Colloquium

Genetic and functional differences between multipotent neural and pluripotent embryonic stem cells

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Stem cells (SCs) are functionally defined by their abilities to self-renew and generate differentiated cells. Although much effort has been focused on defining the common characteristics among various types of SCs, the genetic and functional differences between multipotent and pluripotent SCs have garnered less attention. We report a direct genetic and functional comparison of molecularly defined and clonally related populations of neural SCs (NSCs) and embryonic SCs (ESCs), using the Sox2 promoter for isolation of purified populations by fluorescence-activated cell sorting. A stringent expression profile comparison of promoter-defined NSCs and ESCs revealed a striking dissimilarity, and subsequent chimera analyses confirmed the fundamental differences in cellular potency between these populations. This direct comparison elucidates the molecular basis for the functional differences in pluripotent ESCs and multipotent NSCs.

Materials and Methods

Production of Transgenic Mice and Isolation of Cells. The Sox2 promoter fragment used in these studies consists of the 5.5-kb BglII fragment immediately 5’ to the Sox2 gene. A pBluescript KS+ clone of this region was the kind gift of Angie Rizzino (18). Details regarding construction of the P/Sox2-GFP-Ires-Puro vector are available on request.

Transgenic ESC clones were derived from ROSA26-4 (19) ESCs as described (20) and were microinjected into blastocysts to verify ESC clones that exhibited telencephalic-restricted enhanced GFP (EGFP) expression.

Chimeric fetuses were generated via blastocyst injection of P/Sox2-GFP ESCs, collected at embryonic day (E) 14, and examined by epifluorescent microscopy to select fetuses exhibiting high degrees of chimera (≥75%). The telencephalon was dissected free of the meninges and remaining brain tissue, diced with a scalpel blade, and digested for 5 min at 37°C in 1 ml of 0.25% wt/vol trypsin/0.54 mM EDTA. Then, 3 ml of ovomucoid trypsin inhibitor (Sigma) at 0.7 mg/ml in DMEM/F12 (1:1) was added, and the tissue was partly dissociated by trituration with a fire-polished Pasteur pipette. The suspension was pelleted for 2 min at 500 × g. The cell pellet was resuspended and further dissociated with a fire-polished Pasteur pipette in PBS. The suspension was then filtered through a 35 μM cell strainer (Falcon) into a tube containing 0.001% vol/vol DNase I and 5 μg/ml propidium iodide for analysis by fluorescence-activated cell sorting (FACS).

Flow Cytometry. Sorting and analysis of fluorescent cells were performed on a FACSStar Plus cell sorter equipped with an argon laser (488 nm, 100 mW) and using CELLQUEST software (BD Biosciences). Cells were gated on forward and side scatter and dead cells were excluded by eliminating propidium iodide-positive events. Immediately after sorting, cells were pelleted for 3 min at 500 × g and resuspended in 200 μl of medium, and the density of viable cells was determined by using a hemocytometer. For isolation of total RNA, cells were sorted directly into RNeasy lysis buffer (Qiagen, Valencia, CA) at 4°C to preserve the integrity of the RNA. Cells were homogenized by passing lysate over a QIAshredder column (Qiagen), and total RNA was prepared according to the manufacturer’s protocol.

Real-Time Quantitative PCR (Q-PCR). P/Sox2-EGFP-positive cells were isolated by using FACS from neural stem/progenitor cultures derived from E14 chimeric fetuses after 5 days in vitro (DIV). Gates were set to collect nonoverlapping populations of negative, low, and high P/Sox2-EGFP fluorescence intensity.

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Abbreviations: SC, stem cell; EGFP, enhanced GFP; En, embryonic day n; ESC, embryonic SC; FACS, fluorescence-activated cell sorting; ICM, inner cell mass; NSC, neural stem cell; NSCf, cultured NSC; NSCc, freshly isolated NSC.

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Real-time quantitation was carried out as described (21). The Sox2 primer sequences were forward 5′-CACAGATGCAACCGATGCA and reverse 5′-GGTGCCCTGCTGCCGAGTA.

