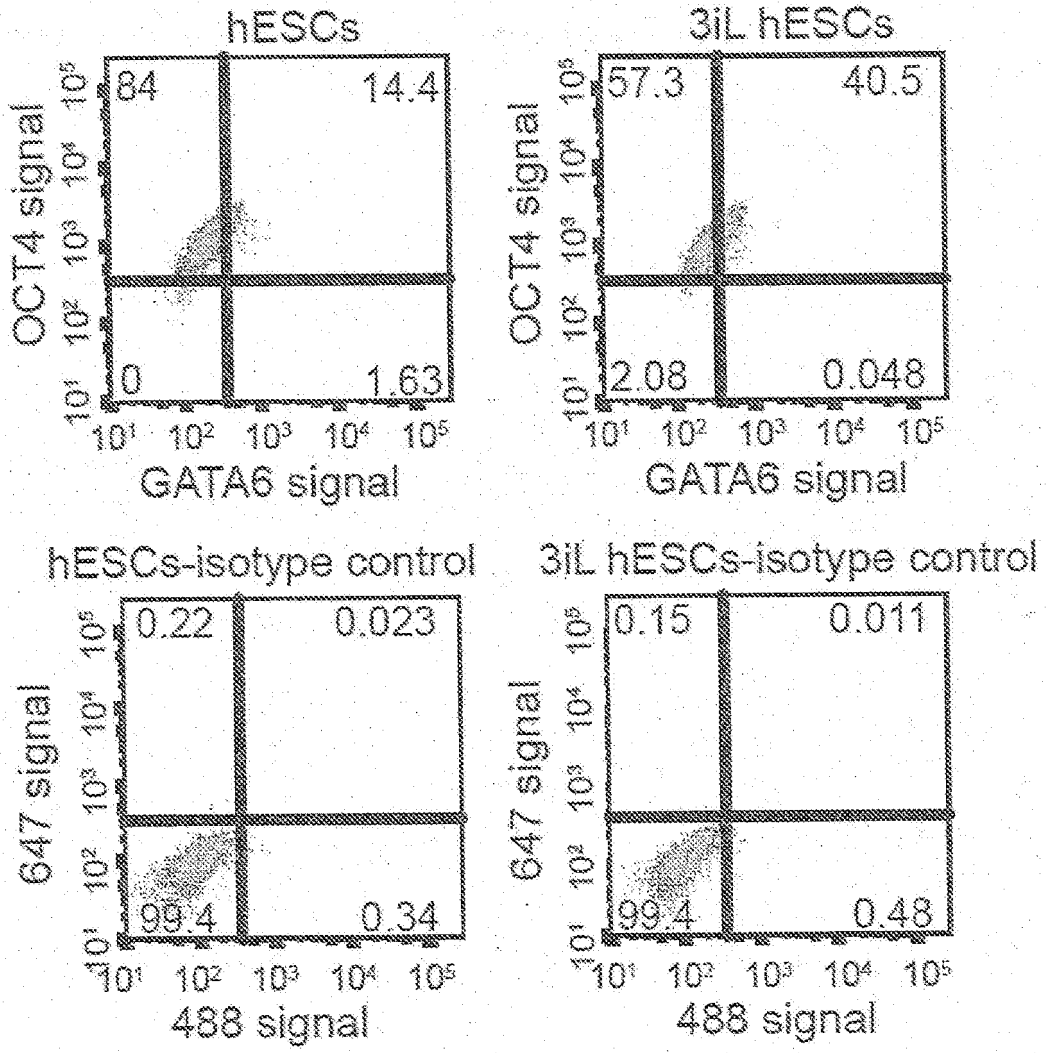

hESCs
84
14.4
0
1.63
OCT4 signal
GATA6 signal
3iL hESCs
57.3
40.5
2.08
0.048
OCT4 signal
GATA6 signal
hESCs-isotype control
0.22
0.023
99.4
0.34
647 signal
488 signal
3iL hESCs-isotype control
0.15
0.011
99.4
0.48
647 signal
488 signal

Fig. 8

N

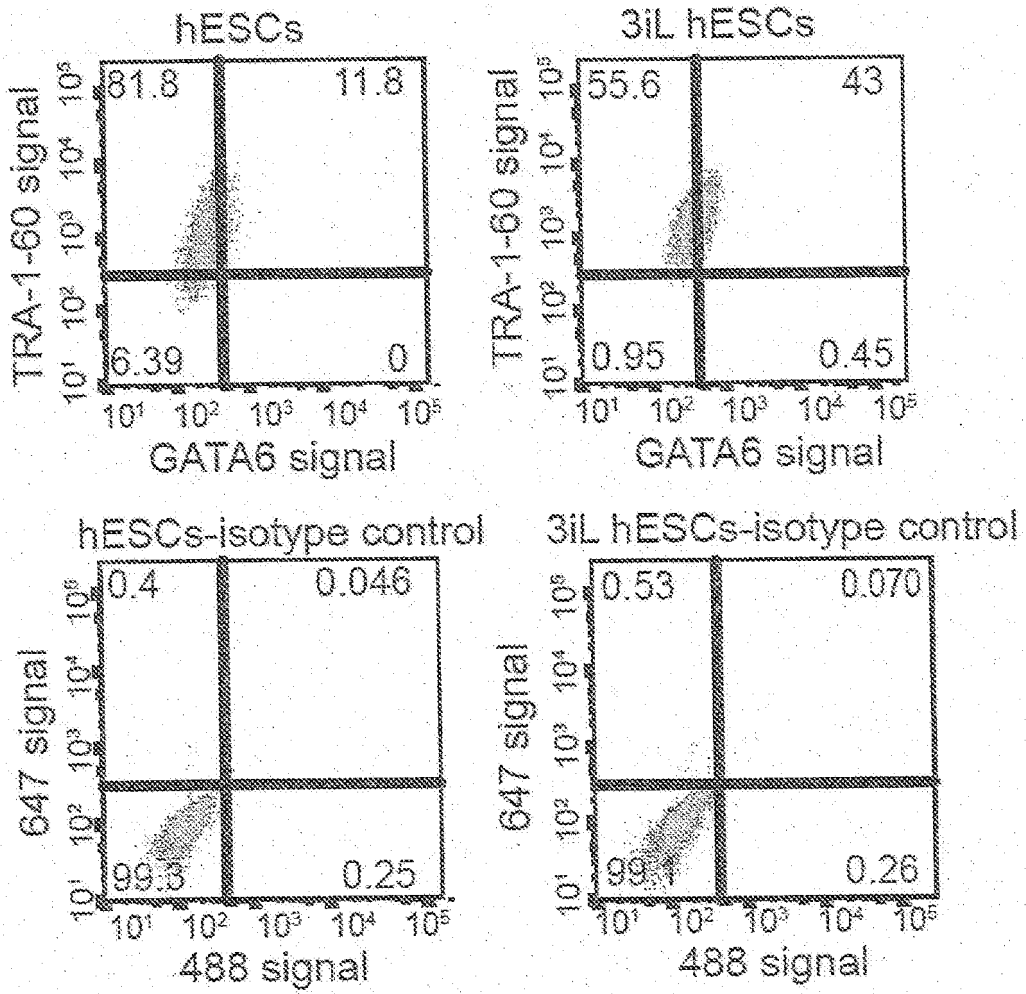

hESCs
81.8
11.8
6.39
0
TRA-1-60 signal
GATA6 signal
3iL hESCs
55.6
43
0.95
0.45
TRA-1-60 signal
GATA6 signal
hESCs-isotype control
0.4
0.046
99.3
0.25
647 signal
488 signal
3iL hESCs-isotype control
0.53
0.070
99.1
0.26
647 signal
488 signal

