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An *in situ* hybridization-based screen for heterogeneously expressed genes in mouse ES cells

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Abstract

We previously reported that *Zscan4* showed heterogeneous expression patterns in mouse embryonic stem (ES) cells. To identify genes that show similar expression patterns, we carried out high-throughput *in situ* hybridization assays on ES cell cultures for 244 genes. Most of the genes are involved in transcriptional regulation, and were selected using microarray-based comparisons of gene expression profiles in ES and embryonal carcinoma (EC) cells versus differentiated cell types. *Pou5f1* (*Oct4*, *Oct3/4*) and *Krt8* (*EndoA*) were used as controls. Hybridization signals were detected on ES cell colonies for 147 genes (60%). The majority (136 genes) of them showed relatively homogeneous expression in ES cell colonies. However, we found that two genes unequivocally showed *Zscan4*-like spotted expression pattern (spot-in-colony pattern; *Whsc2* and *Rhox9*). We also found that nine genes showed relatively heterogeneous expression pattern (mosaic-in-colony pattern: *Zfp42/Rex1*, *Rest*, *Atf4*, *Pa2g4*, *E2f2*, *Nanog*, *Dppa3*/Pgc7/*Stella*, *Esrrb*, and *Fscn1*). Among these genes, *Zfp42/Rex1* showed unequivocally heterogeneous expression in individual ES cells prepared by the CytoSpin. These results show the presence of different types or states of cells within ES cell cultures otherwise thought to be undifferentiated and homogeneous, suggesting a previously unappreciated complexity in ES cell cultures.

Keywords

ES cells; EC cells; pluripotent stem cells; heterogeneous gene expression; homogeneous gene expression; Zscan4; Pou5f1; Oct4; Oct3/4; Krt8; EndoA; Whsc2; Nelfa; Rhox9; Zfp42; Rex1; Rest; Zfp42; Rex1; Rest; Atf4; Pa2g4; E2f2; Nanog; Dppa3; Pgc7; Stella; Esrrb; Fscn1

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1. Results and discussion

1.1. Rationale for the study

Mouse ES cells remain undifferentiated when cultured in the presence of leukemia inhibitory factor (LIF) (Niwa, 2007). The undifferentiated state of ES cells is usually verified by positive staining for expression of alkaline phosphatase and *Pou5f1* (aka: *Oct4*, *Oct3*/4). It has been thought that these undifferentiated ES cells are relatively homogeneous and retain capacities for pluripotency and self-renewal (Niwa, 2007). Therefore, the analysis of ES cells is typically population-based. For example, we have analyzed the global gene expression profiles of ES cells, comparing populations of ES cells cultured in conditions which promote or prevent cellular differentiation (Aiba et al., 2006; Sharova et al., 2007).

However, the presence of heterogeneous cell populations in undifferentiated ES cell cultures is now increasingly recognized. Recent reports have demonstrated that surface markers, such as *Ssea1*, *Pecam1*, and *Icam1*, are expressed heterogeneously in mouse ES cell cultures, and their expression patterns appear to be modulated by differentiation processes in these cultures (Cui et al., 2004; Li et al., 2005). Furthermore, in the case of *Pecam1*, overall expression levels and isoform distributions are indicative of ES cell differentiation state (Cui et al., 2004; Li et al., 2005) as well as embryonic developmental potential (Furusawa et al., 2004). The presence of *T*-positive cells in ES cell colonies has also been reported (Suzuki et al., 2006). Recently, we have reported that a gene named Zscan4, which is expressed exclusively in 2-cell embryos and ES cells, shows heterogeneous expression in ES cells: only up to 10% of ES cells cultured in undifferentiated conditions express *Zscan4* by *in situ* hybridization (Falco et al., 2007). We suspect the existence of other transcription-regulating genes that show similar expression patterns.

The goals of this study are to find additional evidence for heterogeneous cell populations within undifferentiated ES cell cultures by identifying such genes and characterizing their expression patterns. We report here a two-step approach to identify transcription factor genes responsible for heterogeneity in ES cell cultures, possibly with roles in regulating early differentiation.

1.2. Identification of transcription factor genes expressed predominantly in ES/EC cells

First, we used a microarray-based comparison of gene expression profiles in ES and embryonal carcinoma (EC) cells vs. differentiated cell types to identify enriched transcripts in ES and EC cells. Previously published Microarray data sets (Carter et al., 2003; Aiba et al., 2006) generated using the NIA Mouse 22K Microarray (Carter et al., 2003) were processed using the NIA Array Analysis Tool (Sharov et al., 2005b). Expression profiles for undifferentiated cultures of 129/SvEv and R1 ES cells were combined with those of F9 and P19 embryonal carcinoma (EC) cells to represent stem cell gene expression patterns. Expression profiles for trophoblast stem (TS) cells, neural stem/progenitor (NS) cells, differentiated cells (DC) from the NS cells, and E12.5 whole placenta were included to represent gene expression from differentiation-committed or differentiated cells (Figure 1A). Hierarchical clustering of tissues was carried out using the average distance method, and all tissues representing stem cell gene expression were grouped into a single compact branch of the dendrogram, along with TS cells (Figure 1A), suggesting that as a cultured, oligopotent stem cell line, TS cells have a gene expression profile that bears many similarities to EC and ES cell profiles, which are not related to pluripotency. Sets of genes expressed predominantly in ES/EC cells were identified for clusters 10, 12, 13, and 14 as described previously (Sharov et al., 2005b) and these lists were combined to form a list of 541 genes.

Based on GO annotations (Ashburner et al., 2000), protein motif homology, and literature reports, a list of 2,727 known/putative transcription factor and/or DNA-binding genes was

identified, 2,025 of which are identified and annotated in the NIA Mouse Gene Index v5.0 (NMGI5) (Sharov et al., 2005a). This list was used to identify ~300 transcription factor candidates from the ES/EC-enriched gene list. In a similar process, data sets comparing gene expression in ES and TS cells on the NIA Mouse 44K Microarray (Carter et al., 2005), a whole-genome platform which allows estimation of absolute expression levels, were filtered to identify 126 ES-enriched transcription factor candidate genes. In this case, genes with absolute expression levels estimated at ≥1 copy/cell in ES cells and < 1 copy/cell in TS cells were considered "ES-enriched". Genes predominantly expressed in the inner cell mass (ICM) of mouse blastocysts were previously identified using whole-mount in situ hybridization (Yoshikawa et al., 2006) and 61 of these genes were included in the candidate list. Finally, unpublished gene lists from experiments performed in our laboratory were added, as well as individual genes of interest under study. All gene lists were compiled into a non-redundant master list of 344 candidate genes.

1.3. Clone selection and verification

For each candidate gene that is represented in NMGI4, transcript assembly models (Sharov et al., 2005a) were consulted for manual selection of 319 cDNA clone templates for *in situ* hybridization probes. When possible, cDNA clones covering 3'UTRs of genes were selected to give higher specificity, and clones with inserts ≤1 Kb in length were preferred. In 38 cases where a given gene was not represented in NMGI4 or a suitable cDNA clone for that gene was not available in NIA libraries, clones were selected from other sources (e.g., Open Biosystems Inc. collections). All bacterial clones were sequence-verified prior to use as ISH probe templates. Finally, we obtained digoxigenin-labeled riboprobes for 254 genes.

1.4. High-throughput in situ protocol

Previously we reported the development of a protocol for a high-throughput whole mount in situ hybridization of preimplantation mouse embryos and the expression patterns of 98 genes in mouse blastocysts (Yoshikawa et al., 2006). We adapted this protocol to mouse ES cell culture (see the detailed protocol in Supplement). We tested *Pou5f1*, which is known to be expressed in undifferentiated ES cells, and *Krt8* (*EndoA*), which is known to be expressed in trophectoderm and visceral endoderm. The transcripts of *Pou5f1* were detected in the undifferentiated colonies of ES cells, whereas the transcripts of *Krt8* were detected in the flatter cells that surround undifferentiated colonies (Fig. 2, Fig. 3). To increase the throughput of the procedure, we adapted the method to gelatin-coated 12-well microtiter plates. This particular culture condition was not necessarily best suited to undifferentiated ES cells. In fact, we frequently observed cells with features of differentiation, such as flatter cell morphology and the expression of Krt8, along with undifferentiated cells in typical compact colonies. Therefore, in each plate, the expression patterns of 10 genes were examined, together with *Pou5f1* and *Krt8* as controls. We also scored *in situ* hybridization results of cells only within compact ES cell colonies (see Fig. 2 and 3 for examples).

1.5. In situ hybridization screen

We then carried out *in situ* hybridization of 254 genes (Figure 2). Based on the visual inspection of high-resolution images of these results, we classified the expression patterns of 254 genes by their signal intensities (Table 1). Six categories of signal intensity (non-detectable - 97/40%, very faint – 56/23%, faint – 36/15%, medium – 27/11%, strong – 13/5%, and very strong – 15/6%) were identified, with decreasing membership at higher signal intensities. In fact, almost half of the clones hybridized produced no discernable signal, but this aspect of the results is not surprising, given that many transcription factor/DNA-binding gene products are expressed at low levels, compared to metabolic and structural proteins. Because ISH is not as sensitive as other transcript detection methods, we might expect that some low-abundance transcripts

may be missed, and some detection sensitivity is sacrificed for the localization information which is provided by ISH. For example, it is well known that *Foxd3* (Hanna et al., 2002) and *Klf4* (Nakatake et al., 2006) are expressed in mouse ES cells and play an important function, but we could not detect any hybridization signals for these genes (Figure 3). This is clearly a limitation of the current methodology.

