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# A Focused Microarray for Screening Rat Embryonic Stem Cell Lines

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Here, we describe a focused microarray for screening rat embryonic stem cells (ESCs) and provide validation data that this array can distinguish undifferentiated rat ESCs from rat trophoblast stem (TS) cells, rat extraembryonic endoderm cells, mouse embryonic fibroblast feeder cells, and differentiated rat ESCs. Using this tool, genuine rat ESC lines, which have been expanded in a conventional rat ESC medium containing two inhibitors (2i), for example, glycogen synthase kinase 3 (GSK3) and mitogen-activated protein kinase (MEK) inhibitors, and leukemia inhibitory factor, and genuine rat ESCs, which have been expanded in rat ESC medium containing four inhibitors (4i), for example, GSK3, MEK, Alk5, and Rho-associated kinase inhibitors were compared; as were genuine rat ESCs from 4 different strains of rats. Expression of *Cdx2*, a gene associated with trophoblast determination, was observed in genuine, undifferentiated rat ESCs from 4 strains and from both 2i and 4i ESC derivation medium. This finding is in contrast to undifferentiated mouse ESCs that do not express *Cdx2*. The rat ESC focused microarray described in this report has utility for rapid screening of rat ESCs. This tool will enable optimization of culture conditions in the future.

## Introduction

THE RAT LAGS BEHIND THE MOUSE in the field of functional genomics [1,2]. After 2 decades of trying, genuine rat embryonic stem cells (ESCs) were produced [3,4]. Recently, genuine rat ESCs were used to produce a knockout rat [5]. Several lines of evidence support the contention that the mechanism of self-renewal and maintenance of the ground state of pluripotency is fundamentally different in mice and humans. The differences that exist between human and rat ESCs or between rat and mouse ESCs are yet to be fully elucidated. For example, we recently reported that rat ESCs maintained using the 2 inhibitor cocktail of PD0325901, a mitogen-activated protein kinase (MEK) inhibitor, and CHIR99021, a glycogen synthase kinase 3 (GSK3) inhibitor plus leukemia inhibitory factor (LIF), called two inhibitors (2i) below, express *Cdx2* [6], a gene involved in trophoblast fate determination [7,8]. This finding is in sharp contrast to what is observed in mouse ESCs. Another difference is that rat ESCs cannot be maintained in medium containing only LIF when grown on inactivated mouse embryonic fibroblasts (MEFs), in contrast to mouse ESCs [9]. Finally, as reported for the mouse, strain differences may affect the quality of rat ESCs for producing germline transmission, or may affect the ability of the blastocyst to integrate ESCs and the efficiency of rat ESCs to contribute to the germline is lower than the mouse [3,4,6,10]. Therefore, distinct species differences exist between rat and mouse ESCs. Further work is needed to fully understand the differences between rat and mouse ESCs and to optimize rat ESC culture conditions to increase germline transmission efficiency.

Here, our goal was to develop and validate a rat-specific microarray focused on detection of pluripotency, stem cell and differentiation-associated gene expression for rapidly screening rat ESC lines, and enable the optimization of rat ESC culture. To derive this array, we culled the literature to generate a short list of genes that would discriminate undifferentiated and differentiated ESCs [11,12], and ESCs from extraembryonic endoderm cells (XEN, [13–15]), epiblast stem cells (Epi, [16–18]), and from trophoblast stem (TS) cells [19,20]. The gene list was provided to Qiagen and they manufactured the gene array. Next, we used this array to

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compare the gene expression of genuine rat ESCs produced in our laboratory [6] and from the laboratory of QY [4] using 2i medium and genuine ESCs produced using media containing four inhibitors (4i, the Rho-associated kinase inhibitor Y-27632; the MEK inhibitor PD0325901; the type 1 TGFβ receptor inhibitor A-83-01; and the GSK inhibitor CHIR99021, called 4i below [10]. The 4i genuine rat ESCs were provided by the laboratory of MK and TO. The data show that the array has sensitive quality assurance and quality control elements, good inter-investigator reliability, and good reproducibility between different genuine rat ESC lines. These data confirm that genuine rat ESCs express *Cdx2* since genuine rat ESCs from 3 different labs express the gene with rat ESCs expanded in YPAC medium expressing *Cdx2* at the highest levels. In conclusion, this array discriminates undifferentiated rat ESCs from differentiated rat ESCs and discriminate ESCs from extraembryonic endoderm stem cells (XEN) and TS cells, as well as, other stem cells derived from the developing rat embryo. Therefore, this array is a sensitive, validated tool for rapidly screening rat ESCs lines and for optimizing rat ESC culture conditions.

## Materials and Methods

### Cell lines

Information about samples and sample processing is listed in Table 1. Rat ESC lines used here were derived from Dark Agouti (DA) and transgenic Fischer 344 (F344) rats. ESC derivation, ESC differentiation to embryoid bodies (EBs), and characterization of our DA and F344 ESCs was described previously [6]. In addition, 2i plus LIF genuine rat ESC pellets derived from DA rats were provided by Dr. Q. Ying (University of Southern California, Los Angeles, CA) [4]. Genuine rat ESC pellets derived from Long Evans Agouti and Wistar rats using the 4i medium were provided by Drs. M. Kawamata and T. Ochiya (National Cancer Center Research Institute, Tokyo, Japan) [10]. Rat TS and extraembryonic endoderm stem cells (XEN) were prepared as previously described and were provided by Drs. M. Rumi and M. Soares [19,21]. Mitotically inactivated CF-1 MEFs (passage 3) were obtained and used following the manufacturer's protocol (Globalstem).

### Reverse transcriptase–polymerase chain reaction focused array

The gene list and efficiency data and sample processing is listed in Table 1. The 96-well custom array containing 92 unique elements for evaluation of rat ESCs was manufactured by Qiagen (CAPR10083). We did not independently validate the manufacturer's PCR efficiency assays for each gene. Total RNA was prepared using the RNeasy RNA isolation kit (Qiagen) or TRIZOL (Life Technologies) using the manufacturer's protocol. Complementary DNA was synthesized using Qiagen's $RT^2$ first strand kit following the