**Immunostaining.** Immunocytochemical analysis was performed as described (22).

**Cell Culture.** ESCs were cultured by using standard procedures. Neural stem/progenitor cells were cultured on untreated tissue culture plastic in serum-free media composed of DMEM/F12 (1:1) containing N2 or B27 serum supplements (GIBCO) and 2 mM L-glutamine. Mitogenic stimulation was in the presence of human recombinant EGF and FGF2 (20 ng/ml each; Peprotech, Rocky Hill, NJ) and heparin (5 μg/ml). Differentiation was carried out by plating 1 × 10^5 cells per cm² on glass chamber slides coated with laminin and poly-orithine. Differentiation media consisted of N2 supplemented with 0.5% FBS and 1 μM forskolin. Media were changed every other day, and differentiation proceeded for 6–8 days.

**Expression Profiling.** P/Sox2-EGFP-positive cells were isolated by using FACS from ESC cultures, E14 chimeric fetuses, or neural stem/progenitor cultures derived from E14 chimeric fetuses. Triplicate samples (5 μg total RNA) were processed for labeling, hybridization, and scanning as described (23), using Murine-U74Av2 high-density oligo arrays (Affymetrix, Santa Clara, CA). Initial quality control data analysis was performed with reference to Sandberg et al. (24), and determinations of differentially expressed genes were made by performing gene expression comparisons between each of the three P/Sox2-defined SC populations with three different analysis methods. Empirical [MICROARRAY SUITE 4.0 (Affymetrix) and BULLFROG queries] (24), statistical (Felix Naef algorithms) (25), and model-based (dCHIP 1.0) (26) analysis methods were used. These methods tend to complement each other because they each rely on different sets of assumptions. Using multiple methods generates a list of differentially expressed genes of extremely high confidence because of the requirement that the gene be detected as differential by multiple, independent data analysis methods. A detailed description of all the data analysis procedures is provided in Supporting Text, which is published as supporting information on the PNAS web site, www.pnas.org.

**Chimera Production Assay.** P/Sox2-EGFP-positive cells were isolated from E14 telencephalon or cultured neural stem/progenitor cells by using FACS and were grown overnight in B27 culture plastic in serum-free media composed of DMEM/mGlutamine. Mitogenic stimulation was in the presence of human recombinant EGF and FGF2 (20 ng/ml each; Peprotech, Rocky Hill, NJ) and heparin (5 μg/ml). Differentiation was carried out by plating 1 × 10^5 cells per cm² on glass chamber slides coated with laminin and poly-orithine. Differentiation media consisted of N2 supplemented with 0.5% FBS and 1 μM forskolin. Media were changed every other day, and differentiation proceeded for 6–8 days.

**Qualitative RT-PCR Confirmation.** Total RNA was isolated from P/Sox2-EGFP-expressing ESCs and NSCs as described for expression profiling. Next, 4.0 μg of total RNA for each cell type was DNase-treated (DNA-free, Ambion, Austin, TX) and divided in half for first-strand cDNA synthesis with or without reverse transcriptase by using the SuperScript II kit (Invitrogen). One-twentieth of the cDNA reaction was taken for PCR template and amplified for the following number of cycles: Oct4, 23 cycles ESC and 30 cycles NSC; Klf2, 40 cycles ESC and NSC; Nr5d1, 23 cycles ESC and 30 cycles NSC; Klf5, 40 cycles ESC and NSC; Crtr1, 40 cycles ESC and NSC; Klf4, 30 cycles ESC and NSC; Rlox1, 30 cycles ESC and NSC; Rex2, 23 cycles ESC and 30 cycles NSC; Foxg1, 50 cycles ESC and NSC; NrOb1, 40 cycles ESC and NSC; Zic1, 40 cycles ESC and NSC; Myt1, 23 cycles ESC and NSC. Gapdh and β-actin were amplified for 23 cycles in both ESC and NSC samples. The following primer sets were used:

- Oct4, forward 5’-GTGTTCAAGGACACCAGAC and reverse 5′-GAACATCTGGAACACACCTC; Klf2, forward 5’-CCCAGGAAAGAAGACAGGAG and reverse 5′-AAAACGAGGACCGGGCCAGA; NrOb1, forward 5’-TAGTAGGAGAAACCGGTCGTA and reverse 5′-GCGGTTTAATGGAAAGAGATG; Klf5, forward 5’-ATCTGAACACCGCGCGACAC and reverse 5’-GCTCACCAGTCGACGTG; Crtr1, forward 5’-CTCATCTTGCTGCTTG and reverse 5’-GACAGAGGCCACACAGA; Klf4, forward 5’-ACATGACGGAAGGAAGGAAAA and reverse 5′-CCAAAGCATTCTTTAGGCT; Pent, forward 5’-CGTGGGACAAGAGGGACAAAA and reverse 5′-TCTTCTCTCCTGCGTTTCTGC; Rex2, forward 5′-CCAGAAAGAGAACAGGATGC and reverse 5′-CTCTAGCTCCTGGGCTAATCT; Foxg1, forward 5′-CTCCCATCCCTTGTCTAGTGTGGT and reverse 5′-GTCCACAGGAGTCTGGGTG; Nr2f1, forward 5′-AAGGAACGATTGGAAGAGGACC and reverse 5′-TGAAGAACAGCTGCTGCA; Zic1, forward 5′-GTAAACAGACGAGGCGGCGACAG and reverse 5′-AAAGACCACAGCCAGCAT; Myt1, forward 5′-CAGTGTCCTCTATAGTGTCTCTC and reverse 5′-GCCTGCAAACTCCAAACTTCC; Gapdh, forward 5′-ACCAGCTCCATGCCATCAC and reverse 5′-TCCACCACCTGTGCTGGA; β-actin, forward 5′-CCTTGTGGCTGTAGGAGTTGC and reverse 5′-CCAGAGGCATACAGGGAAC.

**Results**

P/Sox2-EGFP Permits Isolation of NSCs from Fetal Mice. We have used the Sox2 promoter to express the EGFP (Fig. L4) in ESCs and chimeric fetal mice to isolate genetically identical populations of ESCs and NSCs for direct genetic and functional comparison. The transgene exhibits regional specificity to the fetal telencephalon (Fig. 1B) and is exclusively expressed in the immature neuroepithelium, as demonstrated by the lack of EGFP colocalization with the early neuronal marker βIII-tubulin (Fig. 1C). P/Sox2-EGFP-expressing cells were isolated from the telencephalon of E14 chimeric fetal mice by using FACS. The P/Sox2-expressing population was purified in this manner before initiating cultures to eliminate cells that do not express the transgene, because of regional specificity. This purification step also eliminates nontransgenic cells derived from the host blastocyst in these chimeric animals. From cultured populations the proportion of P/Sox2-EGFP+ cells was ~8% (gate M4), and the population of EGFP+ cells was further subdivided into high (M3) and low (M2) expression levels based on fluorescence intensity (Fig. 1D). These positive populations, as well as a population of P/Sox2-EGFP− cells, were individually collected, and the levels of Sox2 mRNA were determined for each by real-time RT-PCR. The fluorescence intensity of P/Sox2-EGFP expresses a positive correlation with the amount of Sox2 mRNA (Fig. 1E). However, a significant level of
Fig. 1. P/Sox2-EGFP identifies a subset of Sox2-expressing cells that are NSCs. (A) Structure of the transgene. (B and C) The 1D2 ESC clone exhibits telencephalic-restricted expression at E14, and the localization of P/Sox2-EGFP expression is in the cell-dense neuroepithelium surrounding the lateral ventricles but not in differentiating cells identified by βIII-tubulin (red). (D) FACS isolation of P/Sox2-EGFP high, low, and negative populations from short-term cultures derived from E14 fetuses. The total proportion of expressing cells represents ~8% of the sorted population that was further subdivided based on high and low expression levels. (E) Quantitation of Sox2 mRNA in populations with varying fluorescence intensity by real-time quantitative PCR, relative to the control Hprt message, exhibits correlation with fluorescence intensity. Notably, the P/Sox2-EGFP+ fraction still contains cells expressing significant levels of Sox2 mRNA, indicating that the transgene marks only a subset of cells that express the Sox2 gene. (F) Selection of P/Sox2-EGFP-expressing cells provides a 20-fold enrichment in neurosphere-initiating activity over nonexpressing cells from cultured populations. (G) All clones derived from single P/Sox2-EGFP cells are multipotent and differentiate into neurons, astrocytes, and oligodendrocytes, as indicated by βIII-tubulin (red), GFAP (purple), and RIP (green) immunoreactivity.