Fig. 8

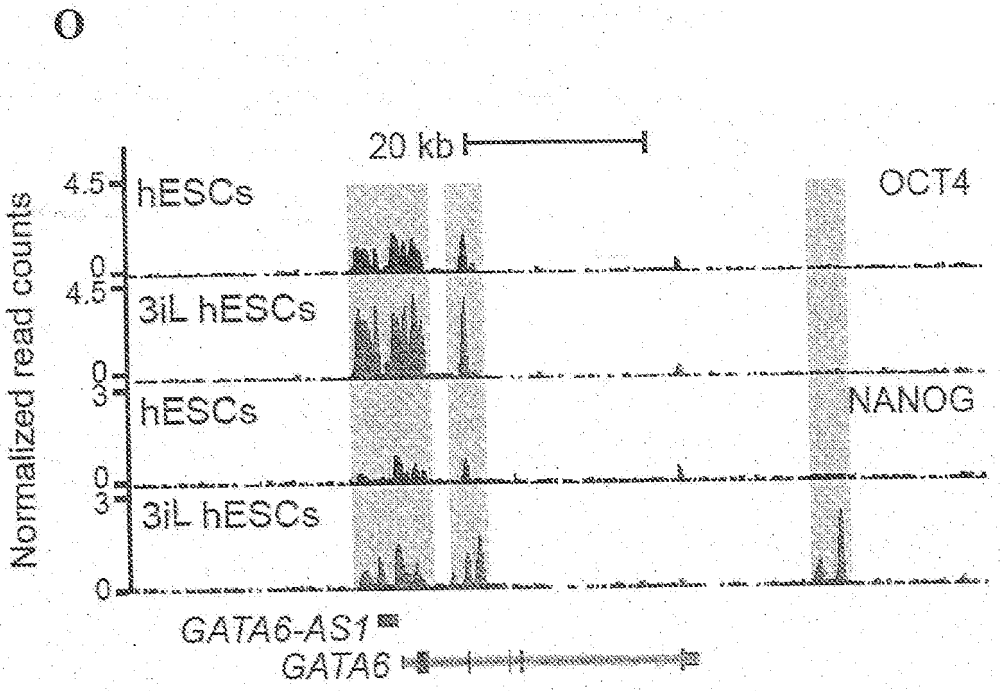

O
20 kb
Normalized read counts
4.5
hESCs
0
4.5
3iL hESCs
0
3
hESCs
0
3
3iL hESCs
0
OCT4
NANOG
GATA6-AS1
GATA6

Fig. 9

A

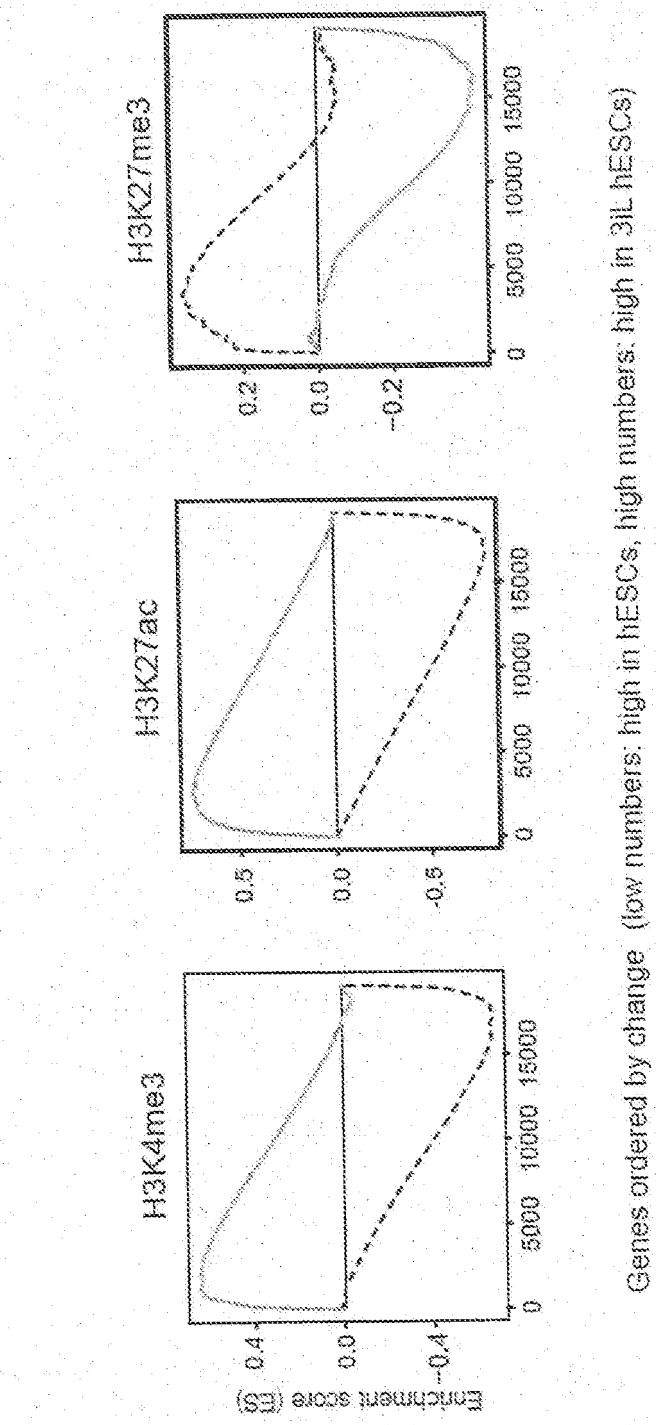

H3K27me3
H3K27ac
H3K4me3
Enrichment score (ES)
0.2
0.0
−0.2
0.5
0.0
-0.5
0.4
0.0
−0.4
0
5000
10000
15000
Genes ordered by change (low numbers: high in hESCs, high numbers: high in 3iL hESCs)
Higher in 3iL hESCs compared to hESCs
Higher in hESCs compared to 3iL hESCs