Table 1. Biological Samples

| *Sample number* | *Sample name* | *Total RNA preparation* | *Source* |
|---|---|---|---|
| Sample 1 & 2 | XEN | TRIZOL | Provided by Michael Soares' lab University of Kansas Medical Center |
| Sample 3 & 4 | inactivated MEF | Rneasy RNA isolation kit (qiagen) per manufacturer's protocol | Purchased from GlobalStem |
| Sample 5 & 6 | DA54.1 p9 chimera | Rneasy RNA isolation kit (qiagen) per manufacturer's protocol | Generated in house as described in Hong et al. [6] |
| Sample 7 & 8 | Fischer 344 2.1.1 p24, unknown | Rneasy RNA isolation kit (qiagen) per manufacturer's protocol | Generated in house as described in Hong et al. [6] |
| Sample 9 & 10 | DA52 p9, GERMLINE | Rneasy RNA isolation kit (qiagen) per manufacturer's protocol | Generated in house as described in Hong et al. [6] |
| Sample 11 & 12 | DAc8, p35, GERMLINE | Rneasy RNA isolation kit (qiagen) per manufacturer's protocol | Provided by Qilong Ying's lab University of Southern California, generated per Li et al. [4] |
| Sample 13 & 14 | DA54.1 EB, 5 d | Rneasy RNA isolation kit (qiagen) per manufacturer's protocol | Generated in house as described in Hong et al. [6] |
| Sample 15 & 16 | DA54.1 EB, 10d | Rneasy RNA isolation kit (qiagen) per manufacturer's protocol | Generated in house as described in Hong et al. [6] |
| Sample 18 & 19 | TS | TRIZOL per manufacturer's protocol | Provided by Michael Soares' lab University of Kansas Medical Center |
| Sample 19 & 20 | DA53.1, p10, GERMLINE | Rneasy RNA isolation kit (qiagen) per manufacturer's protocol | Generated in house as described in Hong et al. [6] |
| Sample 25 & 26 | Fischer 344 38, GERMLINE | Rneasy RNA isolation kit (qiagen) per manufacturer's protocol | Generated in house as described in Hong et al. [6] |
| Sample 31 & 32 | 115 LEA, p6, 4i | Rneasy RNA isolation kit (qiagen) per manufacturer's protocol | Provided by Kawamata and Ochiya [10], National Cancer Center Research Institute, Tokoyo, Japan |
| Sample 33 & 34 | 116 Wistar, p5, 4i | Rneasy RNA isolation kit (qiagen) per manufacturer's protocol | Provided by Kawamata and Ochiya [10], National Cancer Center Research Institute, Tokoyo, Japan |

manufacturer's protocol. The focused array was run using Qiagen's $RT^2$ qPCR MasterMix for the BioRad iQ5 thermal cycler. Thermal cycling and quantitation were performed using a BioRad iQ5 iCycler controlled by Biorad iCycler IQ software version 3.1.7050. Following PCR, the products were subjected to melting point analysis. All biological samples were run in duplicate (technical replicates) independently prepared by different investigators (JH or HH). The array data were uploaded to the gene expression omnibus (GEO) website (www.ncbi.nlm.nih.gov/geo, Accession number GSE30582).

### *Reverse transcriptase–polymerase chain reaction to test for rat genomic DNA contamination*

Total RNA samples used in the array were tested for rat genomic DNA contamination (RGDC). Complementary DNA was synthesized using Superscript III First-Strand Synthesis Supermix kit (Life Technologies) primed with oligo-$dT_{12-18}$ per the manufacturer's protocol. A primer set for rat PBGD was designed that spanned an intron and was validated to discriminate genomic DNA (primer sequence available upon request). PCR was performed using a BioRad iCycler or iQ5: the initial denaturation at 95°C for 3 min, 30 cycles of (94°C for 30 s, 53°C–55°C for 30 s, and 72°C for 1 min), and the final extension at 72°C for 10 min. Following PCR, the products were resolved on a 1% agarose gel with 100 bp DNA ladder (Promega) and imaged.

### *Data analysis and assembly of figures*

No calls or missing data were assigned Ct value of 40.0. Data were loaded into Microsoft Excel 2010 for analysis (raw and transformed data are in Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/scd). The average threshold cycle (Ct), standard deviation, and % covariance were calculated for selection of housekeeping gene (HKG) candidates. Unmanipulated data from technical replicates were plotted in a scattergram and a linear regression line was calculated using SigmaPlot version 12. To evaluate the impact of normalizing the data using HKG, the 5 experimental genes with the lowest standard deviations were inspected using the RefFinder tools available via the Cotton EST database (www.leonxie.com/referencegene.php) that facilitates comparison of HKG using major computational programs such as geNorm [22], Normfinder [23], BestKeeper [24], and the comparative Ct method [25]. Two of these genes (*Gapdh* and *Ldha*) were used to normalize the Ct data and the scattergrams were plotted and linear regression lines and $R^2$ values calculated (see Results section). For simplicity of calculation, we assumed 100% PCR replication efficiency and used $2^{(-\Delta\Delta Ct)}$ to calculate fold change. We did this despite knowing the manufacturer's quality assurance and quality control (QA/QC) specifications PCR efficiency data for each gene in the array (Table 1) because we assumed that experimental amplification efficiency might differ. Kruskal–Wallis Analysis of Variance for Ranks was used to determine overall differences between biological samples. Following significant ANOVA, Student-Newman–Keuls test was used for post hoc testing of planned comparisons. Unless specified differently, 10 or 100-fold increases or decreases in gene expression were selected as critical values for significant differences in gene expression between groups. Significance was set at $\alpha < 0.05$.

## Results

### *QA and QC*

SABioscience/Qiagen $RT^2$ profiler array has 7 wells dedicated to QA and QC.

(1) RGDC QA/QC step. RGDC is a concern since it can affect the interpretation of array findings. To obviate this potential problem, we used DNaseH treatment during isolation of total RNA in some samples. For every sample, SuperArray's $RT^2$ First Strand Kit (Qiagen) was used with its genomic DNA elimination step. RGDC was evaluated by calculating Ct of the RGDC–Ct HKG. RGDC was considered questionable if the difference was <14. Using this criterion, 2 samples [one replicate of Ying ESCs (DAc8) and one replicate of TS cells] had questionable results as to RGDC. To investigate those cases, RGDC was evaluated using reverse transcriptase–polymerase chain reaction (RT-PCR) using a primer set that spans an intron and the amplicon run on a 1% agarose gel along with a rat genomic DNA positive control. As shown in Fig. 1A, RGDC was not confirmed by RT-PCR. The fact that the technical replicate did not flag RGDC and that the RT-PCR did not confirm RGDC in these 2 cases suggest that the RGDC test reported a false positive 2 times in 26 arrays (about 8% error rate).

(2) Reverse transcriptase efficiency (RTE) QC step. RTE can be impacted by poor RNA quality or contaminants in the sample. RTE was evaluated by calculating average Ct of the reverse transcriptase control (RTC)–average Ct of the positive PCR control (PPC) from the triplicate wells on the array. The manufacturer's specification was that the difference should be <5. All samples passed (average $3.9 \pm 0.42$, range = 3.3–4.5).

(3) PCR amplification efficiency QC step. PCR efficiency should be consistent across arrays to reduce the need of making many technical replicates to achieve consistent, reproducible data. PCR efficiency was evaluated by calculating the average Ct of the triplicate PPC wells in the array. The manufacturer's specifications state that the PPC Ct values should be an average of $20 \pm 2$ cycles across in an experiment. We observed an average of $19.5 \pm 0.2$, meeting the PCR efficiency specification. In addition, the manufacturer has designed the PCR primers and tested their efficiency. The average efficiency of the primers in this array was $108.4\% \pm 10.1\%$ (range 90.5%–146.8%). Primer efficiency information provided by Qiagen is found in Table 1.