Sox2 mRNA exists in the P/Sox2-EGFP− population. Because all nontelencephalic and nontransgenic cells derived from the host blastocyst were previously eliminated on initiation of these cultures, expression of Sox2 mRNA in the fluorescence-negative fraction indicates that the 5.5-kb element defines only a subset of cells within a larger population that expresses the Sox2 gene.

P/Sox2 permits the isolation of NSCs from cultured telencephalic progenitor populations, as evidenced by an increase in the number of neurospheres generated from P/Sox2-EGFP+ over P/Sox2-EGFP− cells. We observed that 95% of the neurosphere-initiating activity was found in the P/Sox2-EGFP-expressing population (Fig. 1F). It was not feasible to perform enrichment experiments from freshly isolated cells because of the chimeric nature of the fetuses and the regional specificity of expression of the P/Sox2 transgene. Thus, significant numbers of neurosphere-initiating cells were observed in the EGFP+ population in assays of freshly isolated cells.

In addition, P/Sox2-expressing cells are bona fide NSCs, exhibiting the hallmarks of self-renewal and multilineage differentiation when cultured as clonal isolates. P/Sox2-EGFP cells were isolated by using FACS directly from the telencephalon of E14 fetuses or from cultured populations derived from E14 telencephalon. Cells were plated individually into wells of 96-well plates by limiting dilution. Progenitor clones derived from single cells were dissociated after 7–14 DIV and replated into 96-well plates, where some wells were observed to contain a single cell. Wells with single cells that gave rise to secondary clones demonstrated the self-renewal capacity of P/Sox2-EGFP cells. The secondary clones were expanded and differentiated to verify that progeny of neuronal, astrocytic, and oligodendrocytic lineages were produced (Fig. 1G). We observed that 50% (25/50) of single P/Sox2-EGFP+ cells isolated from culture gave rise to primary clones, and single cell-derived secondary clones were generated from every primary clone. Furthermore, all of the secondary clones exhibited a multipotent character. Thus, given the constraints on cell survival after FACS isolation, at least 50% of P/Sox2-EGFP cells are SCs in vitro; this finding validates the P/Sox2 approach for prospective isolation.

Expression Profiling of P/Sox2 Defined SCs. The availability of P/Sox2 as a common molecular identifier for both ESCs and NSCs allows for their isolation and thereby permits a direct transcriptional profiling comparison between pluripotent and multipotent SCs, respectively. An overview of the experimental design for transcriptional profiling, as well as some of the observed transcripts that one might expect, is illustrated in Fig. 2.
three populations of P for identification of differential transcripts. This procedure we then subjected to a very stringent matrix analysis paradigm yielded a very precise fingerprint of each SC population that ‡Complete lists are provided in Table 2.

*The number and manner in which the highly confident, differentially expressed genes vary among ESC and NSCf or ESC and NSCc populations.

Table 1. Stringent matrix analysis defines a highly confident set of differentially expressed genes