Fig. 9

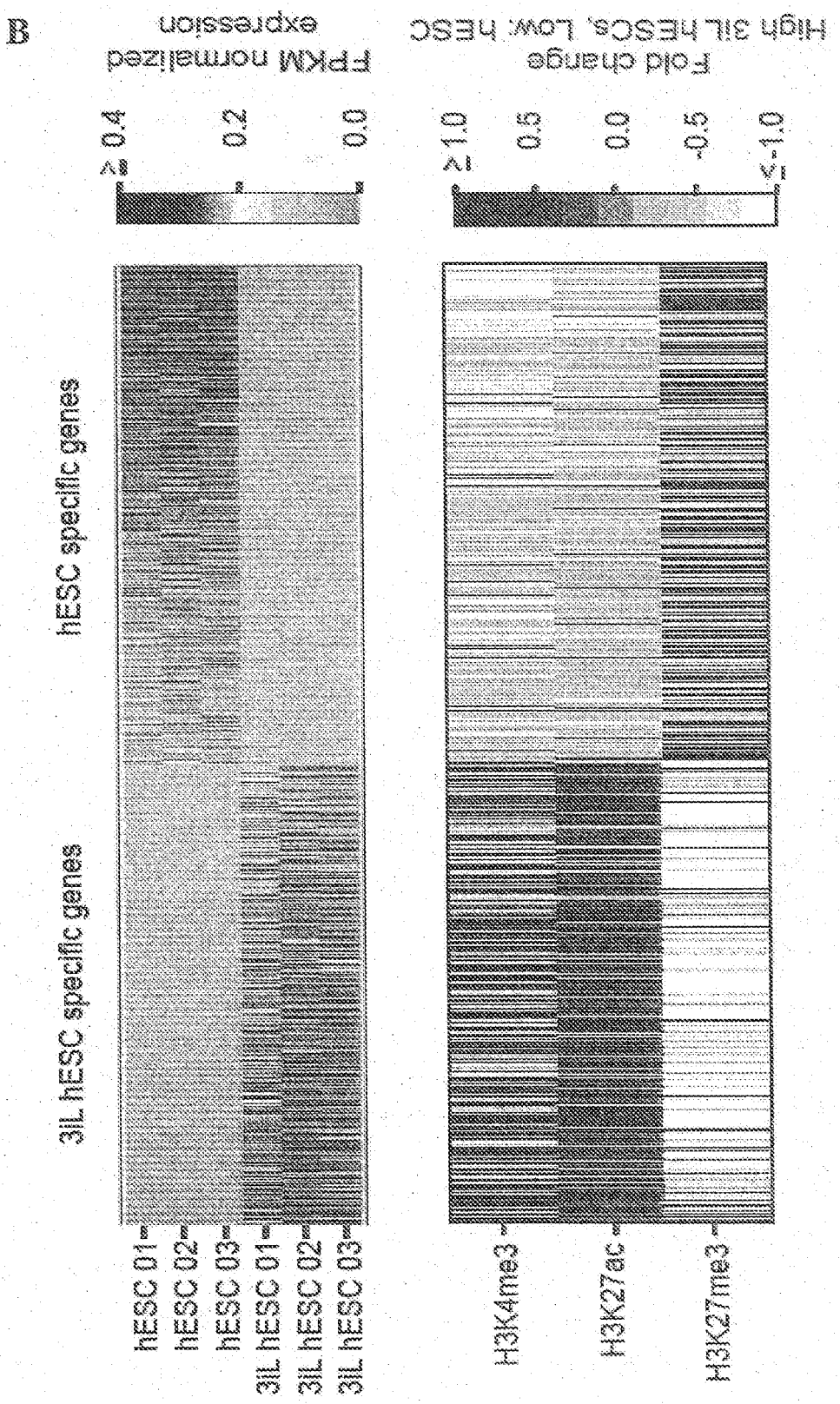

B
FPKM normalized expression
≥ 0.4
0.2
0.0
Fold change High 3iL hESCs, Low hESC
≥ 1.0
0.5
0.0
-0.5
≤ -1.0
3iL hESC specific genes
hESC specific genes
hESC 01
hESC 02
hESC 03
3iL hESC 01
3iL hESC 02
3iL hESC 03
H3K4me3
H3K27ac
H3K27me3

Fig. 9

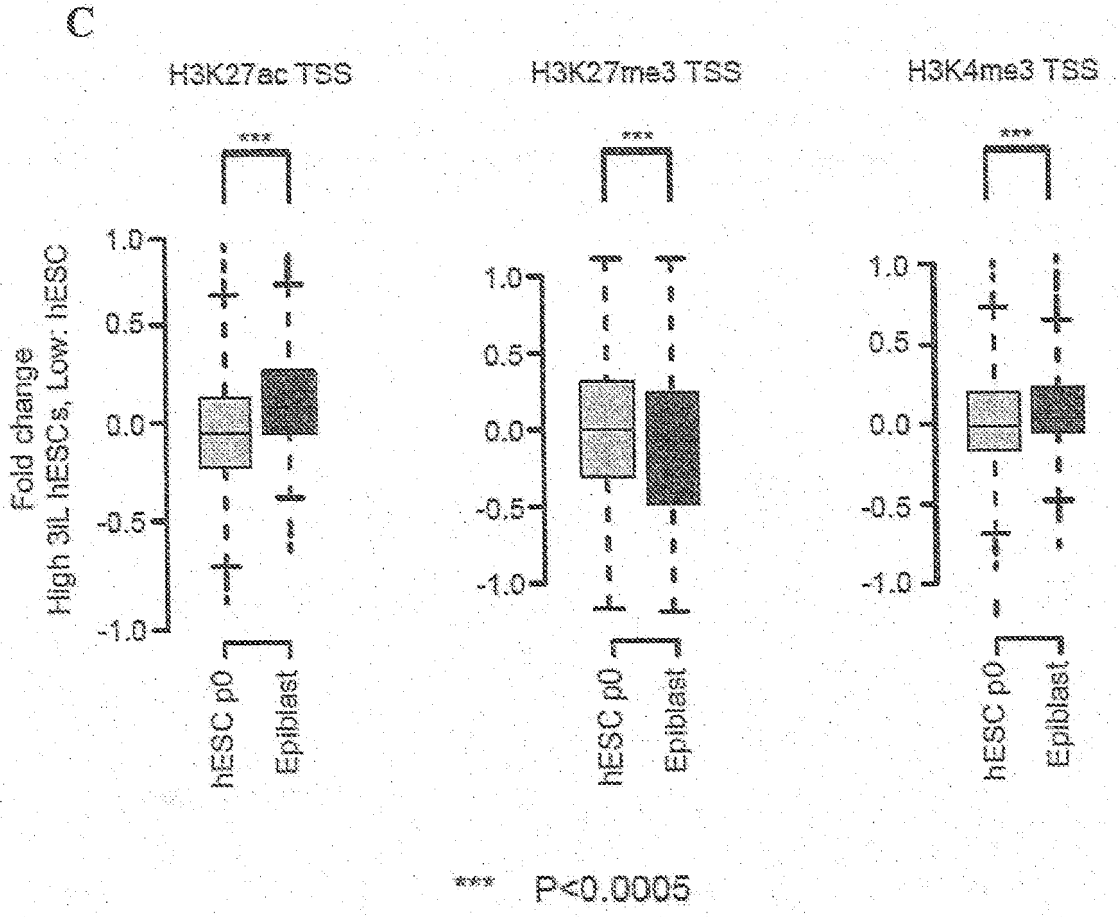

C
H3K27ac TSS
H3K27me3 TSS
H3K4me3 TSS
***
Fold change
High 3iL hESCs, Low: hESC
1.0
0.5
0.0
-0.5
-1.0
hESC p0
Epiblast
*** P<0.0005