(4) Production of a single PCR product QA/QC step. Following amplification, the products were subjected to a melting point analysis to confirm that a single product was produced. Based upon the melting point analysis, PCR produced a single product in every case (data not shown).

### *Selection of the HKG and evaluation of technical replicates*

To select the HKG, the standard deviation of Ct values for each gene in the array was sorted from lowest to highest (see Supplementary Table S1). Of the experimental genes, *Actb*, *Ctnnbl1*, *Ldha*, *Hdac2*, and *Gapdh* had the lowest standard deviations (0.72, 0.73, 0.78, 0.82, and 1.06, respectively). Next, we compared the effect of normalization on the regression $R^2$ values between 9 biologically independent pairs of technical replicates and compared the $R^2$ of raw Ct values and the $R^2$

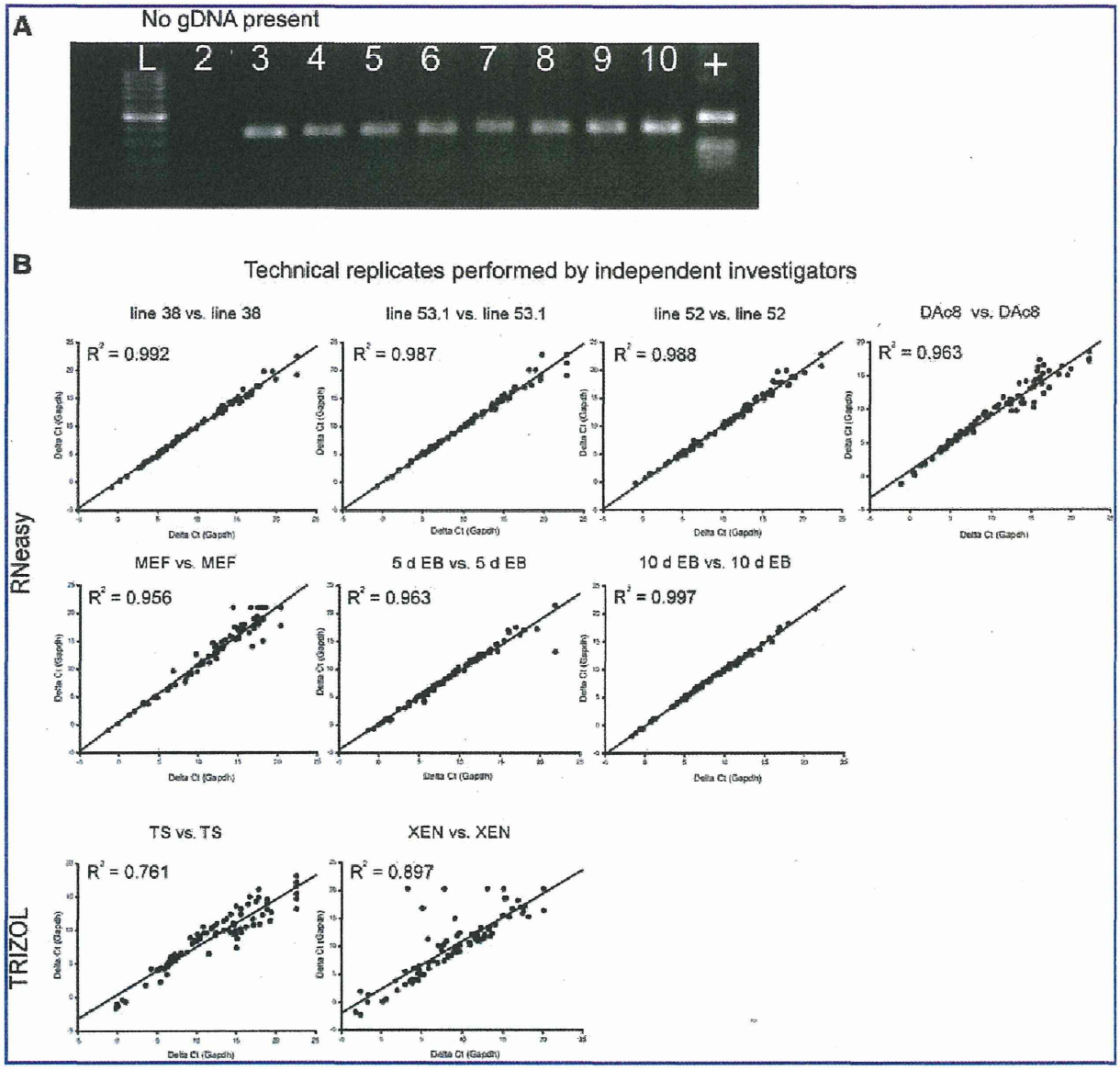



**FIG. 1.** **(A)** Evaluation of rat genomic DNA contamination (RGDC). Reverse transcriptase–polymerase chain reaction (RT-PCR) was performed using a primer pair that spans an intron to evaluate whether RGDC was present. No evidence of RGDC was found, indicating that the array RGDC well provided false positive in 2 instances (2 out of 18 arrays). Lane 2 is RNA sample prepared from inactivated mouse embryonic fibroblasts (MEFs). Lane 3 is RNA sample from undifferentiated genuine rat embryonic stem cells (ESCs). Lane 4 is RNA sample prepared from rat extraembryonic endoderm (XEN) cells. Lanes 5–7 are RNA samples from 3 different ESC lines prepared in-house [Dark Agouti (DA) line 1 (line 53.1), Fischer 344 (line 38), DA line 2 (line 52), respectively]. Lane 8 is an RNA sample prepared from rat trophoblast stem (TS) cells. Lanes 9 and 10 are RNA samples prepared from DA ESCs differentiated to 5 day embryoid bodies (EBs, lane 9) and 10 day EBs (lane 10). A rat genomic DNA sample positive control is labeled "+" (amplicon at 354 bp for cDNA and 433 bp for genomic DNA). Lane 1 (L) is a standard 100 bp ladder. **(B)** Comparison of technical replicates performed by independent investigators using scattergram and regression line with the $R^2$ value. *Top panel* is RNA samples isolated using the RNeasy kit (RNeasy). *Bottom panel* is RNA samples isolated using the TRIZOL method (TRIZOL). Note that the RNeasy isolation kit produced consistently higher $R^2$ values than the TRIZOL method. Scattergrams of ΔCt values (*Gapdh* used to normalize data).

after normalization with *Ldha* and *Gapdh*. There was no significant difference between the $R^2$ values of technical replicates not normalized and those normalized using *Ldha* or *Gapdh* (no normalization $R^2 = 0.940 \pm 0.083$, range = 0.735–0.997; *Gapdh* normalized $R^2 = 0.945 \pm 0.074$, range 0.761–0.994; and *Ldha* normalized $R^2 = 0.945 \pm 0.074$, range 0.761–0.994). Using the tools found at the Cotton EST database (see Methods section), *Gapdh* and *Ldha* were selected as the HKG. The geometric mean of *Gapdh* and *Ldha* was used as HKG for normalization (see Supplementary Table S1).