The observed expression patterns grouped the 147 positive genes into three groups (Table 1): (i) 136 genes showed relatively homogeneous expression in ES cell colonies. Magnified images for representative genes are shown in Fig. 3. These expression patterns resembled that of *Pou5f1*: strong expression in the center of ESC colonies, with reduction or absence of expression at the more differentiated, epithelioid edges of colonies and isolated cells. (ii) 2 genes showed *Zscan4*-like spotted expression patterns (spot-in-colony pattern: *Rhox9* and *Whsc2*; Fig. 3); (iii) 9 genes showed heterogeneous expression patterns (mosaic-in-colony pattern: *Zfp42*, *Atf4*, *Dppa3*, *Esrrb*, *E2f2*, *Fscn1*, *Pa2g4*, *Nanog*, and *Rest*; Fig. 3). Although the expression patterns of these genes were distinctive compared to those of homogeneously expressed genes (Fig. 3), we note that, except for *Zscan4*, *Rhox9*, *Whsc2*, and *Zfp42*, these classifications were rather subjective and should be taken with caution. In fact, among 9 genes that showed mosaic-in-colony expression pattern, only *Zfp42/Rex1* showed unequivocal heterogeneous expression pattern at the single cell level, when *in situ* hybridization was carried out on ES cells trypsinized and attached to a glass slide by the CytoSpin (Fig. 4). Some other genes also appeared to be heterogeneous (e.g., Atf4, Dppa3, Nanog, Pa2g4, and Rest), but the further confirmation by more quantitative methods will be required.

These results show the presence of different types or states of cells in ES cell cultures, suggesting the previously unrecognized complexity of ES cell cultures grown under standard, non-differentiating conditions. We will briefly describe the features of these genes.

1.6. Genes with spotted expression patterns (spot-in-colony pattern)

Among genes we examined in this study, only three genes (Zscan4, *Rhox9, and Whsc2*) showed highly heterogeneous “spotted” expression patterns in undifferentiated ES cell cultures.

Zscan4, which encodes SCAN domain and four zinc-finger domains, was identified previously for the heterogeneous spot-in-colony expression pattern (Falco et al., 2007). *Zscan4* is expressed exclusively in mouse 2-cell embryos and ES cells. Reducing the level of *Zscan4* transcripts delays the progression from 2-cell embryos to 4-cell embryos.

Probes for *Rhox9* (originally identified as *Psx2*) detected a sparse, heterogeneous expression pattern. Rhox homeobox genes are located in a cluster on the X chromosome and are the result of very recent gene duplications (Maclean et al., 2005; MacLean et al., 2006). As a consequence, *Rhox6/Psx1* and *Rhox9/Psx2* are virtually identical at the DNA sequence level, and our *in situ* hybridization probes cannot distinguish between the two genes. Quantitative RT-PCR analysis using primer pairs that can distinguish *Rhox6* and *Rhox9* showed that *Rhox9* constituted 85% of transcripts and *Rhox6* constituted 15% of transcripts in undifferentiated ES cells (data not shown). Therefore, transcripts detected by in situ hybridization were most likely *Rhox9*. *Rhox6* is known to be a marker of the trophectoderm lineage (Han et al., 1998; Chun et al., 1999), but *Rhox9* is expressed specifically in female germ cells (Takasaki et al., 2001).

Whsc2/NelfA mRNA was strongly expressed in a sparse heterogeneous pattern (two to four non-adjacent cells per colony). *Whsc2* is a mammalian homolog of the *Drosophila* NELFA gene (Wright et al., 1999; Wu et al., 2005), which complexes with other Nelf proteins and can repress transcription by pausing RNA Polymerase II elongation (Wu et al., 2005). In *Drosophila* and mammals, it has been reported to be expressed ubiquitously (Wright et al.,

1999; Yamaguchi et al., 1999; Mariotti et al., 2000). NELFA is involved in the regulation of immediate early expression of JunB in HepG2 cells (Aida et al., 2006).

1.7. Genes with heterogeneous expression patterns (mosaic-in-colony pattern)

Zfp42/Rex1 has been identified as a marker for undifferentiated EC cells (Hosler et al., 1989) and often called a pluripotency marker gene. The expression of *Zfp42* is much lower in ES and embryonic germ (EG) cells derived from the C57BL/6 mouse strain than in those derived from the 129 mouse strain (Sharova et al., 2007). The mosaic expression pattern of *Zfp42* has also been shown recently (Yayoi Toyooka and Hitoshi Niwa, personal communication).

RE1-silencing transcription factor (*Rest*) negatively regulates many neuronal genes in stem and progenitor cells (Chong et al., 1995). The expression of this gene was not detected in either ICM or TE in blastocysts by whole mount *in situ* hybridization (Yoshikawa et al., 2006). It has been shown that REST binds to the promoter region of *Pou5f1* in ES cells (Boyer et al., 2005; Loh et al., 2006) and its expression is suppressed when *Pou5f1* was repressed in mouse ES cells (Matoba et al., 2006). Reduction of *Rest* transcripts by siRNA in mouse ES cells does not seem to affect the undifferentiated state of ES cells (Loh et al., 2006).

Activating transcription factor 4 (*Atf4*) is a member of ATF/CREB (activating transcription factor/cyclic AMP response element binding protein) family of basic region-leucine zipper (bZip) transcription factors (Ameri and Harris, 2007). *Atf4* is induced by oxidative and other stresses and known to be involved in multiple processes, including hematopoiesis, lens and skeletal development, and fertility (Ameri and Harris, 2007).

E2F transcription factor 2 (*E2f2*) is one of the eight E2F family member genes, *E2f1-E2f8* (DeGregori and Johnson, 2006). E2Fs have diverse target genes and functions, including cell cycle regulation, apoptosis, and development (DeGregori and Johnson, 2006).

Nanog was originally isolated as a gene whose overexpression maintains the undifferentiated state of ES cells in the absence of LIF and has been used as a marker for pluripotency (Chambers et al., 2003; Mitsui et al., 2003). Heterogeneous expression of Nanog in mouse ES cells has been shown recently (Singh et al., 2007).

Dppa3/Pgc7/Stella was originally isolated as a marker for early germ cell differentiation (Saitou et al., 2002; Sato et al., 2002; Bortvin et al., 2003). Using a GFP marker under the regulation of the *Stella* promoter, it has been shown that GFP was expressed in a heterogeneous manner, i.e., a “salt-pepper” fashion (Payer et al., 2006).

Estrogen related receptor beta (*Esrrb*) plays an important role in the development of primordial germ cells (PGCs) (Mitsunaga et al., 2004). Recently it has been shown that *Esrrb* is one of the pluripotency-related genes (Ivanova et al., 2006; Loh et al., 2006).

Fascin homolog 1, actin bundling protein (Fscn1) has been shown to be expressed in neural and mesenchymal derivative cells (De Arcangelis et al., 2004).

1.8. Genes with homogeneous expression patterns within ES cell colonies

These genes will provide useful markers for undifferentiated ES cells. It will be important to study how these gene expression patterns change during the differentiation of ES cell cultures.

2. Experimental Procedures

2.1. Candidate gene/clone list assembly

Gene expression microarray data from previously published studies (Carter et al., 2005; Aiba et al., 2006) was analyzed using the NIA Microarray Analysis Tool (Sharov et al., 2005b) to generate lists of genes enriched in ES cell cultures grown in non-differentiating conditions. Official gene symbols and NIA Mouse Gene Index 5.0 (NMGI5) identifiers (Sharov et al., 2005a) were assigned to each gene, and NMGI5 locus and transcript models were used to select cDNA clones to be used as probe templates. Preference was given to NIA cDNA clones covering 3'UTRs, with an insert length ≤ 1 kb. In cases where suitable NIA cDNA clones were not available for a given gene, clones were selected and ordered from Open Biosystems Incorporated (OBI).

2.2. RNA probe preparation

cDNA clones were arrayed and cultured in 96-well microtiter plates. Inserts were PCR amplified directly from 2 μl bacterial culture in 100 μl PCR reactions (5 units RedTaq DNA polymerase in 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.1 mM $MgCl_2$; 0.01% gelatin; 200 μM each dNTP; 0.2 μM each primer). Reactions were cycled as follows: 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 47 °C for 30 seconds, extension at 72 °C for 4 minutes, followed by the final extension at 72 °C for 10 minutes. NIA cDNA clones (in pSPORT-based backbones) were amplified using M13 forward (-20) (5'-GTAAAACGACGGCCAGT-3') and M13 reverse (5'-GGAAACAGCTATGACCATG-3') primers. OBI cDNA clones in backbones without M13 forward and reverse sites were amplified using T7 (5'-GTAATACGACTCACTATAGGGC-3') and T3 (5'-AATTAACCCTCACTAAAGGG-3') primers. PCR products were purified using a QIAquick PCR purification kit (Qiagen), eluted in 100 μl of buffer, and quantitated using a ND1000 spectrophotometer (NanoDrop Technologies Inc.). Digoxigenin-labeled RNA probes were transcribed from the PCR product templates using DIG RNA Labeling Mix (Roche) and the appropriate RNA polymerase. Ethanol-precipitated probes were re-suspended in water and quantitated by agarose gel electrophoresis or by running an RNA 6000 Nano Assay on a 2100 Bioanalyzer (Agilent Technologies).

2.3. Cell culture

ES cells (line 129.3, derived from 129Sv/J strain mice) were cultured at 37°C in 5% CO_2 without feeders on gelatin-coated 12-well plastic plates using standard ES cell culture medium (DMEM, 15%FBS, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 2 mM glutamate, 0.1 mM β-mercaptoethanol, 50U/ml penicillin, 50 μg/ml streptomycin,1,000 U/ml ESGRO LIF). Cells were seeded at 2×10^4 cells/well and cultured for 3 days before ISH processing.

2.4. In situ hybridization

Cells were fixed in 4% PFA/PBS at 4°C overnight. After digestion with proteinase K, cells were hybridized overnight with 10 μl Digoxigenin-labeled riboprobe at 62°C overnight. Cells were then washed, blocked, incubated with alkaline phosphatase-conjugated anti-Digoxigenin antibody, and incubated with NBT/BCIP detection buffer for 30 minutes. See supplemental data for full protocol details.