Fig. 9

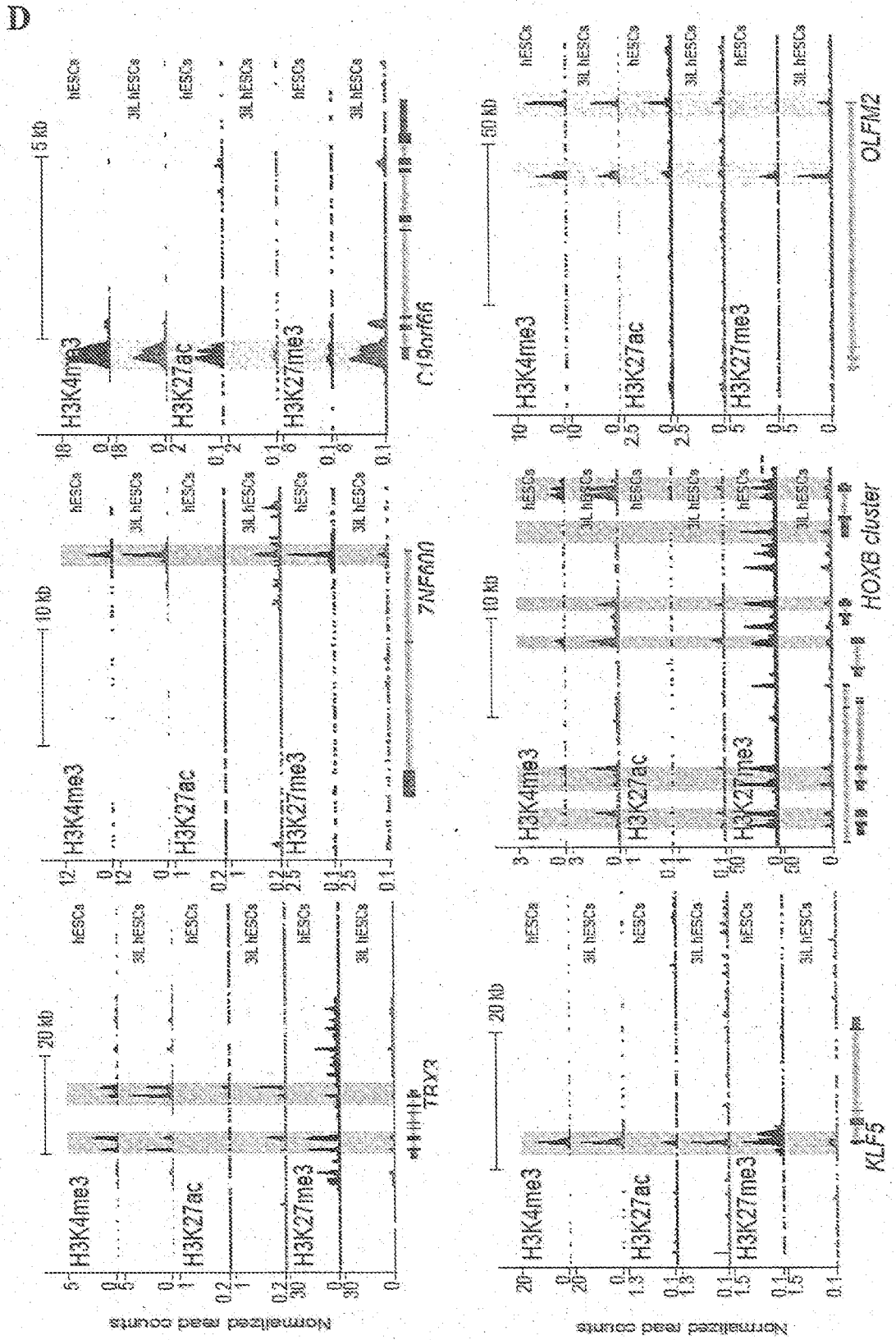

D
H3K4me3
H3K27ac
H3K27me3
hESCs
3BL hESCs
Normalized read counts
TRY3
ZNF800
C19orf66
KLF5
HOXB cluster
OLFM2

Fig. 10

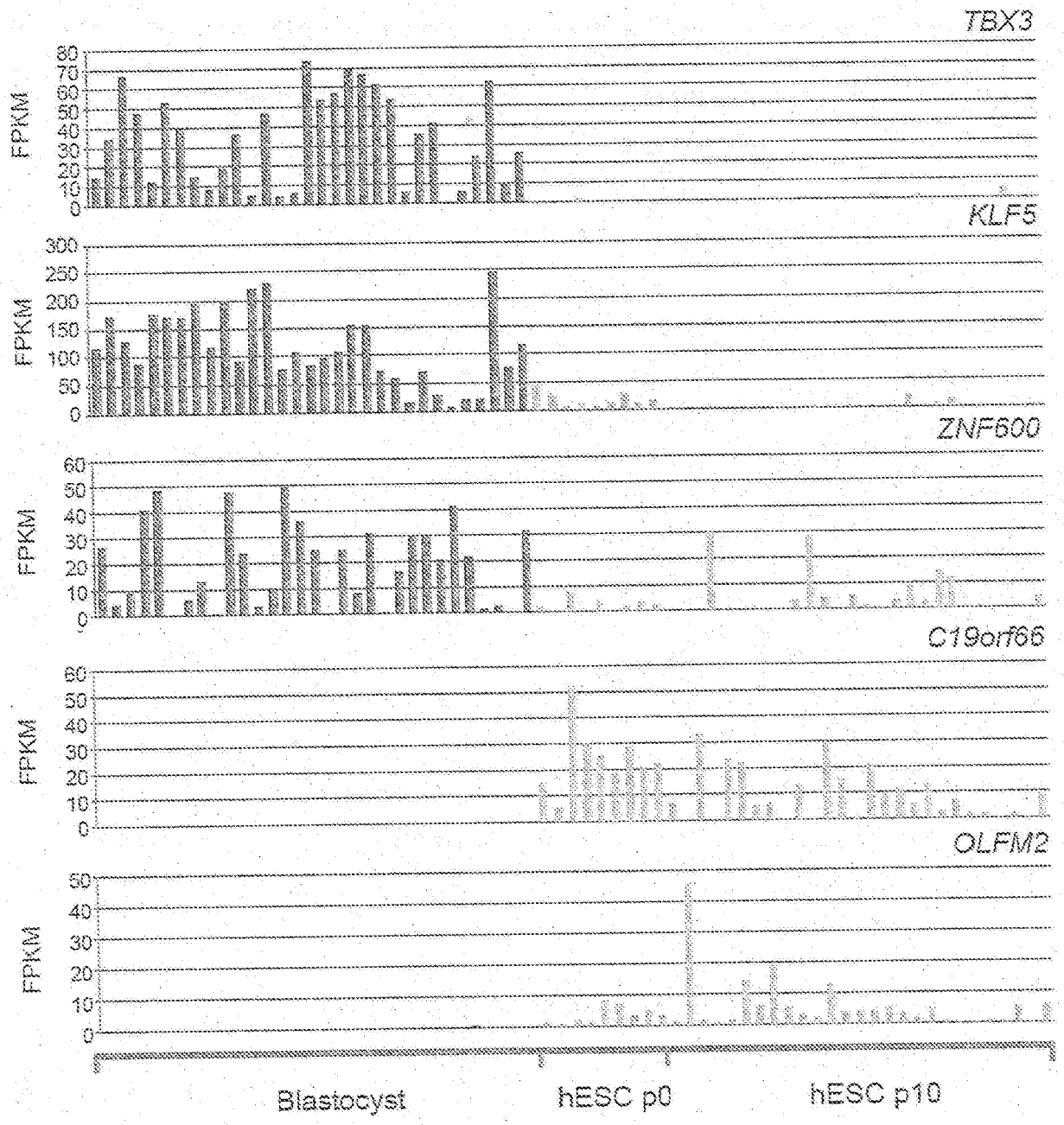

TBX3
FPKM
KLF5
FPKM
ZNF600
FPKM
C19orf66
FPKM
OLFM2
FPKM
Blastocyst
hESC p0
hESC p10

Fig. 11

A

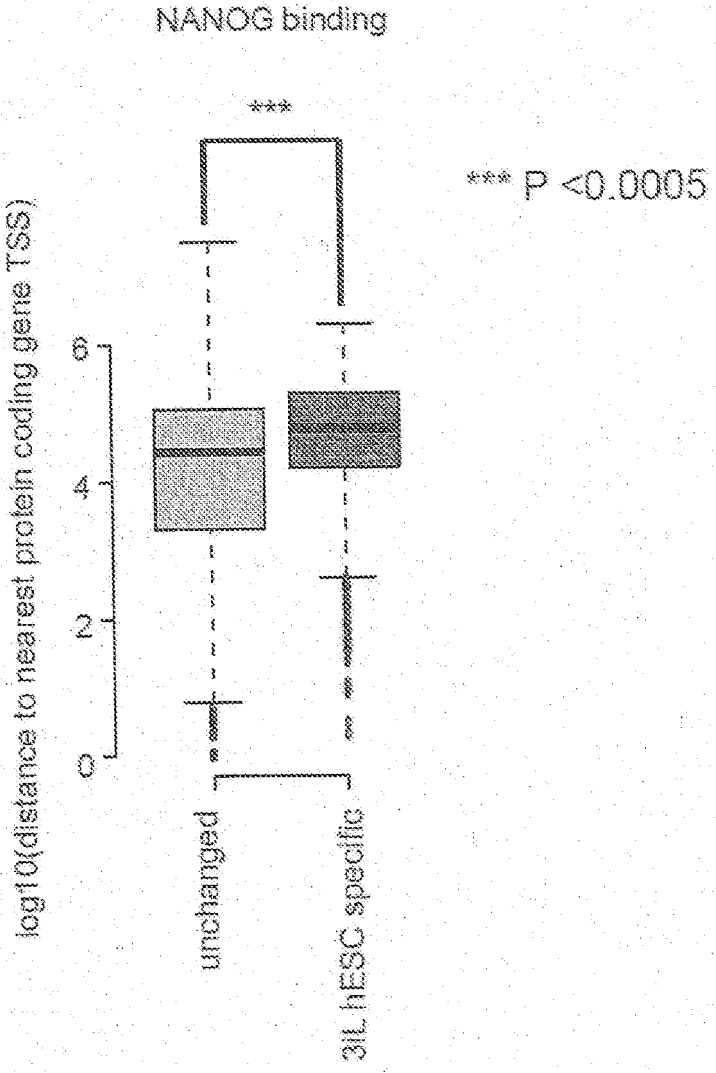

NANOG binding
***
*** P <0.0005
log10(distance to nearest protein coding gene TSS)
0
2
4
6
unchanged
3iL hESC specific