As shown in Fig. 1B, based upon the $R^2$ values cited above, the technical replicates performed by different investigators showed good reproducibility across 9 biological samples evaluated. We observed that total RNA sample preparation using the RNeasy column method tended to produce tighter reproducibility than the TRIZOL method (TS and XEN samples were prepared using the TRIZOL method).

### *The array can discriminate undifferentiated rat ESCs from differentiated ESCs, XEN, TS, and MEFs*

As shown on the top of Fig. 2, when overall gene expression of 3 genuine undifferentiated rat ESC samples expanded in 2i plus LIF from the Weiss lab (lines 52 and 53.1

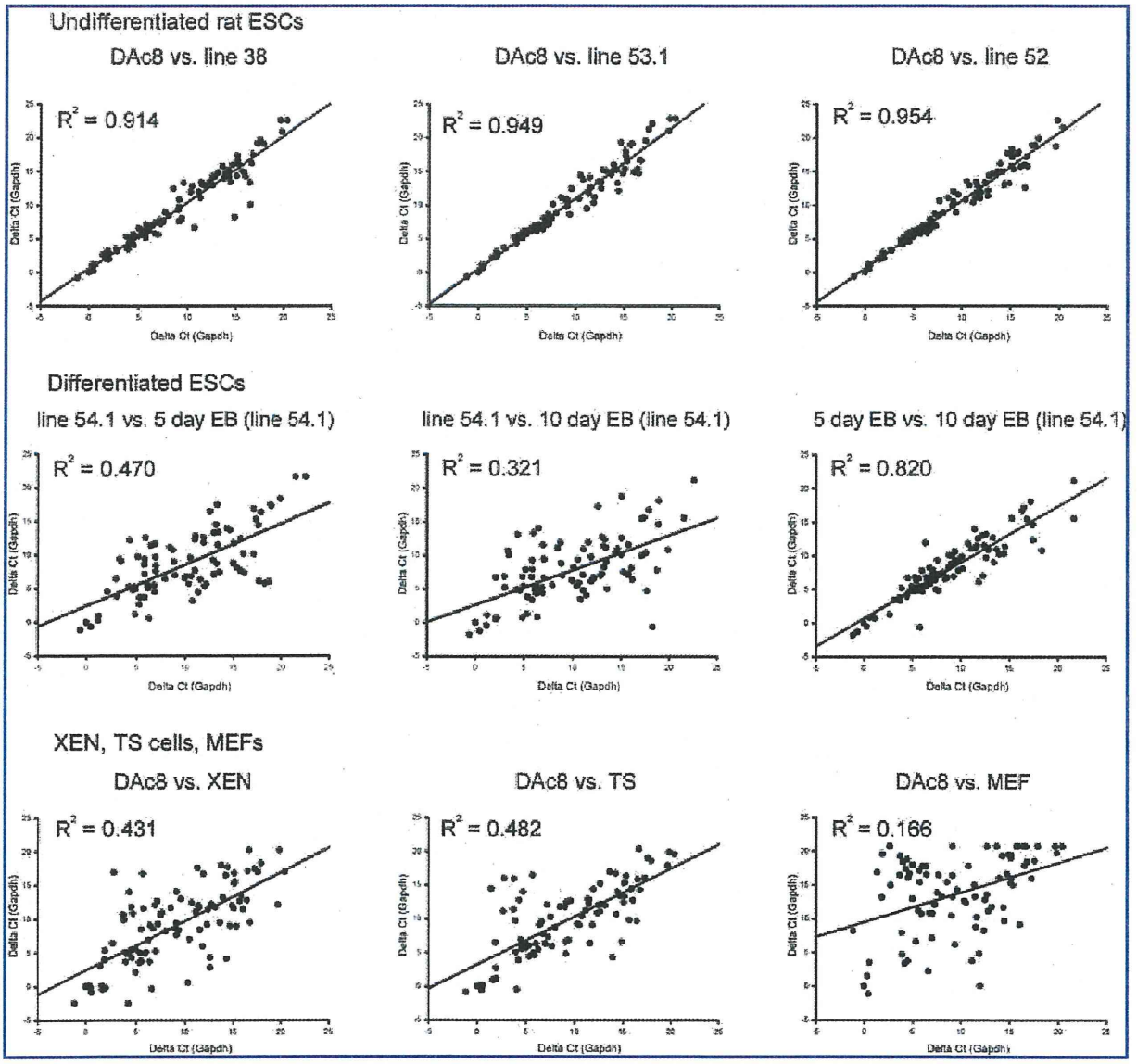



**FIG. 2.** Comparison of biological samples using scattergram and regression line with the $R^2$ value. *Top panel:* Rat ESC lines derived in-house (DA ESC lines 53.1 and 52 and transgenic Fischer 344 ESC line 38) compared with QY's genuine rat ESCs (DAc8). Statistically, there are no significant differences between overall gene expression patterns of the ESC lines. There was a trend for genuine DAc8 versus DA lines 53.1 and 52 to have higher coherence ($R^2$ values > 0.949) than genuine DAc8 versus F344 line 38 ($R^2$ value = 0.914). *Center panel:* (*left and middle graphs*) Comparison of the undifferentiated parental DA ESC cell line (line 54.1) with the 5 day and 10 day differentiated EBs derived from line 54.1. (*Right graph*) Comparison of 5 day and 10 day differentiated EBs. Note that statistical testing revealed that the undifferentiated DA line 1 (line 54.1) was significantly different from the same ESC line after 10 day EB formation (*middle graph*) and that gene expression by 5 day EBs was significantly different from 10 day EBs (*right graph*). *Bottom panel:* Comparison of DAc8 genuine rat ESCs to cells from the XEN, *left graph*; TS cells, *middle graph*; and inactivated MEFs, *right graph*]. By inspection, one can observe differences in gene expression between ESCs and non-ESC cell lines based upon y-intercept (expected to be zero in similar lines), scatter about the regression line and $R^2$ values < 0.5.

from DA rats and line 38 derived from transgenic F344 rats) were compared with undifferentiated genuine rat ESCs expanded in 2i plus LIF from QY's lab (derived from DA rats, line DAc8), there was good coherence in terms of gene expression as indicated by $R^2$ values >0.91. There was a tendency for higher correlations between undifferentiated DA (DAc8) versus DA ESC lines 53.1 and 52 (Fig. 2, top panel middle and right) when compared with DA versus F344 line 38 (Fig. 2, top panel left).