<table>
<thead>
<tr>
<th>Category</th>
<th>n = 112</th>
<th>Pluripotent-specific</th>
<th>n = 158</th>
<th>Multi-potent-specific</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transcriptional regulation</td>
<td>21</td>
<td>Oct4, Utf1, Rex1/2, Klf2/5, NrOb1, Crtr1, Pem, Stat6, Tcf7/15, Rarg, E12, Trim25, Tce3, Aire, Gbx2, ESTs highly similar to Tbx3, ESTs moderately similar to Znf41, ESTs weakly similar to Nkx2.5</td>
<td>24</td>
<td>Foxg1, Nr2f1, Myt1, Zic1, Sox2/3/4/11, Bmi1/4, Mash1, Hes5, Hey1, Dlx1, Cux1, Cdx4, ArgGth1, Jun, Lmyc1, Id4, mKft, ESTs highly similar to Kruppel-like Zfp and Aif9, ESTs moderately similar to Zfp2</td>
</tr>
<tr>
<td>Chromatin modulation</td>
<td>0</td>
<td>None detected</td>
<td>2</td>
<td>Sir2/2, ESTs highly similar to Sir2/2</td>
</tr>
<tr>
<td>RNA binding</td>
<td>4</td>
<td>Esg1, Rbm1, Dazl, ESTs similar to Rbpm</td>
<td>3</td>
<td>Rbm, Cugbp2, ESTs moderately similar to Rbmy</td>
</tr>
<tr>
<td>Growth factors</td>
<td>4</td>
<td>Fgf4, Tdgf1, Ebf1, Gaf3</td>
<td>2</td>
<td>Pleiotropin, Igrp5</td>
</tr>
<tr>
<td>Receptors</td>
<td>5</td>
<td>F2rl1 (thrombin R-like 1), Sdf2r2, Folate R1, Epha2, Tacstd1</td>
<td>8</td>
<td>Fgfr2, Notch1, Gpr56, Ptpra/g/o/z, ESTs highly similar to thyroid hormone Ralpha</td>
</tr>
<tr>
<td>Transporters</td>
<td>0</td>
<td>None detected</td>
<td>10</td>
<td>Bip, Fabp, Syst1, Giur-8 (and splice variant), Gria2, Grik5, Ckcn4-2, Kcnk2, Bact</td>
</tr>
<tr>
<td>Kinases and phosphatases</td>
<td>4</td>
<td>Jak3, Hck, ESTs moderately similar to dual specificity kinase, Inpp5d</td>
<td>10</td>
<td>Jak2, Ddr1, Hipk2, Tesk1, Ptk4, Srrk, Ptpn9, ESTs highly similar to Pkc-nu, PTK, and PP2a-beta</td>
</tr>
<tr>
<td>Cell cycle</td>
<td>3</td>
<td>Mkrs3, Stag3, Gste1</td>
<td>9</td>
<td>cyclin D2, p21 activated kinase 3, Nfia/x, Gas1, Gadd45g, Septin 5, ESTs highly similar to Rgs3 and candidate mediator of p53-dependent G0 arrest</td>
</tr>
<tr>
<td>Other signaling molecules</td>
<td>15</td>
<td>Ptc1, Upp, Annexin 1/11, Cish3, Serpine1, Gjb3, Spint1, Tgfpi, A2a2, Ndr1, Ly75, Caplpactin (Calzizzarin), ESTs highly similar to Rgs5, ESTs moderately similar to Interferon</td>
<td>18</td>
<td>Smo, Fzd2, Delta-like 1, Itga, Pcat15, Rgs2, Rab11a, Arh, Doublecortin, Dab1, Pika, Edz2, adenylyl cyclase 6, Apba2/b1, ESTs highly similar to Zfp289/B7H3</td>
</tr>
<tr>
<td>Metabolism</td>
<td>12</td>
<td>Slc2a3/7a3/7a7/27a2/29a1/38a4, Gp4t2, Pkp2, Cox7a1, Apoc1, Ddx, ESTs moderately similar to Slc39a4</td>
<td>7</td>
<td>Smp2, Pam, Amsae, Pahb1b2, dihydroxyimididine-like (Dpys) 3/4, ESTs moderately similar to Dpys4t</td>
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<tr>
<td>Cell adhesion</td>
<td>6</td>
<td>Icam1, Jcam1, Ctgf, Sp1, Endoglin, Jup,</td>
<td>9</td>
<td>Vcam1, Jcam3, Alcarm, Podha4/6, Catenin alpha2/alpha3, Neurophilin, Kit ligand</td>
</tr>
<tr>
<td>Cytoskeleton</td>
<td>5</td>
<td>Acta2, Tuba2, CD2-associated protein, Epb4, Id4a, ESTs highly similar to Desmoyokin</td>
<td>2</td>
<td>Kifs3a, Gephrin</td>
</tr>
<tr>
<td>Toxic response</td>
<td>2</td>
<td>Mdr1, Epha2</td>
<td>0</td>
<td>None detected</td>
</tr>
<tr>
<td>Visceral traffic</td>
<td>1</td>
<td>Stxb3</td>
<td>10</td>
<td>Nrg1/2, Secretogranin 3, Sgn1</td>
</tr>
<tr>
<td>Other</td>
<td>7</td>
<td>B3gnt1, Fbxo15, Elf1a, Pco1, Crip, Col18a1, Nidogen</td>
<td>10</td>
<td>B4gal2, Ehb1, Gdp1, Pnap, Mfap2, Ubl4, ESTs highly similar to Ubp3, ESTs moderately similar to Sting, ESTs weakly similar to protein disulfide isomerase</td>
</tr>
<tr>
<td>Unknown</td>
<td>23</td>
<td>Bc13, Tcf1, Trap1a, Stra8, Mov10, Esau protein, Tes19, Tesx0, Tcstv1, two EST clusters highly similar to Tcstv1, ESTs highly similar to Cgi-83, ESTs moderately similar to Plackophillin2/NDSP2/DC12, ESTs weakly similar to WAP, seven EST clusters of little or no homology</td>
<td>40</td>
<td>None detected</td>
</tr>
</tbody>
</table>