Fig. 11

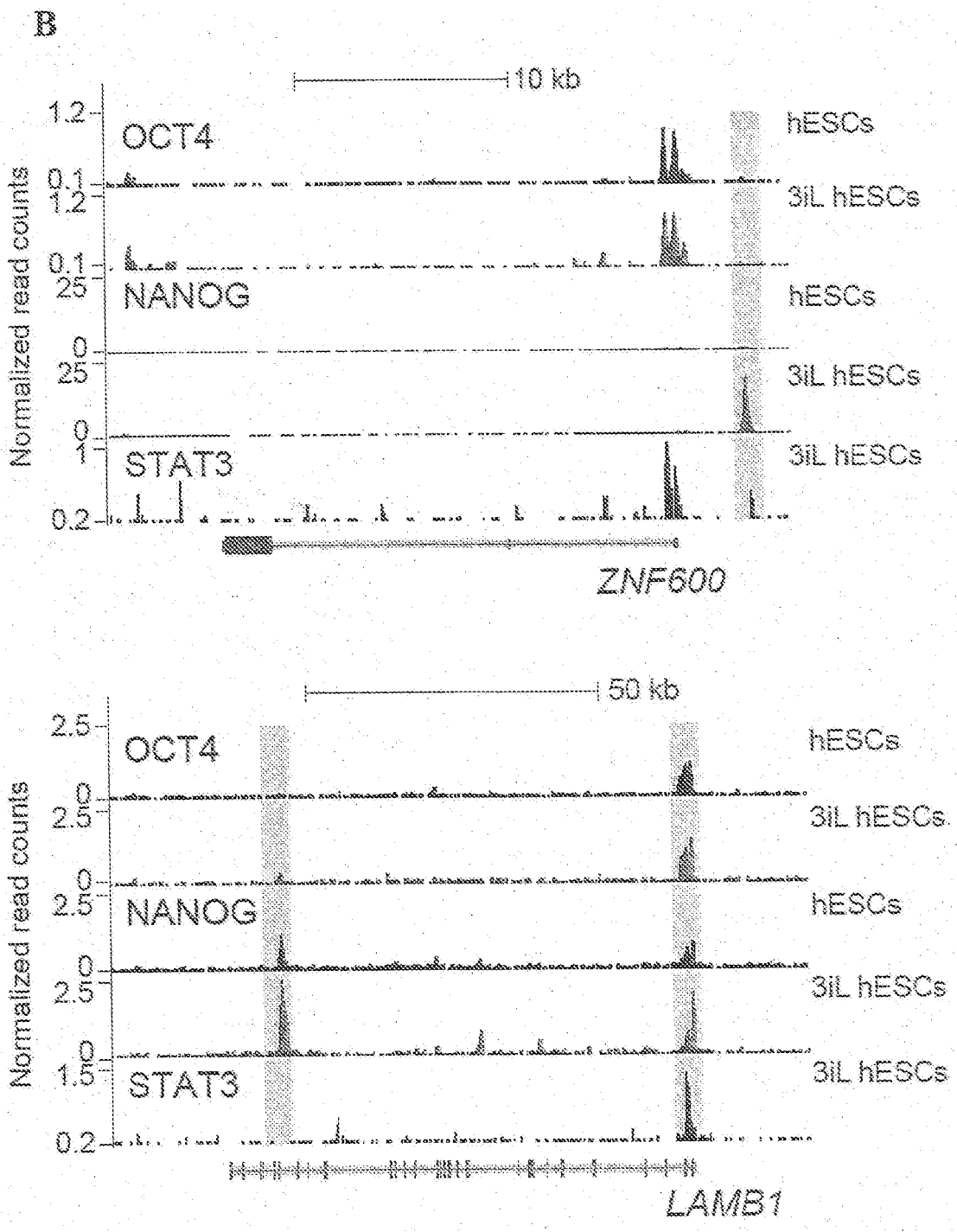

B
10 kb
Normalized read counts
OCT4
NANOG
STAT3
hESCs
3iL hESCs
ZNF600
50 kb
LAMB1

Fig. 12

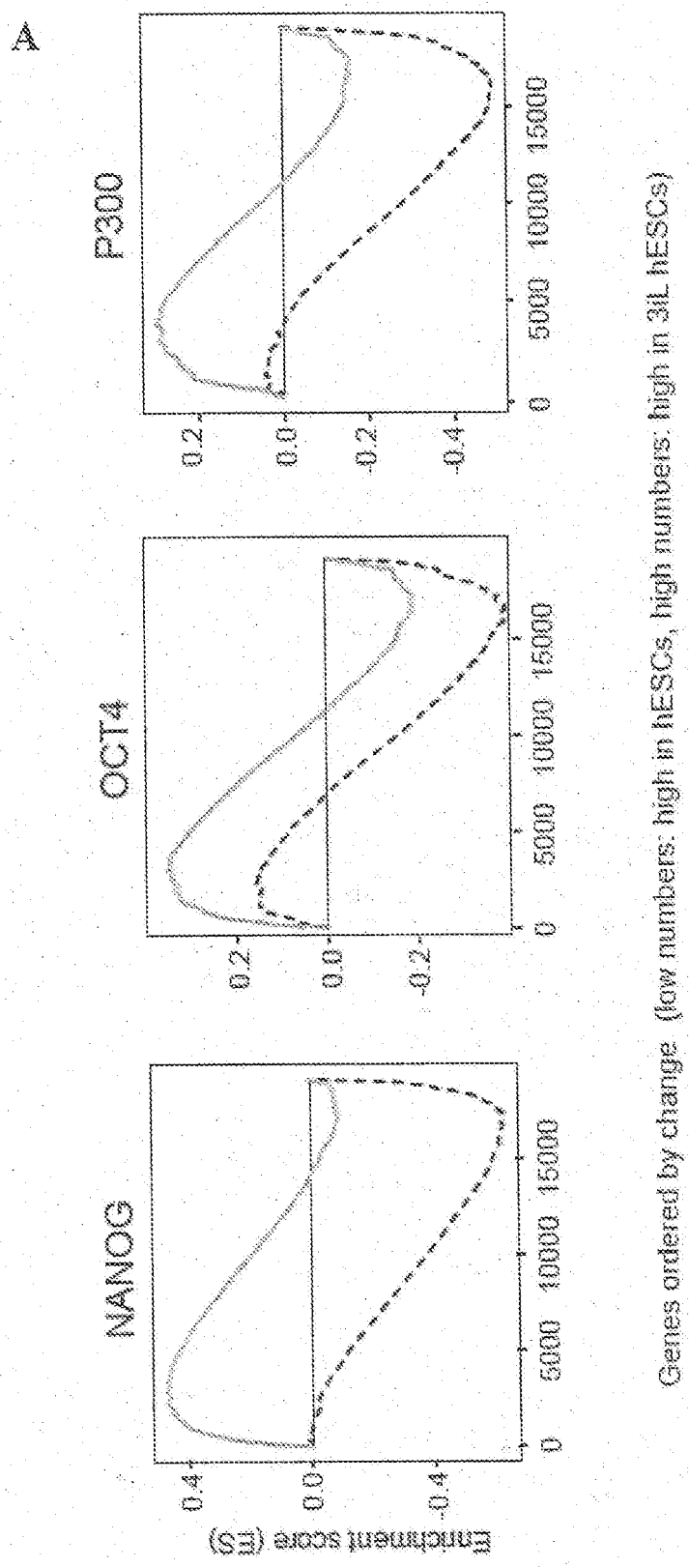

A
NANOG
OCT4
P300
Enrichment score (ES)
0.4
0.0
-0.4
0.2
-0.2
0
5000
10000
15000
Genes ordered by change (low numbers: high in hESCs, high numbers: high in 3iL hESCs)
Higher in 3iL hESCs compared to hESCs
Higher in hESCs compared to 3iL hESCs