To statistically evaluate the samples, a Kruskal–Wallis One-Way Analysis on Ranks was used, which indicated overall significant differences between the 8 biological samples. Next, Student-Newman–Keuls method was used for planned multiple comparisons, and no significant differences were found between the 4 independently derived 2i plus LIF genuine rat ESC samples. In contrast, multiple comparisons revealed a significant difference between the undifferentiated ESC line and the same cells after 10 days of differentiation to EBs, which is supported by the observation of poor correlation between undifferentiated and differentiated samples (Fig. 2, middle panel). As shown in Fig. 2 bottom panel, the overall gene expression patterns shown in the scattergrams for undifferentiated ESCs poorly correlated with rat XEN, rat TS, and inactivated MEFs, too. These suggest differences in gene expression between undifferentiated ESCs and the other cell types.

To evaluate differences in gene expression between cell types, 10-fold and 100-fold differences in expression were selected as arbitrary thresholds. When inactivated MEFs were compared with undifferentiated ESCs (both those derived in 2i plus LIF and those derived in 4i), 47 genes were expressed at>10-fold higher levels in undifferentiated ESCs and 8 genes were found to be expressed at>10-fold higher levels in MEFs (see Supplementary Table S2). Further, 24 genes were expressed at>100-fold higher levels in undifferentiated ESCs and 2 genes were expressed at>100-fold higher levels in MEFs (see Fig. 3A). Notably, genes *Stella*, *Utf1*, *Pou5f1* (*Oct4*), *Gdf3*,*Bex1*, and *Pecam1* were expressed at very high levels in ESCs, about 161666–9717-fold higher levels and the genes *Ptn and Inhba* were expressed 1556-123-fold higher levels in MEFs (see Fig. 3A).

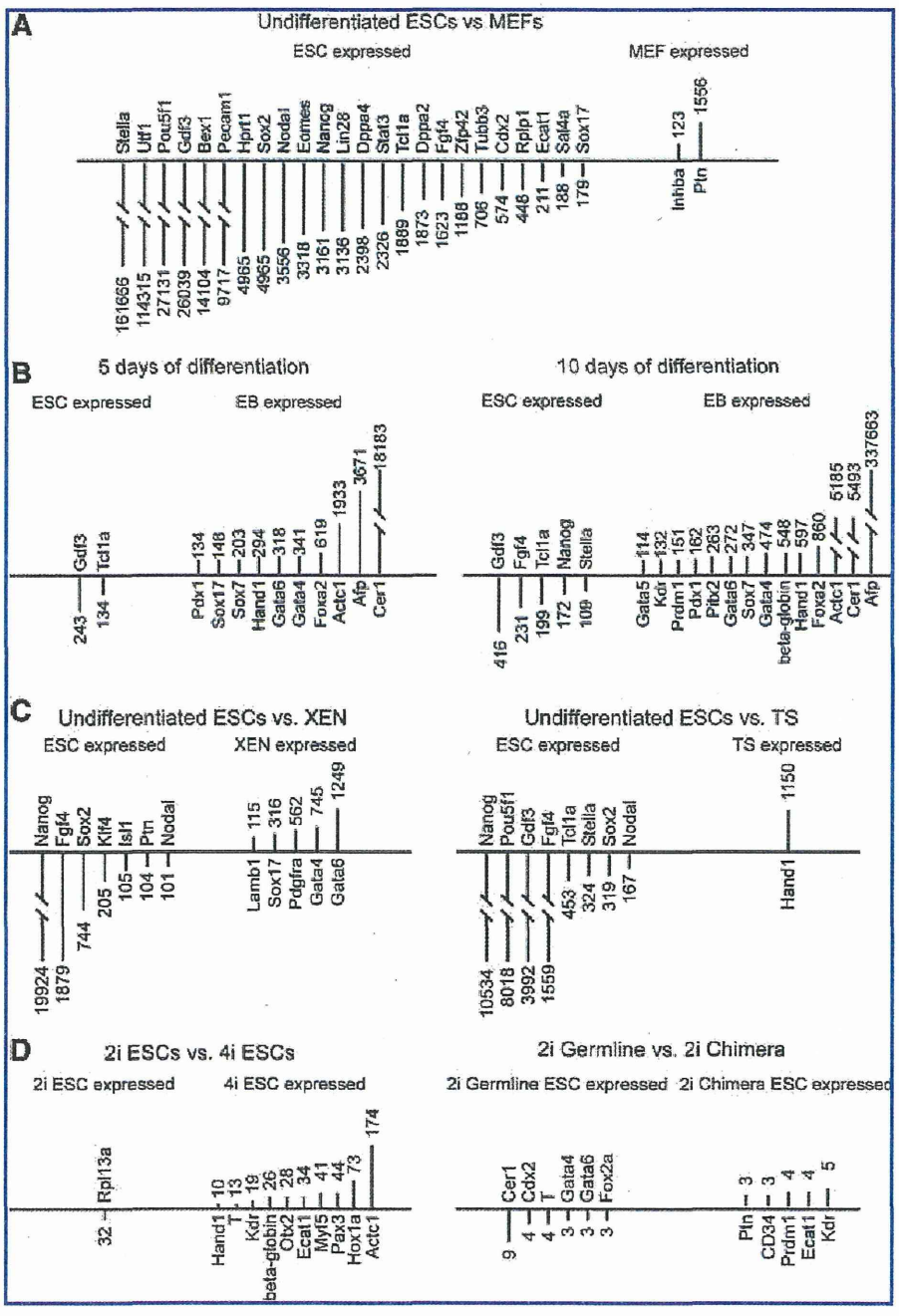
*Comparison of undifferentiated ESCs with differentiated ESCs.* When gene expression of undifferentiated ESCs was compared with gene expression after 5 days differentiation to EBs, 33 genes had greater than 10-fold expression differences between the 2 groups, and 10 genes were expressed higher in undifferentiated ESCs and 23 genes were expressed>10-fold higher levels in EBs after 5 days of differentiation (see Supplementary Table S3). Twelve genes showed>100-fold differences in gene expression between undifferentiated ESCs and EBs after 5 days of differentiation. As shown in Fig. 3B (left), genes *Gdf3* and *Tcl1a* were expressed at>100-fold higher levels in undifferentiated ESCs (range 243- to 134-fold higher) and the genes *Cer1*, *Afp*, *Actc1*, *Foxa2*, *Gata4*, *Gata6*, *Hand1*, *Sox7*, *Sox17*, and *Pdx1* were expressed>100-fold higher levels in the 5 day differentiated ESCs (range of 18183- to134-fold higher). When gene expression of undifferentiated ESCs was compared with gene expression after 10 day differentiation to EBs, 45 genes had>10-fold expression differences between groups (see Supplementary Table S3). Ten genes were expressed at>10-fold higher levels in undifferentiated ESCs and 35 genes were expressed at>10-fold higher levels in EB differentiated for 10 days. Figure 3B (right) shows the 19 genes that had>100-fold expression difference between these groups. Note that the genes *Gdf3*, *Fgf4*, *Tcl1a*, *Nanog*, and *Stella* were expressed> 100-fold in undifferentiated ESCs (range of 416–109-fold higher) and the genes *Afp*, *Cer1*, *Actc1*, *Foxa2*, *Hand1*, *beta-globin*, *Gata4*, *Sox7*, *Gata6*, *Pitx2*, *Pdx1*, *Prdm1*, *Kdr*, and *Gata5* were expressed at>100-fold higher levels in EBs after 10