2.5. Cytospin Cell Preparation

Cells were cultured as described in the section 2.3. The cells were harvested with 1X accutase and centrifuged at 1000 rpm for 5 minutes, washed with PBS, and fixed overnight in 4% PFA in PBS at 4 °C. The cells were washed twice with PBS, counted, and resuspended in PBS at a

concentration of 1×10^6 cells/ml. Cells (100,000) were attached to coated microscope slides (Shandon, cat#5991056) in a Shandon, Cytospin3 centrifuge at 500 rpm (low acceleration) for 5 min and dried overnight on a slide warmer at 37 °C. ISH was performed on the slides as described in the section 2.4.

2.6. Image acquisition and processing

ISH preparations were imaged under bright-field optics on an inverted microscope with DIC/phase-contrast optics. Cells were photographed at 4x, 10x, and 20x magnification. TIFF images were imported into an Open Microscopy Environment (OME) (Goldberg et al., 2005) server and manually annotated. Images were divided into 5 categories based on apparent signal strength, and four categories based on the type of staining pattern observed (Table 1). All images are available at the Jackson Laboratory Gene Expression Database (GXD) (http://www.informatics.jax.org) and the public OME site (http://ome.grc.nia.nih.gov).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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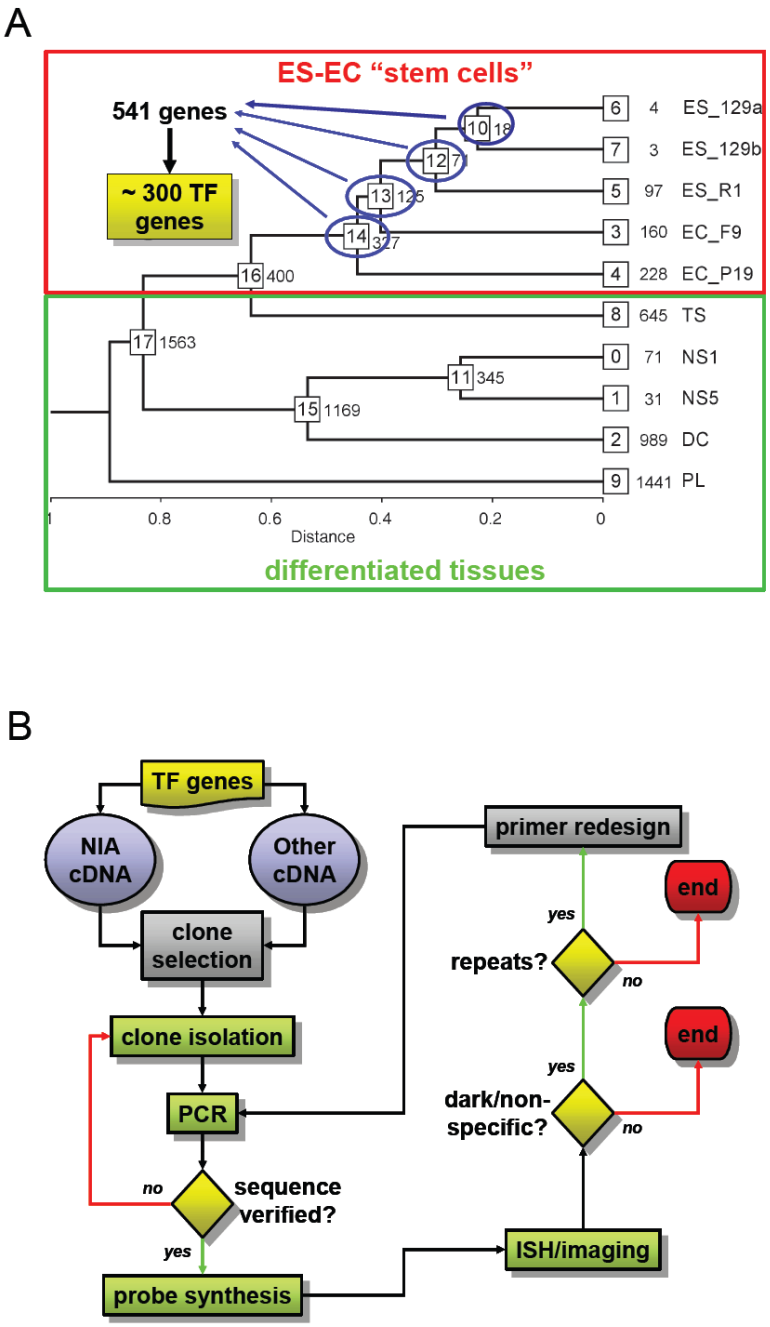
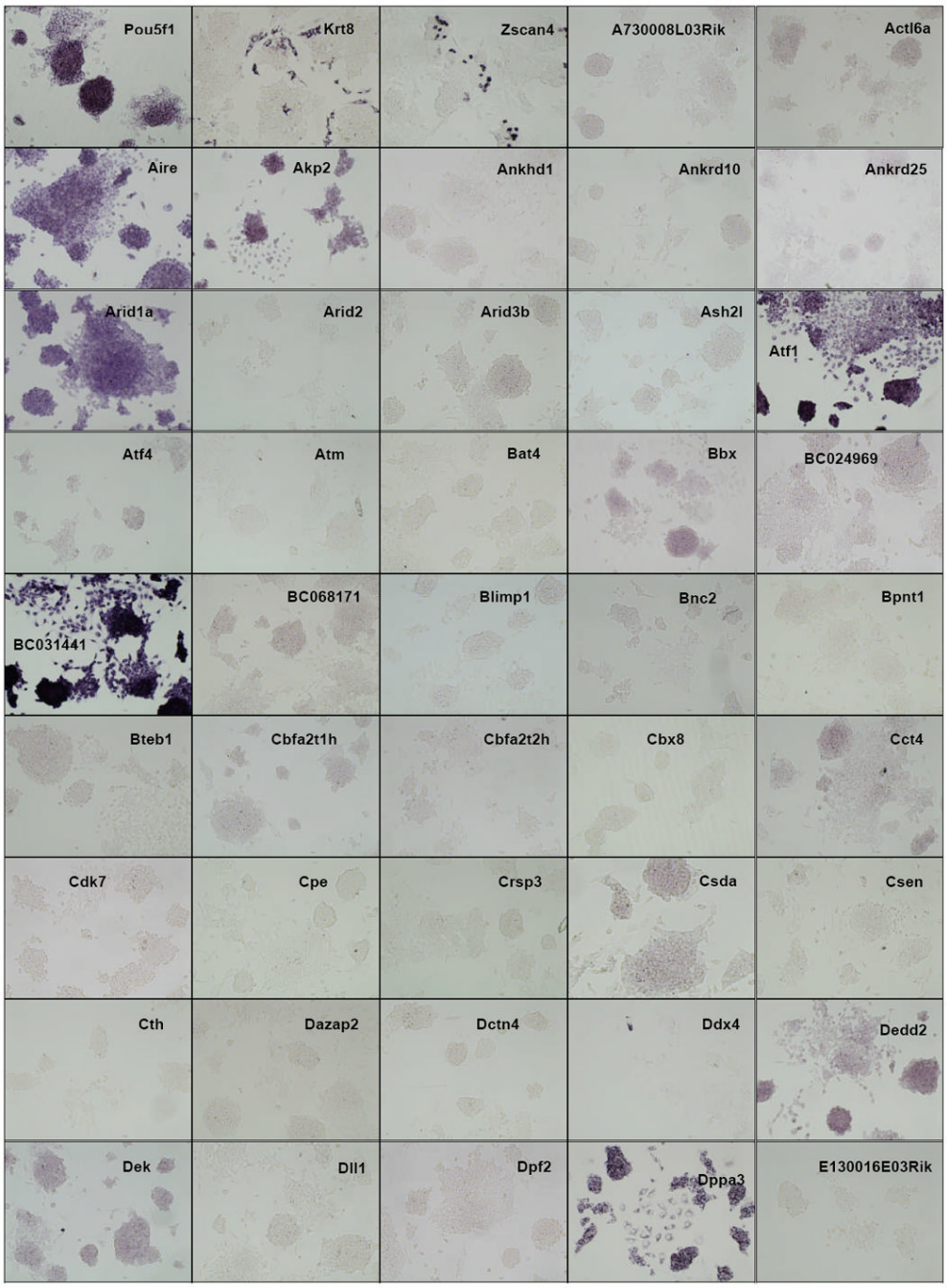