Fig. 12

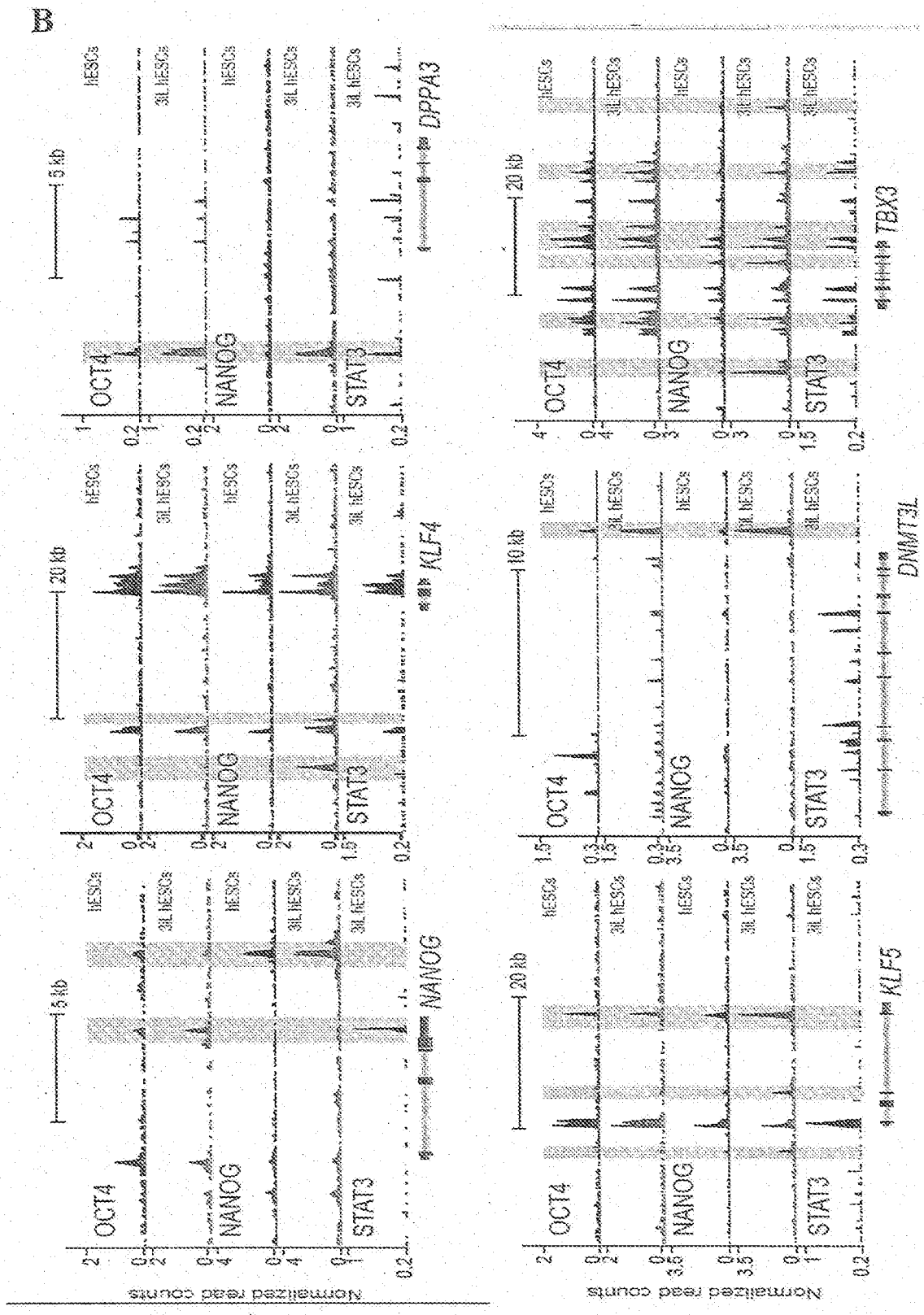

B
5 kb
20 kb
10 kb
hESCs
3IL hESCs
OCT4
NANOG
STAT3
Normalized read counts
NANOG
KLF4
DPPA3
KLF5
DNMT3L
TBX3

Fig. 12

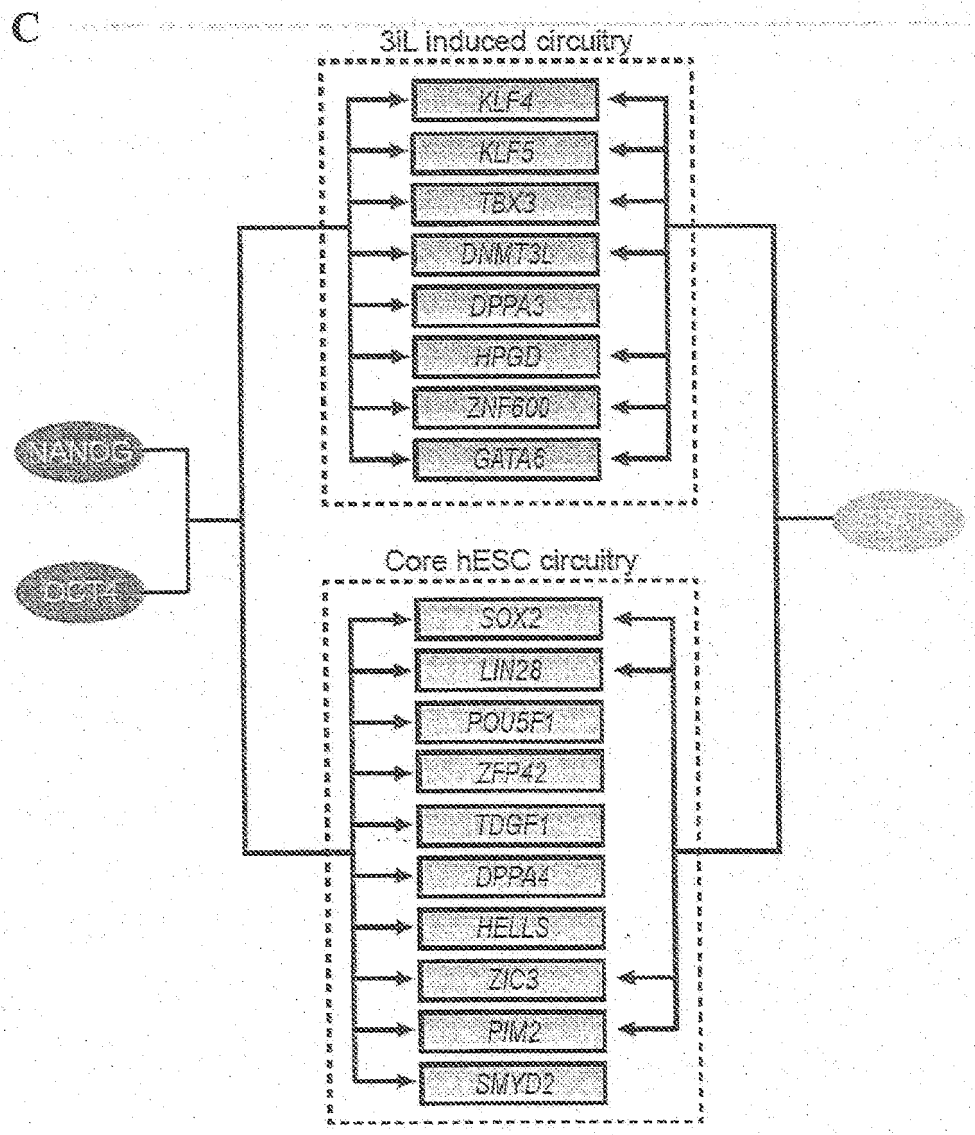

C
3iL induced circuitry
KLF4
KLF5
TBX3
DNMT3L
DPPA3
HPGD
ZNF600
GATA6
NANOG
OCT4
Core hESC circuitry
SOX2
LIN28
POU5F1
ZFP42
TDGF1
DPPA4
HELLS
ZIC3
PIM2
SMYD2