**FIG. 3.** Differences in gene expression detected by the array. **(A)** List of genes that showed>100-fold expression differences between undifferentiated ESCs [all undifferentiated ESCs: both two inhibitors (2i) plus LIF and four inhibitors (4i) ESCs included] and inactivated MEFs. On the *left*, genes that were highly expressed in ESCs are shown in descending order. On the *right*, genes that were highly expressed in MEFs. For a complete, ordered list, see Supplementary Table S1. **(B)** Effect of differentiation for 5 or 10 days on gene expression. **(B)** *Left:* List of genes that showed>100-fold expression differences between undifferentiated ESCs (all undifferentiated ESCs: both 2i plus LIF and 4i ESCs included) versus 5 days of differentiation. Two genes that are highly expressed in undifferentiated ESCs are shown on the *left*, and 10 genes that were expressed by differentiation are listed on the *right*. **(B)** *Right:* List of genes that showed>100-fold expression differences between undifferentiated ESCs (all undifferentiated ESCs: both 2i plus LIF and 4i ESCs included) versus 10 days of differentiation. Five genes that are highly expressed in undifferentiated ESCs are shown on the *left*, and 14 genes that were expressed by differentiation are listed on the *right*. For a complete, ordered list, see Supplementary Table S2. **(C)** Comparison of undifferentiated ESCs with other cells derived from rat embryos. **(C)** *Left:* List of genes that showed>100-fold expression differences between undifferentiated ESCs (all undifferentiated ESCs: both 2i plus LIF and 4i ESCs included) versus XEN cells. Seven genes that were more highly expressed in ESCs are shown on the *left* and 5 genes that were more highly expressed in XEN cells are shown on the *right*. For a complete, ordered list, see Supplementary Table S3. **(C)** *Right:* List of genes that showed>100-fold expression differences between undifferentiated ESCs (all undifferentiated ESCs: both 2i plus LIF and 4i ESCs included) versus TS cells. Eight genes that were more highly expressed in ESCs are shown on the *left*, and one gene, *Hand1*, which was highly expressed in TS cells is shown on the *right*. For a complete, ordered list, see Supplementary Table S4. **(D)** Comparison of derivation methods and comparison of 2i germline versus 2i chimera ESC lines. **(D)** *Left:* List of genes that showed>10-fold expression differences between undifferentiated ESCs derived and expanded in 2i plus leukemia inhibitory factor (LIF) conditions (2i ESCs) and ESCs derived and expanded in YPAC conditions (4i ESCs). On the *left*, note that one gene, Rpl13a was more highly expressed in 2i ESCs. On the *right*, 10 genes were more highly expressed in 4i ESCs, including Actc1, which was expressed 174-fold higher. For a complete, ordered list, see Supplementary Table S5. **(D)** *Right:* List of genes that showed>3-fold expression differences between undifferentiated 2i ESCs that contribute to the germline (Germline 2i ESCs) and undifferentiated 2i ESCs that contribute to chimera only (2i chimera ESCs). On the *left* is listed 6 genes that were more highly expressed in Germline 2i ESCs. On the *right* are listed 5 genes that were more highly expressed in Chimera 2i ESCs. For a complete, ordered list, see Supplementary Table S6.

days of differentiation (range 337663- to 114-fold higher). Thus, as ESCs undergo differentiation over time, more lineage specification and lineage specific genes are expressed in EBs, as one would expect.

*Comparison of undifferentiated ESCs with XEN cells.* As one might expect from inspection of Fig. 2 (bottom), 34 genes showed > 10-fold expression differences between undifferentiated rat ESCs and XEN cells, and 18 genes were expressed > 10-fold higher in undifferentiated ESCs and 24 genes were expressed at > 10-fold higher levels in XEN cells (see Supplementary Table S4). As shown in Fig. 3C (left), genes *Nanog*, *Fgf4*, *Sox2*, *Klf4*,*Isl1*, *Ptn*, and *Nodal* showed > 100-fold higher expression in undifferentiated ESCs (range of 19924- to 101-fold higher). In contrast, the genes *Gata6*, *Gata4*, *Pdgfra*, *Sox17*, and *Lamb1* were expressed at 100-fold higher levels in XEN cells (range 1249- to 115 times higher expression).

*Comparison of undifferentiated ESCs with TS cells.* Twenty-four genes showed > 10-fold expression difference between undifferentiated rat ESCs and TS cells. Of these 24 genes, 16 genes were expressed > 10-fold higher in undifferentiated ESCs and 8 genes showed > 10-fold higher expression in XEN



(see Supplementary Table S5). As shown in Fig. 3C (right), *Nanog*, *Pou5f1* (*Oct4*), *Gdf3*, *Fgf4*, *Tcl1a*, *Stella*, *Sox2*, and *Nodal* were expressed at > 100-fold higher levels in undifferentiated rat ESCs (ranging from 10534 to 167 times higher expression); *Hand1* was expressed at > 100-fold higher levels in TS cells (1150 times higher expression).

*Comparison of 2i plus LIF with 4i derivation methods.* When germline ESCs derived in 2i plus LIF were compared with germline ESCs derived in 4i, 12 genes were found to exceed the > 10-fold difference in expression threshold, and only one gene was found to have > 100-fold difference in expression (see Supplementary Table S6). As shown in Fig. 3D (left), the gene *Rpl13a* was expressed 32-fold higher levels in 2i plus LIF germline ESCs and the genes *Actc1*, *Hox1a*, *Pax3*, *Myf5*, *Ecat1*, *Otx2*, *beta-globin*, *Kdr*, *T*, and *Hand1* were expressed at higher levels in 4i ESCs (range of 174- to 10-fold higher, respectively).

*Comparison of germline to chimera ESCs.* When germline ESC derived in 2i plus LIF were compared with ESCs derived in 2i plus LIF that only formed chimera, no genes were found to meet the criteria levels of > 10-fold difference in gene expression (see Supplementary Table S7). As shown in Fig. 3D (right), in germline 2i plus LIF ESCs, the gene *Cer1* was found to be expressed at 9-fold higher level. In chimera ESCs, *Kdr* was found to be expressed at 5-fold higher levels.