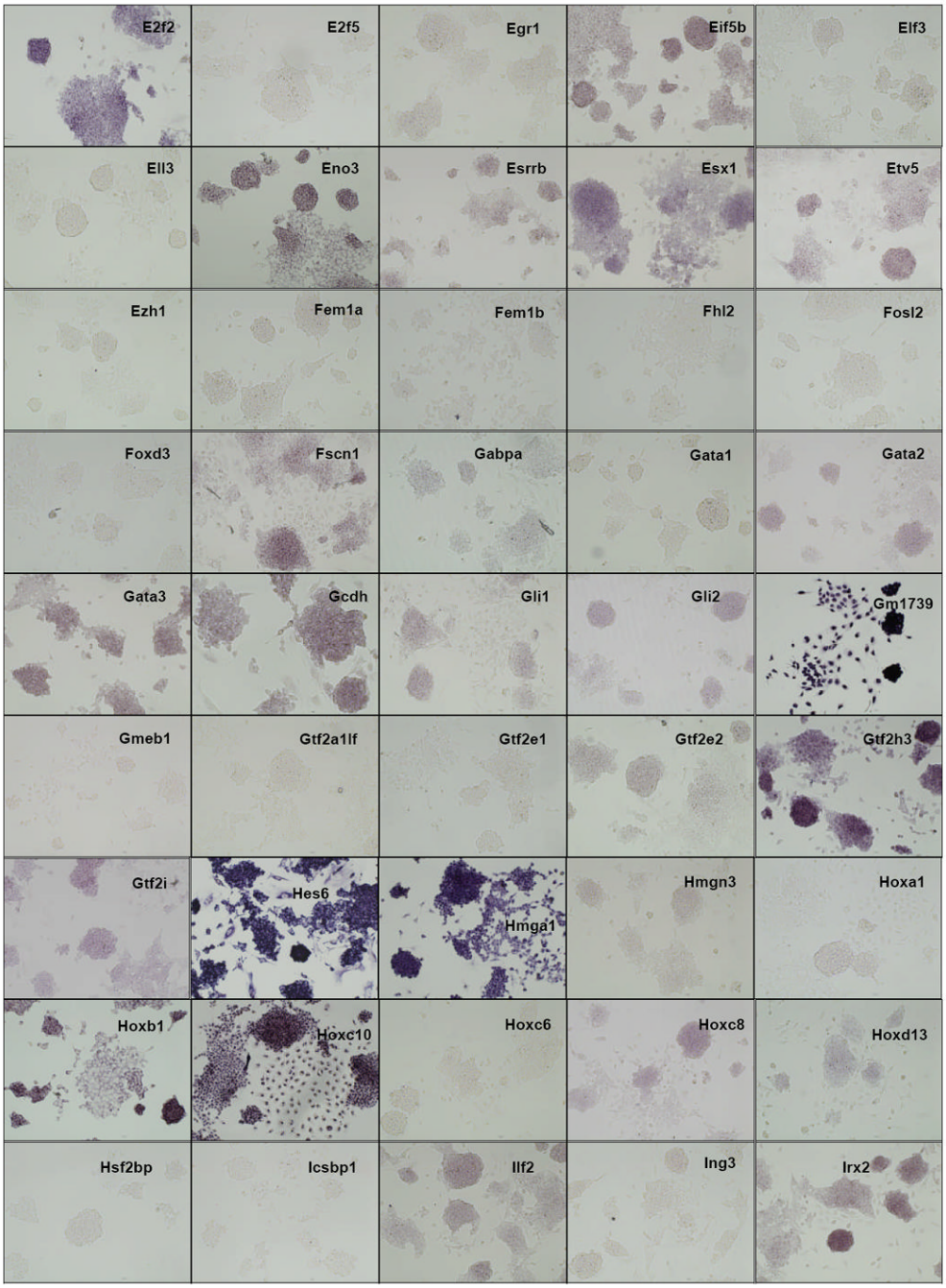
Figure 1. (A) Selection of ES/EC-enriched genes

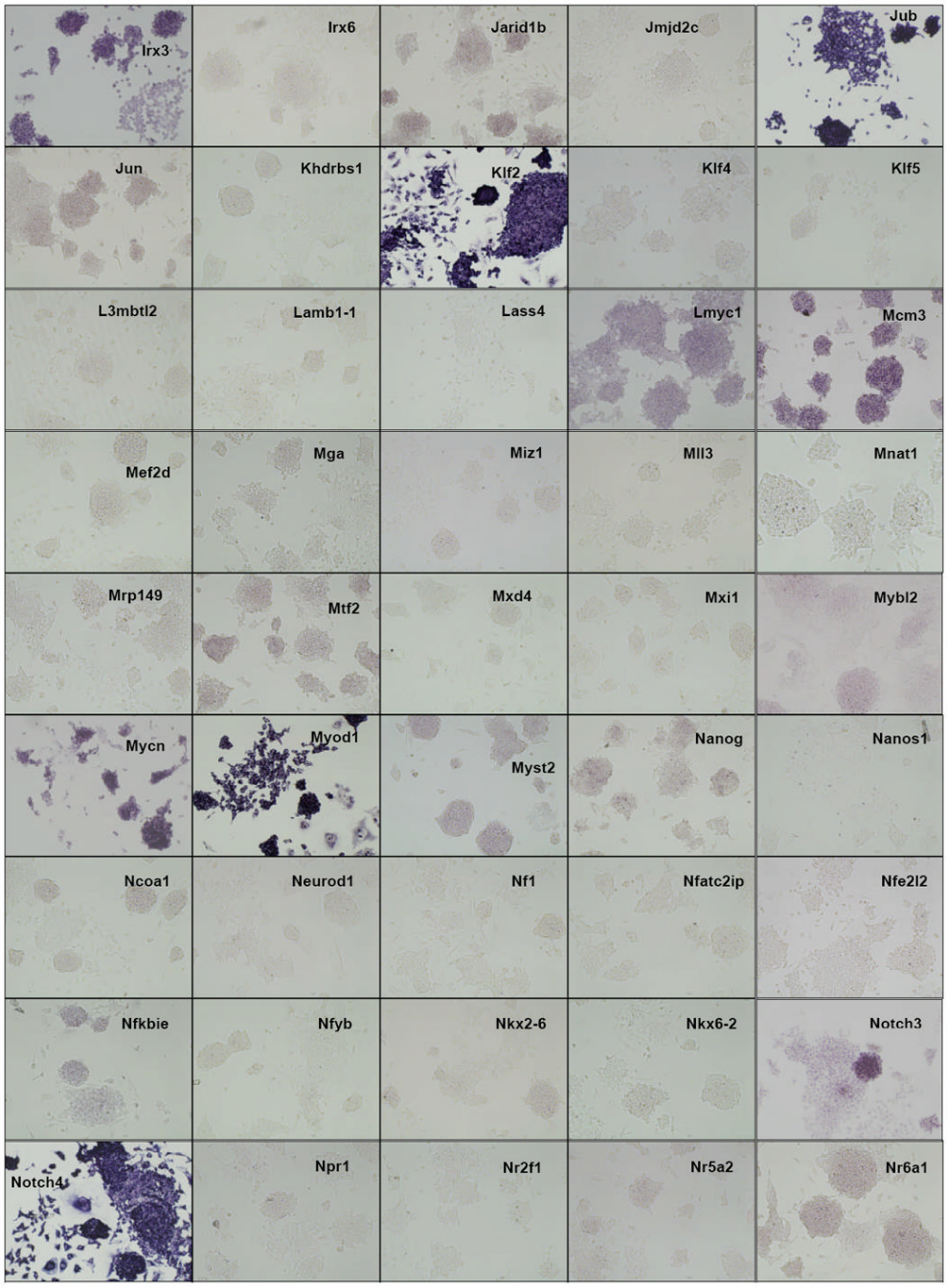
Using the NIA Microarray Analysis Tool (lgsun.grc.nia.nih.gov/ANOVA/), data were compiled from 30 two-channel fluorescent hybridizations of linearly amplified cRNA target mixtures on NIA/Agilent 22K v1.1 60-mer oligo arrays, representing 10 separate tissues and cell cultures in triplicate against a common universal reference. Nodes 12, 13, and 14 on the hierarchical clustering tree above represent transcripts which are enriched in ES cells only, ES cells and F9 EC cells, and ES and EC cells, respectively, relative to TS and differentiated tissues. Of these 541 gene transcripts, approximately 300 encode known or putative transcription factors, DNA binding proteins, or contain motifs associated with DNA-binding and/or transcription-regulating activity. (ES_129a, ES_129b = 129Sv-derived ES cells cultured

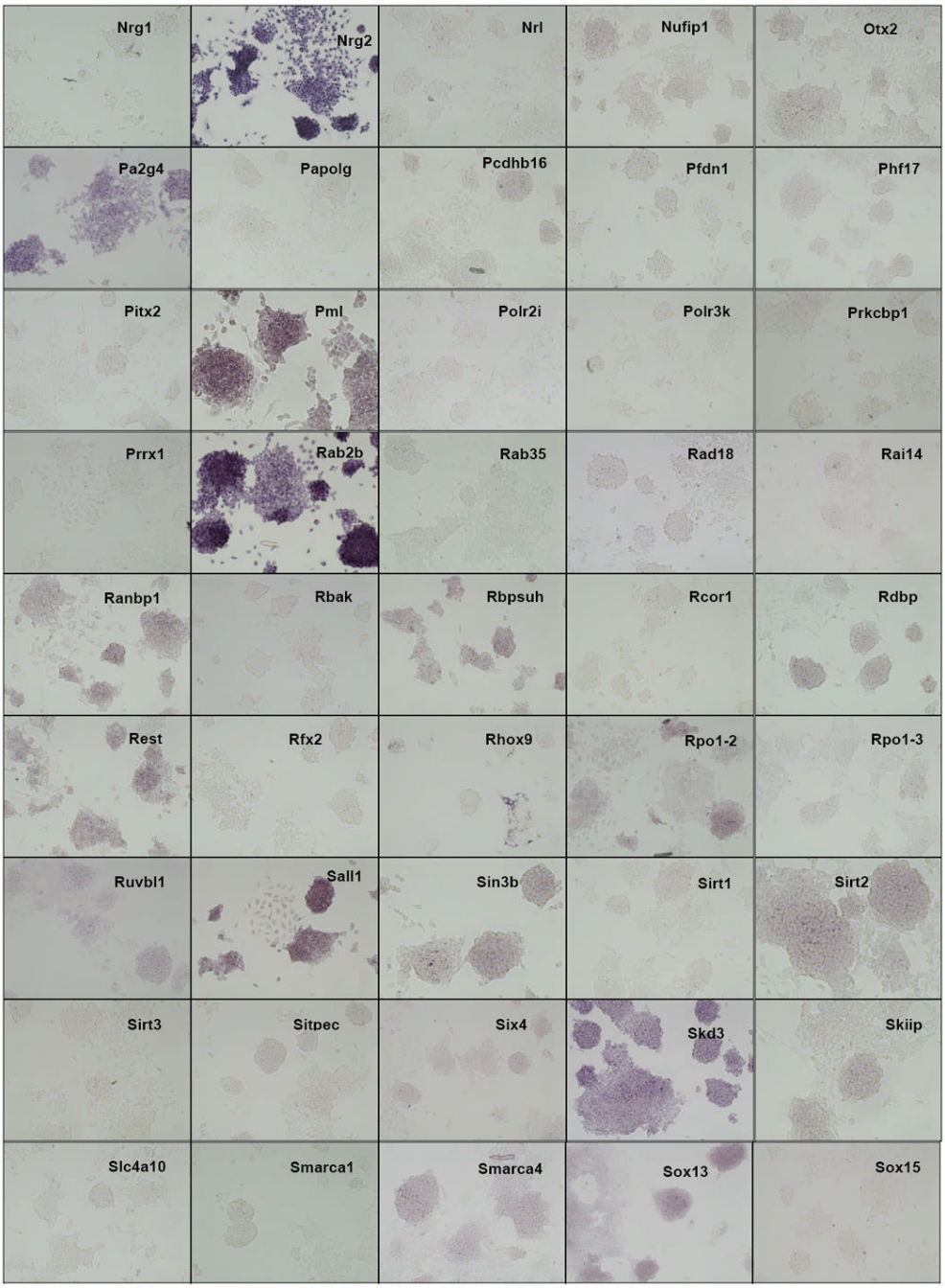
in two different conditions as described (Aiba et al., 2006); ES_R1 = R1 ES cell line; EC_F9, EC_P19 = F9 and P19 embryonic carcinoma cells; TS = trophoblast stem cells; NS1, NS5 = neural stem/progenitor cells cultured for 1 and 5 days; DC = differentiated neural stem cells; PL = E12.5 whole placenta.