There are 112 genes determined to be present only in ESCs and differentially expressed at the 1.4-fold change level between ESCs and both NSC populations. Conversely, there are 158 genes present only in NSCf and NSCc and differentially expressed with ESCs. Transcription factors in bold were validated by RT-PCR for presence/absence (see Fig. 4).
two of three independent methods. This method of comparison highlights the large numbers of genes that may play important roles in defining these two different levels of SC potency. A logical place to begin further investigation is with those genes that are not just enriched but completely specific to each SC class, based on absence of expression using gene chip determinations. The complete lists of pluripotent-specific (n = 112) and multipotent-specific (n = 158) genes are provided in Table 2. A fully searchable database for all classifications of differential as well as nondifferential genes is also provided (Table 3, which is published as supporting information on the PNAS web site).

**Chimera Analysis of P/Sox2 SC Populations.** The large degree of difference in transcriptional profiles between populations of SCs that are clonally related and defined by identical molecular criteria was surprising. However, the transcriptional profiles represent a highly accurate molecular fingerprint of these SC populations. Despite these drastic differences, we wanted to test functionally for pluripotent behavior in the fetus-derived P/Sox2 populations by using chimera analyses. Functional confirmation of the pluripotent and multipotent nature of these SC populations is crucial in light of the previous reports suggesting NSC potency. A logical place to begin further investigation is with those genes that are not just enriched but completely detectable in the ICM. Absence by Qualitative RT-PCR. Functional verification of the pluripotent and multipotent character of these SC populations identifies the differentially transcribed genes as molecular correlates of this difference. Of particular interest on this list are genes that are present in ESCs and absent from NSCs, because these genes may function specifically in the establishment or maintenance of the pluripotent state. Transcription factors were chosen for the first level of analysis because of their commonly described roles in regulation of cell fates and differentiation. As might be expected, the gene encoding the transcription factor Oct4 is at the top of the list with regard to fold change, because it is highly expressed in ESCs and absent from NSCs. The pivotal role of Oct4 in both specification and maintenance of the pluripotent phenotype has already been shown (15). Groups of transcription factors that exhibited a gene chip-predicted present/absent pattern in either ESCs or NSCs (bold in Table 2) were chosen for further analysis.

To verify the presence and absence of these genes in their respective compartments, we used qualitative RT-PCR with up to 40 cycles of amplification for a much more sensitive method of detecting rare transcripts. In addition, for RT-PCR verification we used samples that were prepared by FACS isolation in the same manner but independent from those used for transcriptional profiling. All genes except Zic1 exhibited strong differential expression by RT-PCR in the same direction as predicted by gene chip analysis. The detection of completely ESC-specific transcripts was verified for seven of eight genes, with Klf4 being the exception, because a small amount of message was detectable in the NSC population (Fig. 4). The RT-PCR results confirmed the differential nature of these genes

lack the hallmark abilities of pluripotent ESCs supports the use of these genetic comparisons for further studies of SC potency.
but highlighted the need for additional methods of verification for presence/absence determinations.