## Gene expression of selected genes

As shown in Fig. 4, when the fold expression was calculated and normalized using *Ldha* and *Gapdh* as HKG and MEFs as the control cell using the $2^{-\Delta\Delta Ct}$ method, undifferentiated rats ESCs expressed the transcription factors *Gdf3*, *Nanog*, *Pou5f1* (*Oct4*), and *Sox2* at relatively high levels and these clearly distinguish undifferentiated ESCs from the other groups. As we reported [6], *Cdx2* is expressed by undifferentiated rat ESCs (see Fig. 4, bottom left). Here, we noted that genuine rat ESCs expanded in 2i plus LIF express *Cdx2* at a lower level than TS cells. In contrast, genuine rat ESCs expanded in 4i medium (YPAC) express *Cdx2* at > 3-fold higher level than rat ESCs expanded in 2i plus LIF and at a higher level than rat TS cells. Thus, a tremendous variability in *Cdx2* expression was observed in genuine rat ESCs. It is also noted that differentiation of rat ESCs that were grown in 2i plus LIF conditions to EBs resulted in an increase of *Cdx2* expression. This might suggest that rat ESCs may easily differentiate along the extraembryonic lineage. This notion is supported by the induction of *Hand1*, *Eomes* and, *Fgfr2* by EB formation (*Eomes* and *Ffg42* shown in Fig. 4).

*The rat ESC array list.* Prior to normalization of the gene array data, PPC and RTC had the lowest variation followed by experimental genes *Gapdh*, *Ldha*, *Hdac2*, *Ctnnbl1*, and *Actb* (see Table 2). As presented above, these genes were evaluated as potential HKG using the Cotton EST database RefFinder tools and *Gapdh* and *Ldha* were selected. As shown in the bottom of Table 2, following normalization using HKG and averaging of the technical replicates, the variation of *Gapdh* and *Ldha* was < PPC and RTC, as expected. It is interesting to note that after normalization, the variation of *Hdac2*, *Ctnnbl1*,and *Actb* was < PPC and RTC, too (see Supplementary Table S1). This suggests that these 5 genes cluster.

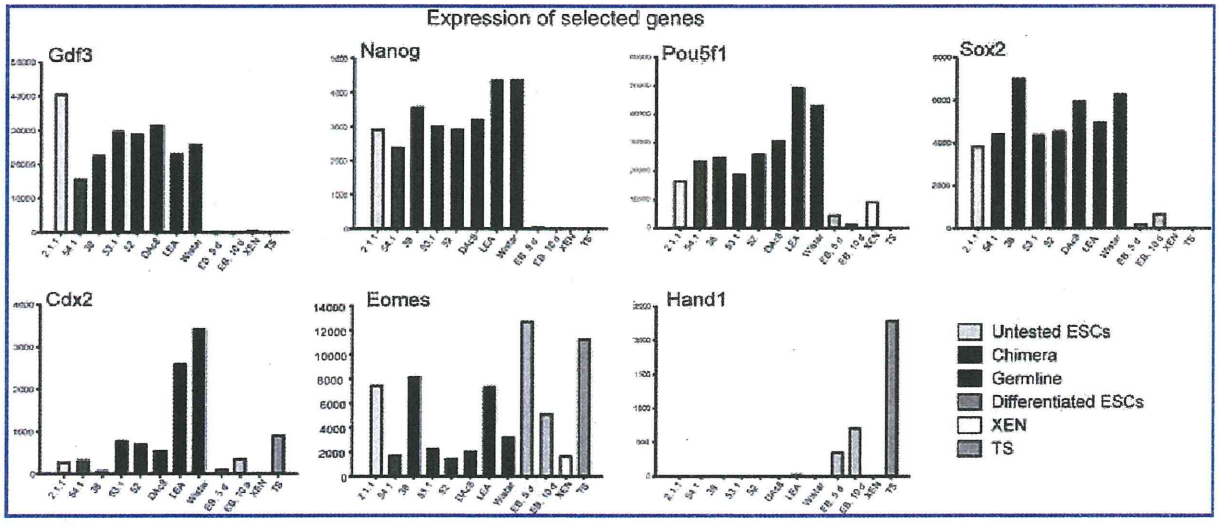



**FIG. 4.** Normalized expression of 7 genes by biological samples. Genes were selected based upon their ability to differentiate between the cell types, for example, undifferentiated rat ESCs, differentiated ESCs and XEN, and TS cells. Note that undifferentiated ESCs expressed the transcription factors *Gdf3*, *Nanog*, *Oct4* (*Pou5f1*), and *Sox2* at 1000-fold higher levels than differentiated ESCs or the XEN or TS cells. Note that the expression of these genes was decreased by differentiation of rat ESCs to EBs. Note that rat ESCs expressed markers of the trophoblast lineage, *Cdx2 and Eomes*, suggesting that rat ESCs may have a capacity to differentiate along this lineage. In support of this notion, differentiation of rat ESCs induces the expression of *Hand1*. Note that ESC derived from DA rats (lines 54.1, 53.1, 52, and DAc8), lines derived from F344 rats (line 2.1.1 and 38), and lines derived from Long Evans Agouti and Wistar (LEA and WIS), express the transcription factors *Gdf3*, *Nanog*, *Oct4* (*Pou5f1*), and *Sox2* at similar levels, and they express *Cdx2*, too. Note that all genuine ESCs: those ESCs derived and maintained in 2i plus LIF and ESCs derived and maintained in 4i conditions, express *Cdx2*; and that ESCs derived in 4i conditions express *Cdx2* at 3–4-fold higher levels than that ESCs derived in 2i plus LIF conditions.

TABLE 2. GENE LIST AND EFFICIENCY, PHYSICAL LAYOUT (WELL MAP)