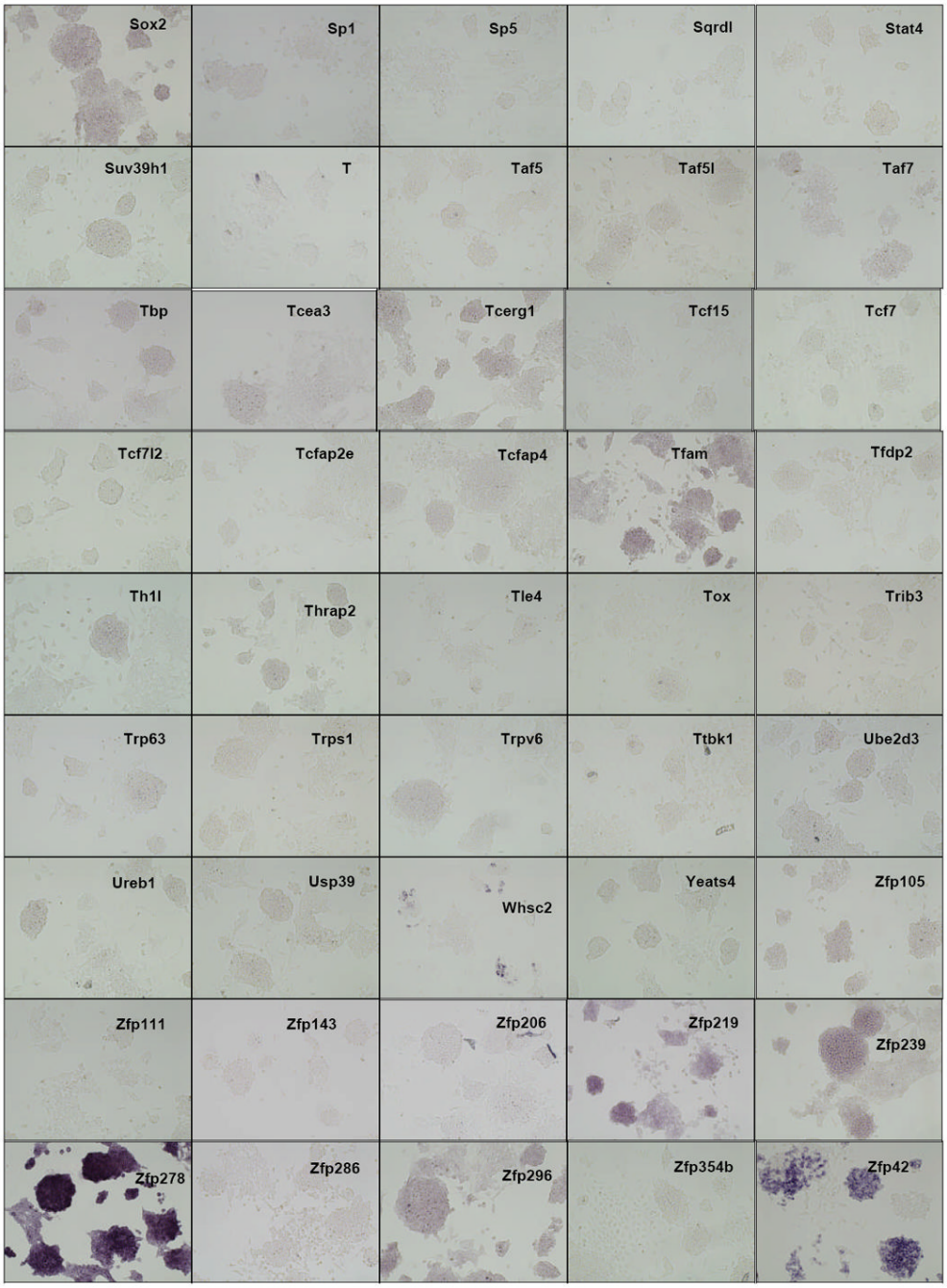
(B) ES cell culture *in situ* hybridization screening workflow. Clones were identified in NIA cDNA libraries representing genes from our list of approximately 300 known or putative transcription factors. For genes not represented, clones were obtained from Open Biosystems. All clones were single-colony purified, PCR amplified with M13 forward and reverse primers, and PCR products were sequenced for verification. Digoxigenin-labeled antisense RNA probes were synthesized from PCR products and hybridized to fixed ES cells grown on gelatin-coated plastic in LIF-supplemented medium for 3 days. Probes producing extremely dark and/or non-specific signals were examined for repeat element sequences, and gene-specific primers were designed to amplify repeat-free probe templates, which were used for subsequent hybridizations.











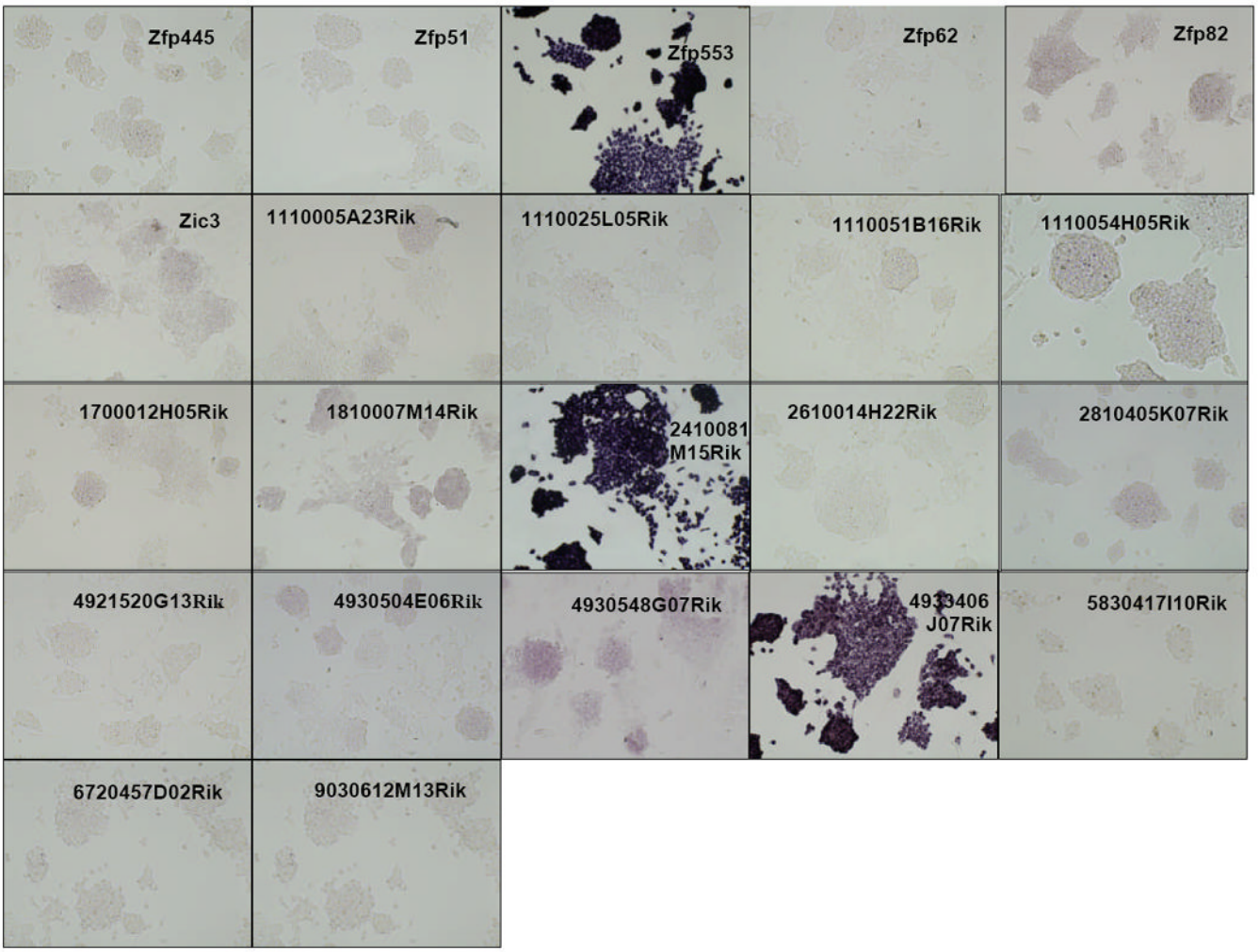
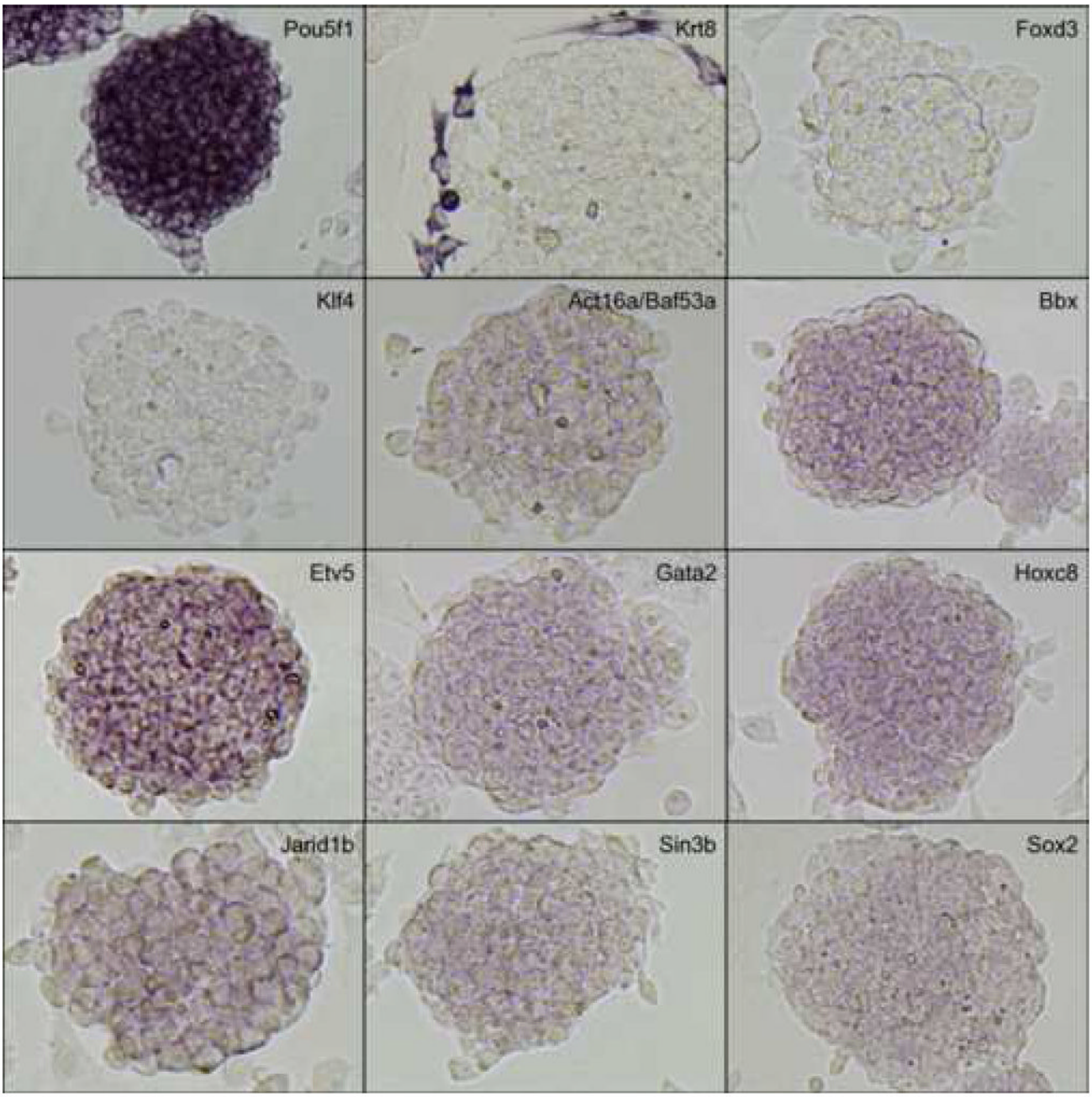