Fig. 12

D

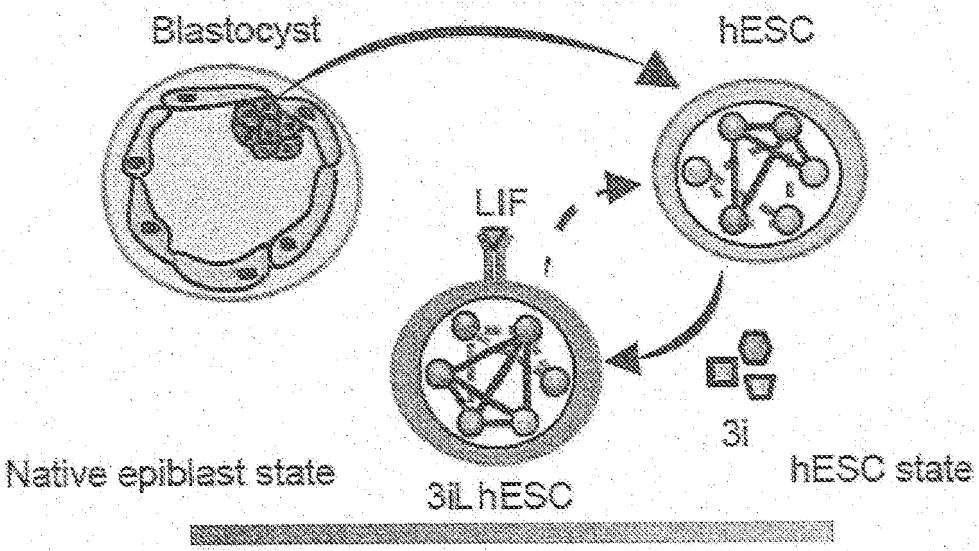
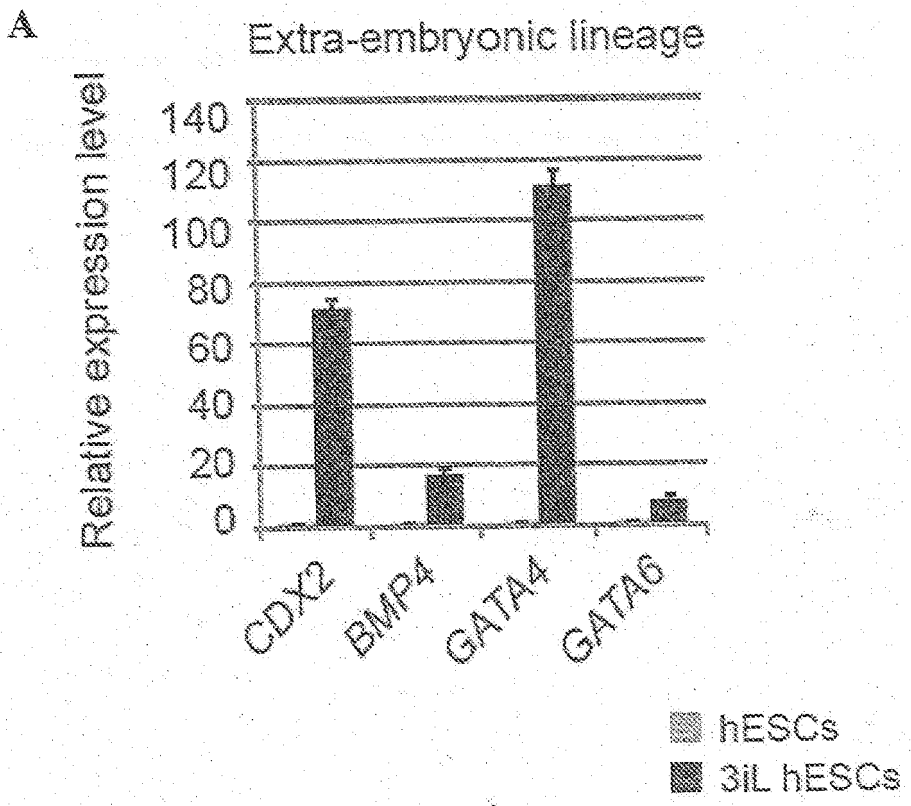

Blastocyst
hESC
LIF
3i
Native epiblast state
3iL hESC
hESC state

Fig.13


A
Extra-embryonic lineage
Relative expression level
140
120
100
80
60
40
20
0
CDX2
BMP4
GATA4
GATA6
hESCs
3iL hESCs

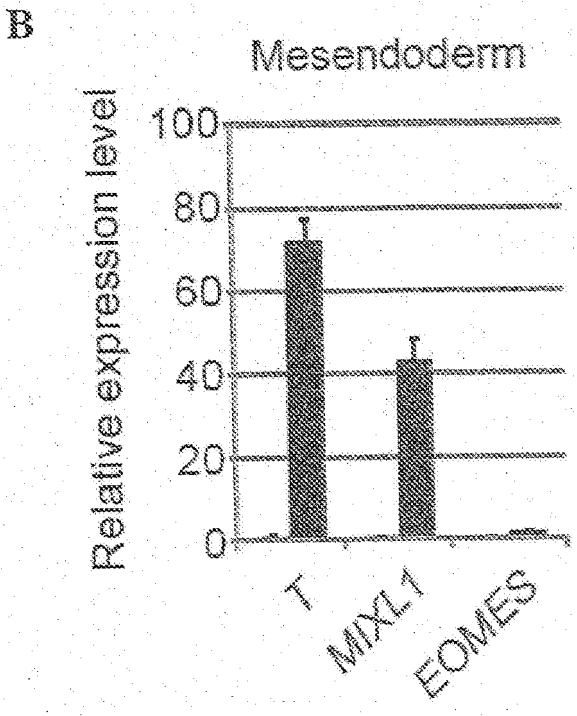
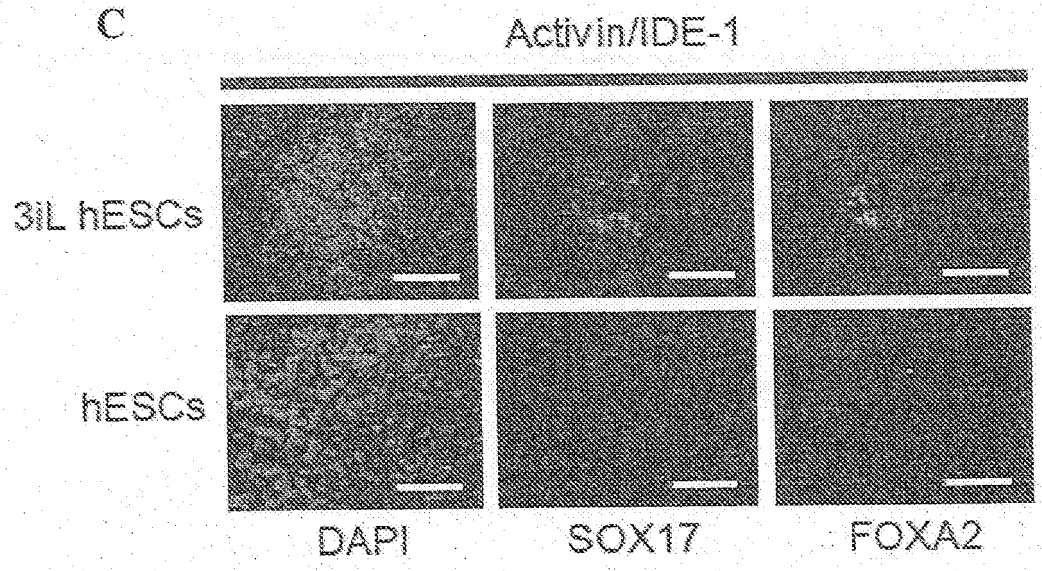