| No. | Position | Cat | Gene symbol | Refseq # | Official full name | Ct | E (%) | R |
|---|---|---|---|---|---|---|---|---|
| 1 | A1 | PPR42970A | *Actc1* | NM_019183 | Actin, alpha, cardiac muscle 1 | 24.56 | 106.8% | 0.9995 |
| 2 | A2 | PPR44288A | *Afp* | NM_012493 | Alpha-fetoprotein | 23.75 | 112.7% | 0.9996 |
| 3 | A3 | PPR06570A | *Actb* | NM_031144 | Actin, beta | 24.80 | 104.6% | 0.9994 |
| 4 | A4 | PPR53512A | *Ctnnbl1* | NM_001024870 | Catenin, beta-like 1 | 25.07 | 108.8% | 0.9997 |
| 5 | A5 | PPR54901A | *LOC689064* | XM_001069372 | Beta-globin | 24.28 | 113.5% | 0.9992 |
| 6 | A6 | PPR44291A | *Bex1* | NM_001037365 | Brain expressed gene 1 | 23.32 | 111.9% | 0.9993 |
| 7 | A7 | PPR57030A | *Prdm1* | XM_228320 | PR domain-containing 1, with ZNF domain | 24.79 | 104.7% | 0.9998 |
| 8 | A8 | PPR06561A | *Bmp4* | NM_012827 | Bone morphogenetic protein 4 | 24.87 | 101.9% | 0.9996 |
| 9 | A9 | PPR59972A | *T* | XM_217890 | T, brachyury homolog (mouse) | 23.74 | 102.7% | 0.9998 |
| 10 | A10 | PPR55773A | *Cd34* | XM_223083 | CD34 molecule | 23.85 | 102.3% | 0.9999 |
| 11 | A11 | PPR51519A | *Cdx2* | NM_023963 | Caudal type homeo box 2 | 23.88 | 109.1% | 0.9995 |
| 12 | A12 | PPR66629A | *RGD1563046* | XR_008686 | Similar to cerberus-like | 24.79 | 146.8% | 0.9989 |
| 13 | B1 | PPR45580A | *Myc* | NM_012603 | Myelocytomatosis oncogene | 24.14 | 107.2% | 0.9991 |
| 14 | B2 | PPR68946A | *Dazl* | NM_001109414 | Deleted in azoospermia-like | 27.48 | 103.5% | 1.0000 |
| 15 | B3 | PPR45645A | *Dkk3* | NM_138519 | Dickkopf homolog 3 (Xenopus laevis) | 25.74 | 109.9% | 0.9995 |
| 16 | B4 | PPR42793A | *Mapk3* | NM_017347 | Mitogen activated protein kinase 3 | 23.26 | 109.6% | 0.9997 |
| 17 | B5 | PPR68945A | *Dppa2* | XM_001063497 | Developmental pluripotency associated 2 | 24.81 | 135.4% | 0.9985 |
| 18 | B6 | PPR68425A | *LOC685378* | XM_001063552 | Similar to developmental pluripotency associated 4 isoform 1 | 21.84 | 109.4% | 0.9997 |
| 19 | B7 | PPR47830A | *Pias1* | XM_217188, XM_001074210, NM_001106829 | Protein inhibitor of activated STAT, 1 | 24.82 | 111.8% | 0.9988 |
| 20 | B8 | PPR57113A | *Eomes* | XM_001061749 | Eomesodermin homolog (Xenopus laevis) | 25.03 | 117.4% | 0.9975 |
| 21 | B9 | PPR66519A | *Ecat1* | XM_001053599 | ES cell-associated transcript 1 | 25.53 | 113.4% | 0.9995 |
| 22 | B10 | PPR55731A | *Cdcp1* | XM_236747 | CUB domain-containing protein 1 | 24.11 | 112.1% | 0.9998 |
| 23 | B11 | PPR59431A | *Esrrb* | NM_001008516 | Estrogen-related receptor beta | 25.91 | 90.9% | 0.9992 |
| 24 | B12 | PPR47773A | *Fbxo15* | XM_341633 | F-box protein 15 | 23.92 | 109.7% | 0.9999 |
| 25 | C1 | PPR52297A | *Fgf4* | NM_053809 | Fibroblast growth factor 4 | 23.99 | 134.5% | 0.9990 |
| 26 | C2 | PPR06647A | *Fgf5* | NM_022211 | Fibroblast growth factor 5 | 24.09 | 94.7% | 0.9998 |
| 27 | C3 | PPR06654A | *Fgfr2* | XM_341940 | Fibroblast growth factor receptor 2 | 26.19 | 101.4% | 0.9999 |
| 28 | C4 | PPR45134A | *Foxa2* | NM_012743 | Forkhead box A2 | 27.63 | 90.5% | 0.9998 |
| 29 | C5 | PPR59440A | *Foxd3* | XM_575873 | Forkhead box D3 | 25.14 | 93.3% | 0.9992 |
| 30 | C6 | PPR48683A | *Gata2* | NM_033442 | GATA-binding protein 2 | 25.02 | 91.4% | 0.9976 |
| 31 | C7 | PPR48066A | *Gata4* | NM_144730 | GATA-binding protein 4 | 26.00 | 91.4% | 0.9997 |
| 32 | C8 | PPR66727A | *Gata5* | NM_001024316 | GATA-binding protein 5 | 24.37 | 106.2% | 0.9994 |
| 33 | C9 | PPR44170A | *Gata6* | NM_019185 | GATA-binding protein 6 | 23.91 | 107.3% | 1.0000 |
| 34 | C10 | PPR53058A | *Gbx2* | XM_346072 | Gastrulation brain homeobox 2 | 23.82 | 108.9% | 0.9996 |
| 35 | C11 | PPR50097A | *Nr6a1* | XM_342427 | Nuclear receptor subfamily 6, group A, member 1 | 24.36 | 102.9% | 1.0000 |
| 36 | C12 | PPR63353A | *Gdf3* | XM_575661 | Growth differentiation factor 3 | 24.13 | 104.1% | 0.9995 |
| 37 | D1 | PPR49194A | *Hand1* | NM_021592 | Heart and neural crest derivatives expressed 1 | 23.84 | 115.6% | 0.9998 |
| 38 | D2 | PPR42588A | *Hdac2* | XM_342149 | Histone deacetylase 2 | 24.09 | 101.9% | 1.0000 |
| 39 | D3 | PPR44530A | *Inhba* | NM_017128 | Inhibin beta-A | 24.49 | 98.6% | 0.9993 |
| 40 | D4 | PPR48904A | *Isl1* | NM_017339 | ISL LIM homeobox 1 | 24.39 | 111.4% | 0.9999 |
| 41 | D5 | PPR06671A | *Kdr* | NM_013062 | Kinase insert domain protein receptor | 24.00 | 106.7% | 0.9997 |
| 42 | D6 | PPR43919A | *Klf4* | NM_053713 | Kruppel-like factor 4 (gut) | 23.21 | 104.9% | 1.0000 |
| 43 | D7 | PPR50208A | *Lamb1* | XM_216679 | Laminin, beta 1 | 24.56 | 113.2% | 0.9995 |
| 44 | D8 | PPR63360A | *Lin28* | XM_575928 | Lin-28 homolog (C. elegans) | 22.98 | 104.5% | 0.9997 |
| 45 | D9 | PPR44878A | *Ascl2* | NM_031503 | Achaete-scute complex homolog 2 (Drosophila) | 26.78 | 110.9% | 0.9978 |
| 46 | D10 | PPR48578A | *Ascl1* | NM_022384 | Achaete-scute complex homolog 1 (Drosophila) | 24.84 | 126.0% | 0.9994 |
| 47 | D11 | PPR65449A | *RGD1564419* | XM_575480 | Similar to hypothetical gene supported by BC025338 | 25.04 | 131.7% | 0.9999 |
| 48 | D12 | PPR63361A | *Myf5* | XM_235101 | Myogenic factor 5 | 23.95 | 110.2% | 0.9996 |
| 49 | E1 | PPR44362A | *Myod1* | NM_176079 | Myogenic differentiation 1 | 24.98 | 112.1% | 0.9990 |
| 50 | E2 | PPR59663A | *Nanog* | XM_575662 | Nanog homeobox | 24.05 | 113.9% | 0.9995 |
| 51 | E3 | PPR44422A | *Nes* | NM_012987 | Nestin | 23.82 | 110.7% | 0.9994 |

(*Continued*→)