Figure 2. ES cell in situ hybridization results

Distribution of gene expression intensity and pattern in ES cell cultures by *in situ* hybridization In situ hybridized ES cells were photographed under standardized conditions, and images from 252 different clones which were successfully isolated, sequence verified, PCR-amplified, transcribed into DIG-labeled probes, and hybridized were classified according to expression intensity and pattern type (Table 1). High-resolution images are available at the public OME site (http://ome.grc.nia.nih.gov).



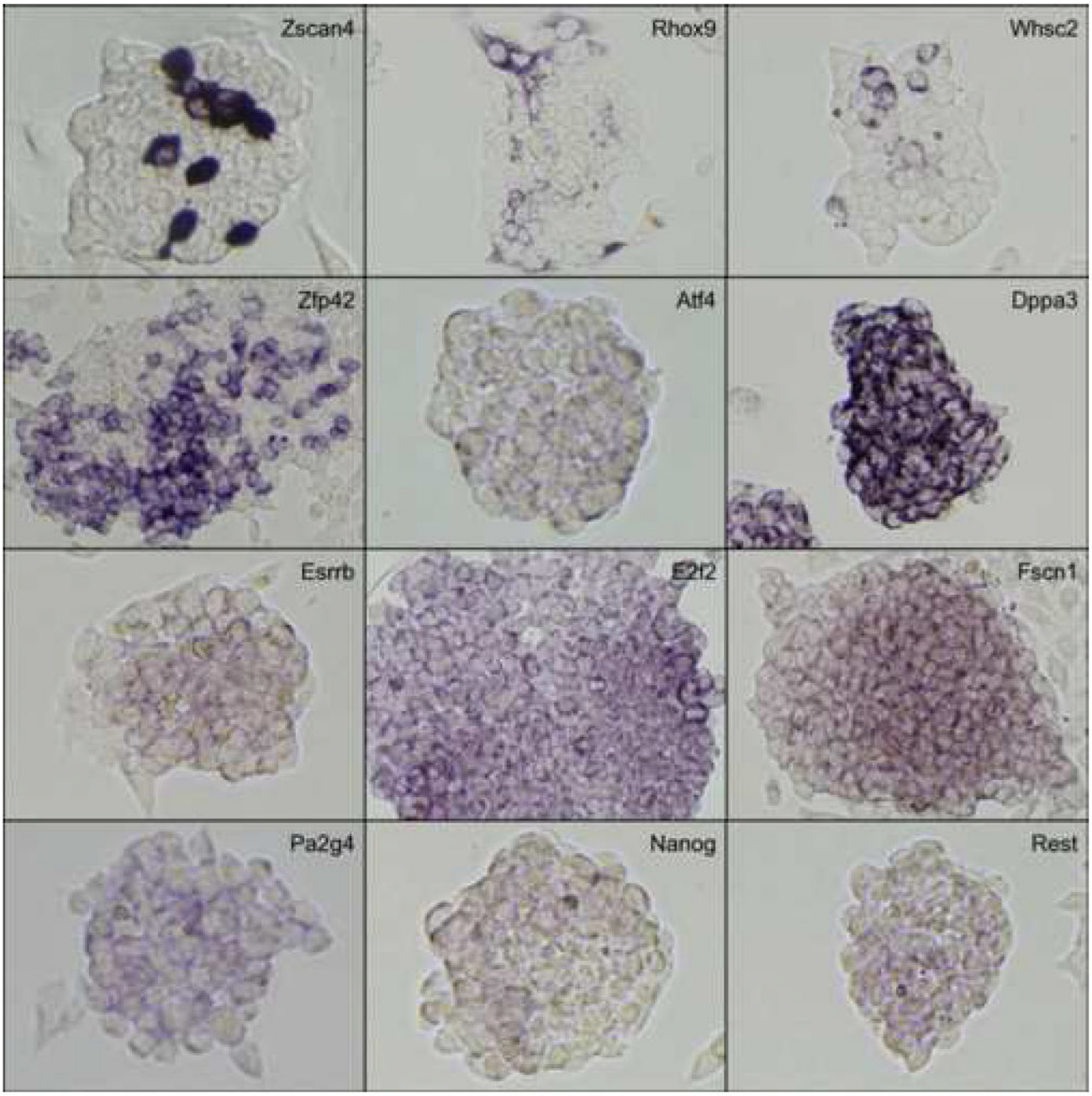


Figure 3. Magnified view of representative genes

In situ hybridization results of representative genes are selected from Fig. 2 and magnified to show the detailed staining patterns. See the text for the interpretation of results.