Discussion

Here we report the development of an approach based on the Sox2 promoter for the isolation and comparison of NSCs and ESCs. The P/Sox2 transgene is expressed exclusively in ESCs and the immature neuroepithelium of the fetal telencephalon, thus allowing prospective isolation of both populations by FACS. Selection of P/Sox2-EGFP cells from fetal mice enriches for neurosphere-initiating activity, and individually plated cells have ESC function. The transcriptional profiles of clonally related NSCs and ESCs defined by P/Sox2-EGFP expression were compared. This comparison revealed an extraordinary degree of difference between these pluripotent and multipotent SC populations. To ensure that the fetus derived P/Sox2-EGFP populations do not exhibit pluripotent character, despite their drastically different genetic profiles, these cells were aggregated with morula-stage embryos or injected into blastocysts. This assay functionally demonstrates the multipotent and pluripotent character of the P/Sox2-defined NSC and ESC populations, respectively, and validates the differentially expressed genes as potential molecular correlates of this functional difference.

Recently, two other groups have reported expression profiling comparisons between ESCs and multipotent SCs (30, 31). This report differs from those in that it focuses on elucidating the differences between these SC populations rather than seeking a common transcriptional profile that may be attributable to “stemness.” In addition, there are several important aspects of our transcriptional profiling approach that contribute to making it the most accurate and reliable comparison of NSCs and ESCs reported to date. First, because P/Sox2 is down-regulated in the differentiating progeny of both ESCs and NSCs, the selection of P/Sox2-expressing cells provides a more homogeneous population for each SC and defines these populations by identical criteria (Fig. 5, which is published as supporting information on the PNAS web site). Purification eliminates the need to remove the “differentiation genes” that are expressed in heterogenous SC populations. Performing direct comparisons between the entire transcriptome of ESCs and NSCs adds power to the comparison, because removing “differentiation genes” from comparative analysis has no biological rationale. The fact that a gene is expressed in both the SC and its differentiating progeny does not exclude it from having functional importance to that SC’s “genetic program.” Second, because the NSCs are isolated from chimeric fetuses, populations are provided that share identical genomes, because they are all derived from the same ESC clone. This feature will eliminate the variability in gene transcription that arises from genomic heterogeneity (24). Third, this isolation method permits, for the first time, the analysis of both freshly isolated and cultured NSC populations. Fourth, because populations can be acquired in sufficient quantity, this method eliminates the need for multiple rounds of in vitro transcription (IVT). Single-round IVT better maintains the 5’ complexity of cRNA and reduces the introduction of data biases that accompany multiple-round IVT protocols (32). Fifth, the use of three independent data analysis methods for determinations of differential transcripts adds an additional level of confidence to the gene lists.

In conclusion, we have presented a transgenic approach that uses a common property of SCs for the purification of multipotent NSC and pluripotent ESC populations to generate direct genetic and functional comparisons between these two SC classes. These comparisons have underscored the genetic dissimilarity between functionally verified multipotent and pluripotent populations, providing new avenues for investigation of the biology underlying the control of cellular potency.

Note Added in Proof. Two groups recently described a novel homeobox transcription factor, Nanog, that plays a functionally essential role in maintaining the pluripotent state of ESCs (33, 34). This gene was one of the most highly pluripotent-specific transcripts detected in our gene chip analyses at the level of ≥2.0-fold change, and in this report is classified as one of the “seven EST clusters of little or no homology.” It may be identified in Table 3 by probe set ID numbers 161072.s_at and 161653.Lat or by query for UniGene number Mm.6047.

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Fig. 4. RT-PCR confirmation of presence/absence in ESC and NSC populations. Groups of SC class-specific transcription factors of high confidence (2.0 or greater fold change) were assayed by RT-PCR as a more sensitive method for detection of rare transcripts. Most of the ESC-specific genes were verifiably absent in NSCs. Only two of four NSC-specific genes were found absent from ESCs. Gapdh and β-actin gene signals are approximately the same, indicating an equivalent input for both cell types.