B
Mesendoderm
Relative expression level
100
80
60
40
20
0
T
MIXL1
EOMES

Fig.13


C
Activin/IDE-1
3iL hESCs
hESCs
DAPI
SOX17
FOXA2

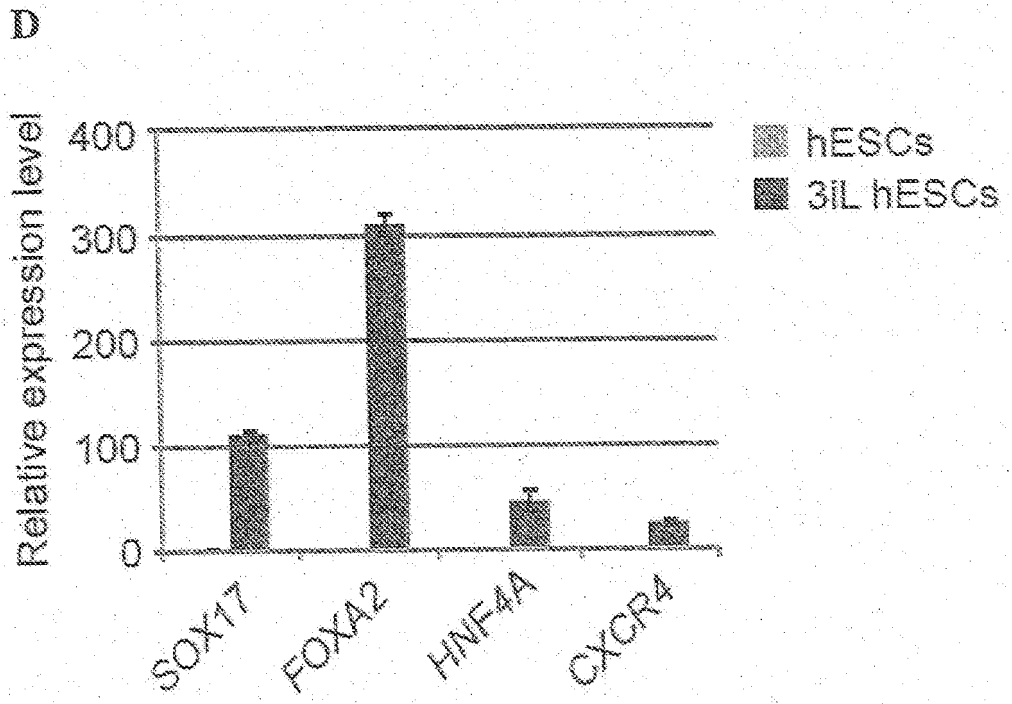
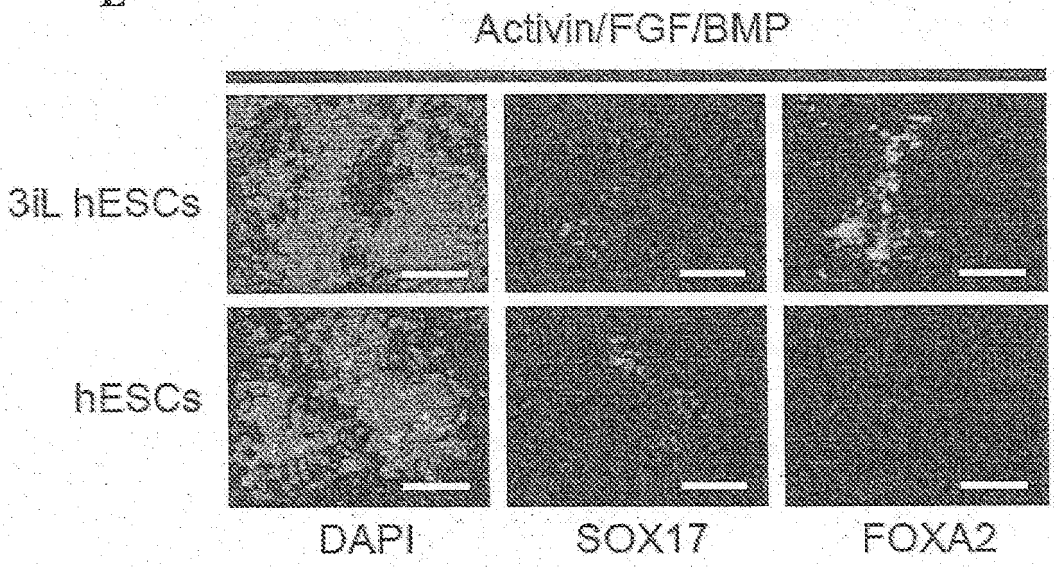
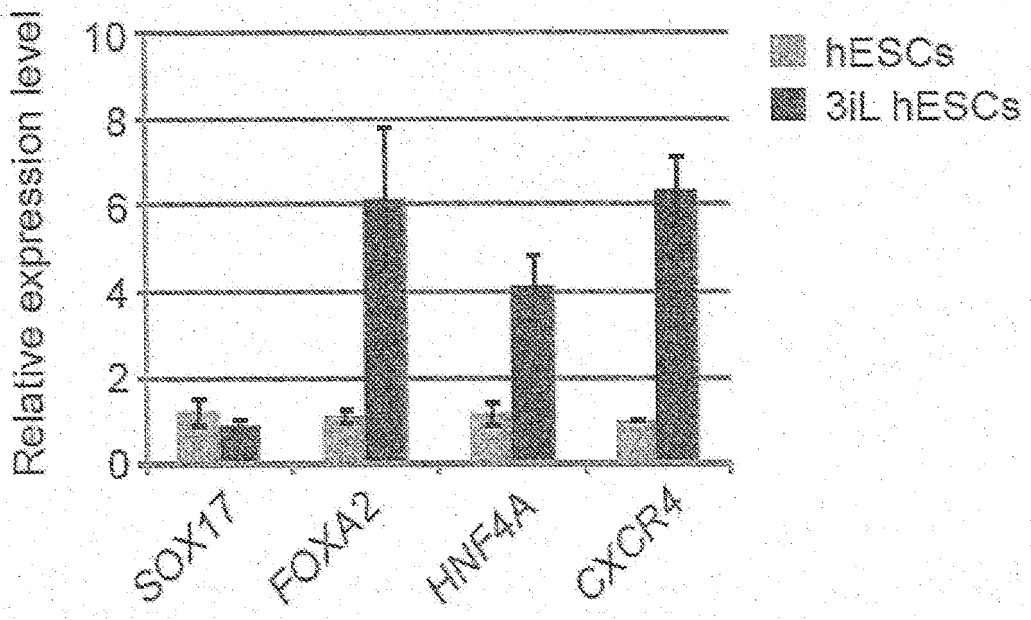

D
Relative expression level
400
300
200
100
0
hESCs
3iL hESCs
SOX17
FOXA2
HNF4A
CXCR4

Fig.13

E


Activin/FGF/BMP
3iL hESCs
hESCs
DAPI
SOX17
FOXA2

F


Relative expression level
10
8
6
4
2
0
hESCs
3iL hESCs
SOX17
FOXA2
HNF4A
CXCR4