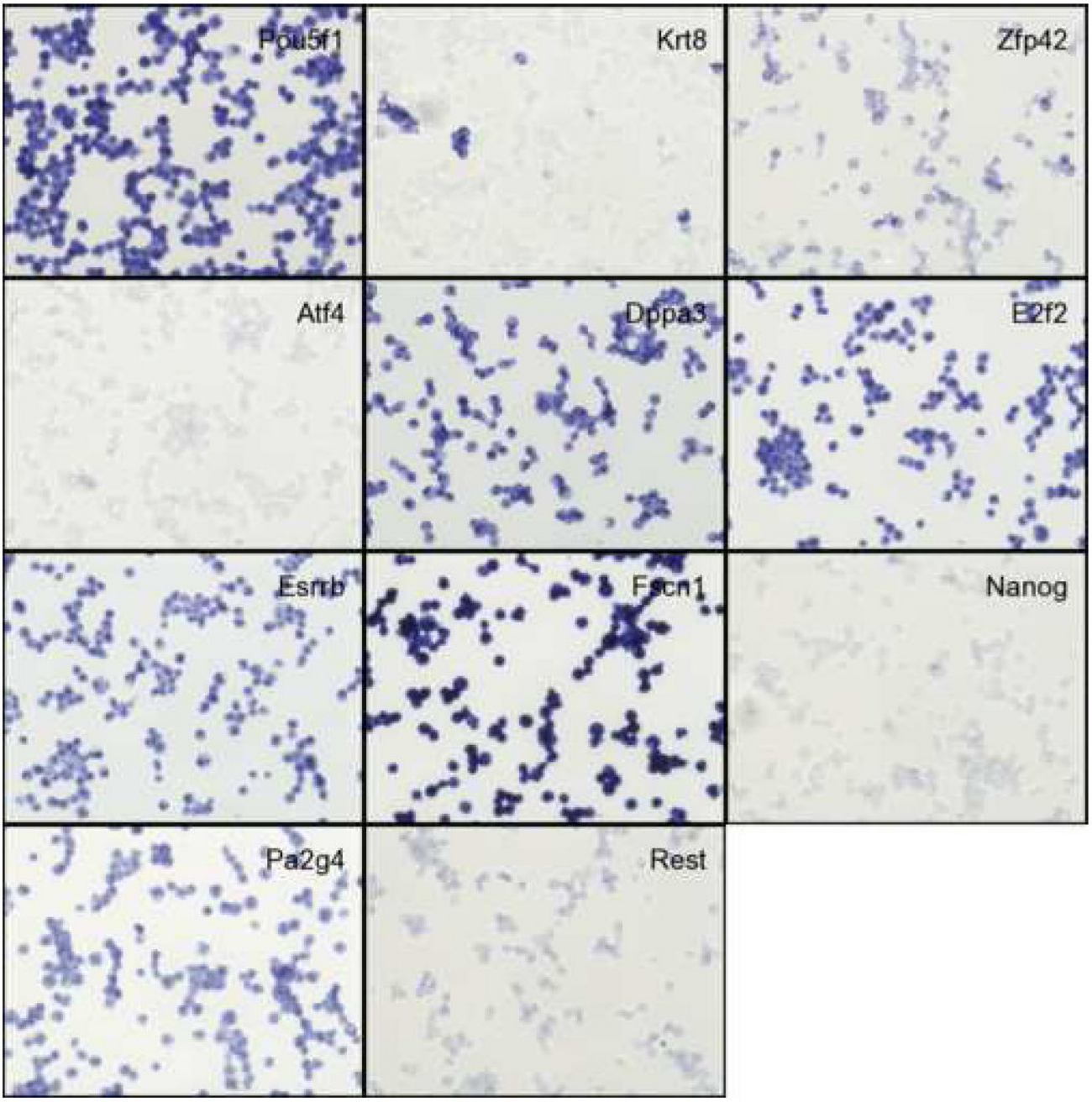


Figure 4. In situ hybridization results of CytoSpin-prepared ES cells

Table 1

A list of all the genes screened and the in situ results

Gene	TF?	cDNA clone IDs	Signal Intensity	Expression Pattern in ES colonies
Pou5f1 (Oct3/4, Oct4)	Yes	**H3028H01**	strong	Homogeneous
Krt8 (EndoA)	No	**H3031C01**	strong	Peripheral
Zscan4 (Gm397)	Yes	**C0407B02**	strong	Spot-in-Colony
A730008L03Rik (Nelfb)	Yes	**K0109B03**	v faint	Homogeneous
Actl6a (Baf53a)	Yes	**H3080C12**	faint	Homogeneous
Aire	Yes	**EMM1002-7515871**	strong	Homogeneous
Akp2	Yes	**C0257F12**	med	Homogeneous
Ankhd1	Yes	**K0400H03**	v faint	Homogeneous
Ankrd10	Yes	**K0122H01**	v faint	Homogeneous
Ankrd25	Yes	**H3123C11**	v faint	Homogeneous
Arid1a (Baf250a)	Yes	**H3023A03**	strong	Homogeneous
Arid2	Yes	**K0917G07**	n/d	n/d
Arid3b	Yes	**C0326H11**	v faint	Homogeneous
Ash2l	Yes	**H3147F02**	v faint	Homogeneous
Atf1	Yes	**H4067F10**	v strong	Homogeneous
Atf4	Yes	**H3124E06**	faint	Mosaic-in-Colony
Atm	Yes	**H8200G06**	n/d	n/d
Bat4	Yes	**H3059D08**	n/d	n/d
Bbx	Yes	**H3026G07**	faint	Homogeneous
BC024969 (Adnp2)	Yes	**C0866H11**	v faint	Homogeneous
BC031441	Yes	**H8230G02**	v strong	Homogeneous
BC068171 (Dkc1)	No	**H3066D04**	v faint	Homogeneous
Blimp1 (Prdm1)	Yes	**EMM1032-597986**	n/d	n/d
Bnc2	Yes	**H3065E10**	v faint	Homogeneous
Bpnt1	Yes	**H3057C10**	n/d	n/d
Bteb1 (Klf9)	Yes	**H3027A04**	v faint	Homogeneous
Cbfa2t1h (Runx1t1)	Yes	**H3125F02**	v faint	Homogeneous
Cbfa2t2h (Cbfa2t2)	Yes	**H3091A05**	v faint	Homogeneous
Cbx8	Yes	**H4002D08**	n/d	n/d
Cct4	No	**H3027F12**	faint	Homogeneous
Cdk7	Yes	**H3002H05**	n/d	n/d
Cpe	No	**H4028H11**	n/d	n/d
Crsp3 (Med23)	No	**H4006A08**	n/d	n/d
Csda	Yes	**C0202D02**	faint	Homogeneous
Csen (Kcnip3)	Yes	**K0906H01**	n/d	n/d
Cth	No	**H3103D05**	n/d	n/d
Dazap2	Yes	**A0813H07**	n/d	n/d
Dctn4	No	**H4070C12**	n/d	n/d
Ddx4 (Mvh)	No	**H3134G04**	n/d	n/d
Dedd2	Yes	**H4061C06**	med	Homogeneous
Dek	Yes	**H3138A12**	faint	Homogeneous
Dll1	No	**MMM1013-9202259**	n/d	n/d
Dpf2 (Req)	Yes	**C0818B07**	n/d	n/d
Dppa3 (Stella, Pgc7)	No	**L0289B08**	strong	Mosaic-in-Colony
E130016E03Rik (Rad54b)	Yes	**H3116F06**	n/d	n/d
E2f2	Yes	**EMM1002-7498698**	med	Mosaic-in-Colony
E2f5	Yes	**H3072H12**	n/d	n/d
Egr1 (Krox24)	Yes	**B0267A11**	v faint	Homogeneous
Eif5b	No	**H3053D03**	med	Homogeneous
Elf3	Yes	**H3015C12**	v faint	Homogeneous
Ell3	Yes	**E0754G01**	n/d	n/d
Eno3	No	**H4027G12**	strong	Homogeneous
Esrrb	Yes	**H4053E01**	med	Mosaic-in-Colony
Esx1	Yes	**EMM1002-6760787**	med	Homogeneous
Etv5	Yes	**C0282F03**	med	Homogeneous
Ezh1	Yes	**H3154F11**	n/d	n/d
Fem1a	Yes	**C0407G11**	v faint	Homogeneous
Fem1b	Yes	**H3029F04**	n/d	n/d
Fhl2	Yes	**H3033C07**	n/d	n/d
Fosl2	Yes	**H3110A12**	n/d	n/d
Foxd3 (Genesis)	Yes	**EMM1002-1111636**	n/d	n/d
Fscn1	No	**H3006D08**	strong	Mosaic-in-Colony
Gabpa	Yes	**C0260F10**	v faint	Homogeneous
Gata1	Yes	**H3038A09**	n/d	n/d
Gata2	Yes	**H3009F04**	faint	Homogeneous
Gata3	Yes	**H3049E04**	med	Homogeneous
Gcdh	Yes	**C0328C11**	med	Homogeneous

Gene	TF?	cDNA clone IDs	Signal Intensity	Expression Pattern in ES colonies
Gli1	Yes	A0243G01	faint	Homogeneous
Gli2	Yes	K0239G06	faint	Homogeneous
Gm1739	No	H3128A09	v strong	Homogeneous
Gmeb1	Yes	K0502H03	n/d	n/d
Gtf2a1lf	Yes	EMM1002-4195952	n/d	n/d
Gtf2e1	Yes	H3105C05	n/d	n/d
Gtf2e2	Yes	H3144B01	faint	Homogeneous
Gtf2h3	Yes	H8201G03	strong	Homogeneous
Gtf2i	Yes	H3140H12	med	Homogeneous
Hes6	Yes	MMM101365220	v strong	Homogeneous
Hmga1	Yes	H3029B11	strong	Homogeneous
Hmgn3	Yes	B0224F03	v faint	Homogeneous
Hoxa1	Yes	EMM1032-608139	n/d	n/d
Hoxb1	Yes	H3031H08	strong	Homogeneous
Hoxc10	Yes	H3120B02	strong	Homogeneous
Hoxc6	Yes	EMM1002-25304	n/d	n/d
Hoxc8	Yes	H4041F10	med	Homogeneous
Hoxd13	Yes	EMM1002-6823925	faint	Homogeneous
Hsf2bp	Yes	H3125B05	n/d	n/d
Icsbp1 (Irf8)	Yes	H3072F09	n/d	n/d
Ilf2	Yes	H3029E05	med	Homogeneous
Ing3	Yes	H4072A09	n/d	n/d
Irx2	Yes	EMM1002-5626242	med	Homogeneous
Irx3	Yes	H3010C07	strong	Homogeneous
Irx6	Yes	EMM1002-1856631	v faint	Homogeneous
Jarid1b	Yes	H3041C04	faint	Homogeneous
Jmjd2c	Yes	H4053F10	n/d	n/d
Jub	No	H3024A03	v strong	Homogeneous
Jun	Yes	H3058C09	faint	Homogeneous
Khdrbs1	Yes	A0501F06	n/d	n/d
Klf2	Yes	EMM1002-7515994	v strong	Homogeneous
Klf4	Yes	H3015B01	n/d	n/d
Klf5	Yes	H3102C04	v faint	Homogeneous
L3mbtl2	Yes	A0354D01	v faint	Homogeneous
Lamb1-1 (Lamb1)	No	H3147C04	n/d	n/d
Lass4 (Trh1)	Yes	H3129C07	n/d	n/d
Lmyc1 (Mycl1)	Yes	H3024G07	med	Homogeneous
Mcm3	Yes	C0403F03	strong	Homogeneous
Mef2d	Yes	C0261E02	v faint	Homogeneous
Mga	Yes	K0110B04	v faint	Homogeneous
Miz1 (Pias2)	Yes	K0232F02	n/d	n/d
Mll3	Yes	H3057C11	n/d	n/d
Mnat1	Yes	C0025B02	n/d	n/d
Mrpl49	No	H3039A04	v faint	Homogeneous
Mtf2	Yes	H3048E07	faint	Homogeneous
Mxd4	Yes	H3131B07	v faint	Homogeneous
Mxi1	Yes	H4077G09	n/d	n/d
Mybl2 (Bmyb)	Yes	H3144E01	faint	Homogeneous
Mycn (Nmyc1)	Yes	H4041A05	v strong	Homogeneous
Myod1 (MyoD)	Yes	EMM1002-1452165	v strong	Homogeneous
Myst2	Yes	H3107B07	faint	Homogeneous
Nanog	Yes	H3050A07	faint	Mosaic-in-Colony
Nanos1	No	K0409A10	n/d	n/d
Ncoa1	Yes	A0400A03	v faint	Homogeneous
Neurod1	Yes	H4035E12	n/d	n/d
Nf1	No	H4070B01	n/d	n/d
Nfatc2ip	Yes	H3046E09	n/d	n/d
Nfe2l2	Yes	C0819F04	n/d	n/d
Nfkbie	Yes	MMM1013-7512133	faint	Homogeneous
Nfyb	Yes	H3146B03	n/d	n/d
Nkx2-6	Yes	EMM1002-1148995	v faint	Homogeneous
Nkx6-2	Yes	EMM1002-21216	n/d	n/d
Notch3	Yes	H4028C12	strong	Homogeneous
Notch4	Yes	EMM1002-6752438	v strong	Homogeneous
Npr1	No	L0927H03	v faint	Homogeneous
Nr2f1 (COUPTF1)	Yes	H3096D07	n/d	n/d
Nr5a2 (LRH1)	Yes	H3087G01	v faint	Homogeneous
Nr6a1 (Gcnf)	Yes	H3026F12	faint	Homogeneous
Nrg1	Yes	EMM1032-582702	n/d	n/d

Gene	TF?	cDNA clone IDs	Signal Intensity	Expression Pattern in ES colonies
Nrg2/LOC381149	No	**EMM1002-5539961**	v strong	Homogeneous
Nrl	Yes	**EMM1002-4982597**	n/d	n/d
Nufip1	Yes	**H3009B11**	v faint	Homogeneous
Otx2	Yes	**H3030H12**	v faint	Homogeneous
Pa2g4	Yes	**H3024B11**	med	Mosaic-in-Colony
Papolg	Yes	**J0235C01**	n/d	n/d
Pcdhb16	Yes	**K0997C06**	v faint	Homogeneous
Pfdn1	Yes	**H3157C01**	n/d	n/d
Phf17	Yes	**H3127B05**	v faint	Homogeneous
Pitx2	Yes	**E0473B09**	n/d	n/d
Pml	Yes	**C0208F04**	med	Homogeneous
Polr2i	Yes	**H3089E01**	n/d	n/d
Polr3k	Yes	**H3083H08**	n/d	n/d
Prkcbp1	Yes	**H3027G06**	n/d	n/d
Prrx1	Yes	**K0403B09**	n/d	n/d
Rab2b	Yes	**A0363E07**	v strong	Homogeneous
Rab35	Yes	**H3136E02**	n/d	n/d
Rad18	Yes	**C0407D04**	n/d	n/d
Rai14	Yes	**K0417A05**	n/d	n/d
Ranbp1	No	**H3104D06**	faint	Homogeneous
Rbak	Yes	**H3091E03**	n/d	n/d
Rbpsuh (Rbpj)	Yes	**H4069C09**	faint	Homogeneous
Rcor1	Yes	**H4030A11**	n/d	n/d
Rdbp	No	**H3025E10**	faint	Homogeneous
Rest	Yes	**H3153C03**	med	Mosaic-in-Colony
Rfx2	Yes	**H8188D01**	n/d	n/d
Rhox9 (Psx2)	Yes	**L0042E08**	faint	Spot-in-Colony
Rpo1-2	Yes	**H8164D08**	med	Homogeneous
Rpo1-3	Yes	**H3121B06**	v faint	Homogeneous
Ruvbl1	Yes	**H3016C12**	v faint	Homogeneous
Sall1	Yes	**H4055D01**	med	Homogeneous
Sin3b	Yes	**C0281D12**	faint	Homogeneous
Sirt1	Yes	**H3119B12**	v faint	Homogeneous
Sirt2	Yes	**C0285H01**	faint	Homogeneous
Sirt3	Yes	**A0352G08**	n/d	n/d
Sitpec (Ecsit)	Yes	**H4049D06**	v faint	Homogeneous
Six4	Yes	**H4056D02**	v faint	Homogeneous
Skd3 (Clpb)	Yes	**C0341A10**	med	Homogeneous
Skiip (Snw1)	Yes	**C0218H03**	v faint	Homogeneous
Slc4a10	Yes	**H4035E11**	n/d	n/d
Smarca1 (Snf2l)	Yes	**H4004G06**	n/d	n/d
Smarca4 (Brg1)	Yes	**C0402B01**	faint	Homogeneous
Sox13	Yes	**L0928B04**	med	Homogeneous
Sox15	Yes	**H3081C12**	n/d	n/d
Sox2	Yes	**C0403H11**	faint	Homogeneous
Sp1	Yes	**H3016H10**	n/d	n/d
Sp5	Yes	**EMM1002-5883694**	n/d	n/d
Sqrdl	No	**H3122H06**	n/d	n/d
Stat4	Yes	**H4077D08**	n/d	n/d
Suv39h1	Yes	**H3141G04**	v faint	Homogeneous
T (Brachyury)	Yes	**H3029B05**	n/d	n/d
Taf5	Yes	**K0278H01**	n/d	n/d
Taf5l	Yes	**B0410D11**	v faint	Homogeneous
Taf7	Yes	**H3071E08**	v faint	Homogeneous
Tbp	Yes	**K0240H01**	v faint	Homogeneous
Tcea3	Yes	**H4005B08**	faint	Homogeneous
Tcerg1	Yes	**H3128B05**	faint	Homogeneous
Tcf15	Yes	**EMM1002-1464550**	n/d	n/d
Tcf7	Yes	**H4003H11**	n/d	n/d
Tcf7l2	Yes	**H4028E05**	n/d	n/d
Tcfap2e	Yes	**H3100H07**	n/d	n/d
Tcfap4	Yes	**MMM1013-9200312**	v faint	Homogeneous
Tfam	Yes	**H8244A10**	med	Homogeneous
Tfdp2	Yes	**H4061E07**	v faint	Homogeneous
Th1l	Yes	**C0208E04**	faint	Homogeneous
Thrap2 (Med13l)	Yes	**H3137F02**	faint	Homogeneous
Tle4	Yes	**H3103G12**	n/d	n/d
Tox	Yes	**K0748A03**	n/d	n/d
Trib3	Yes	**H3035B01**	n/d	n/d

Gene	TF?	cDNA clone IDs	Signal Intensity	Expression Pattern in ES colonies
Trp63	Yes	**E0485F10**	v faint	Homogeneous
Trps1	Yes	**A0909F05**	n/d	n/d
Trpv6	Yes	**H8162C12**	v faint	Homogeneous
Ttbk1	No	**H3137A12**	n/d	n/d
Ube2d3	No	**H3082D12**	v faint	Homogeneous
Ureb1	No	**H3129D11**	v faint	Homogeneous
Usp39	No	**H3005G05**	v faint	Homogeneous
Whsc2 (NelfA)	Yes	**C0288C12**	med	Spot-in-Colony
Yeats4	Yes	**H4002F05**	v faint	Homogeneous
Zfp105	No	**H3154D12**	v faint	Homogeneous
Zfp111	Yes	**K0957E07**	n/d	n/d
Zfp143	Yes	**L0949H07**	n/d	n/d
Zfp206 (Zscan10)	Yes	**H4040D10**	n/d	n/d
Zfp219	No	**H3014D06**	med	Homogeneous
Zfp239	Yes	**H4060F01**	med	Homogeneous
Zfp278 (Patz1)	Yes	**K0852A06**	v strong	Homogeneous
Zfp286	Yes	**C0919G04**	n/d	n/d
Zfp296	No	**H3086D06**	med	Homogeneous
Zfp354b	Yes	**EMM1032-6904991**	n/d	n/d
Zfp42 (Rex1)	Yes	**H3036F04**	strong	Mosaic-in-Colony
Zfp445	Yes	**K0508E08**	v faint	Homogeneous
Zfp51	Yes	**H3122C02**	n/d	n/d
Zfp553	Yes	**C0405D09**	v strong	Homogeneous
Zfp62	Yes	**B0129B06**	n/d	n/d
Zfp82	Yes	**H4054E06**	faint	Homogeneous
Zic3	Yes	**B0716B04**	faint	Homogeneous
1110005A23Rik	Yes	**H3148B10**	v faint	Homogeneous
1110025L05Rik (Bola2)	Yes	**H3085B11**	v faint	Homogeneous
1110051B16Rik	Yes	**E0702B01**	n/d	n/d
1110054H05Rik (Foxk2)	Yes	**C0202E10**	faint	Homogeneous
1700012H05Rik (Rbmxl2)	No	**EMM1002-7533455**	v faint	Homogeneous
1810007M14Rik	Yes	**H4079B04**	faint	Homogeneous
2410081M15Rik	No	**H3093G04**	v strong	Homogeneous
2610014H22Rik (Wbp7)	Yes	**H3159D08**	n/d	n/d
2810405K07Rik (Zfp661)	Yes	**K0914F08**	faint	Homogeneous
4921520G13Rik	Yes	**MMM1013-9335122**	n/d	n/d
4930504E06Rik	No	**K0251E12**	v faint	Homogeneous
4930548G07Rik	Yes	**L0924F06**	med	Homogeneous
4933406J07Rik (Syce1)	No	**H4051E05**	v strong	Homogeneous
5830417I10Rik	Yes	**H3038H08**	n/d	n/d
6720457D02Rik	Yes	**B0142C08**	n/d	n/d
9030612M13Rik	Yes	**H3010E12**	n/d	n/d