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Embryonic Stem Cell Culture Conditions Support Distinct States Associated with Different Developmental Stages and Potency

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SUMMARY

Embryonic stem cells (ESCs) are cell lines derived from the mammalian pre-implantation embryo. Here we assess the impact of derivation and culture conditions on both functional potency and ESC transcriptional identity. Individual ESCs cultured in either two small-mole-cule inhibitors (2i) or with knockout serum replacement (KOSR), but not serum, can generate high-level chimeras regardless of how these cells were derived. ESCs cultured in these conditions showed a transcriptional correlation with early pre-implantation embryos (E1.5–E3.5) and contributed to development from the 2-cell stage. Conversely, the transcriptome of serum-cultured ESCs correlated with later stages of development (E4.5), at which point embryonic cells are more restricted in their developmental potential. Thus, ESC culture systems are not equivalent, but support cell types that resemble distinct developmental stages. Cells derived in one condition can be reprogrammed to another developmental state merely by adaptation to another culture condition.

INTRODUCTION

Embryonic stem cells (ESCs) are karyotypically normal, self-renewing cell lines, derived from the inner cell mass (ICM) of the pre-implantation embryo (Evans and Kaufman, 1981; Martin, 1981). ESCs can be derived and expanded using a variety of conditions, including culture with the cytokine leukemia inhibitory factor (LIF) in the presence of serum (Smith et al., 1988; Williams et al., 1988), in serum-free medium with two small-molecule inhibitors (2i) (Ying et al., 2008), or with knockout serum replacement (KOSR) (Ward et al., 2002). ESCs can be maintained indefinitely in vitro, while retaining the capacity to participate in development and generate all cell types of the embryo including the germ cells (Beddington and Robertson, 1989; Gossler et al., 1986; Lallemand and Brulet, 1990; Robertson et al., 1986; Suemori et al., 1990). They are therefore said to be pluripotent.

Although the first ESCs were derived more than 30 years ago, a number of fundamental questions remain unanswered. At the embryonic stages from which ESCs are derived, the blastocyst is composed of several cell types, the epiblast (Epi), primitive endoderm (PrE), and trophoblast, and, during ESC derivation, subpopulations of embryo-derived cells are selected to expand. While these populations are not the same as the parental embryonic cells from which they are derived (Tang et al., 2010), to what degree do they represent embryonic development? ESC cultures are also heterogeneous (Canham et al., 2010; Chambers et al., 2007; Hayashi et al., 2008; Kobaya-shi et al., 2009; Singh et al., 2007; Toyooka et al., 2008) and this heterogeneity is dynamic, perhaps more dynamic than the blastocyst from which they are derived. However, does this heterogeneity reflect the endogenous cell populations that arise in normal blastocyst development?

The functional potential of ESCs can be assessed using a number of different approaches including in vitro differentiation, teratoma formation, and chimera generation (Beddington and Robertson, 1989; Poueymirou et al., 2007; Robertson et al., 1986; Saburi et al., 1997). Nevertheless, as ESCs are heterogeneous and chimeras are routinely generated by injecting 10–15 ESCs into morula or blastocyst-stage embryos (Bradley et al., 1984; Lallemand and Brulet, 1990) it is difficult to discern the functional properties of individual ESCs or specific ESC subpopulations. Based on the prospective isolation of ESC subpopulations, it has been shown that ESCs cultured in serum and LIF contain dynamic populations of PrE- and Epi-biased cells (Canham et al., 2010). However, these cells are clearly different from the blastocyst from which they are derived, as the PrE-primed cells express elevated levels of PrE RNA, but not protein. ESCs cultured under these conditions also contain a subpopulation that expresses 2-cell embryo (2C)-specific genes (Falco et al., 2007; Macfarlan et al., 2012). Similarly, culture of ESCs in 2i supports a totipotent population of cells that coexpress Epi determinants such as *Nanog* and the RNA for extraembryonic genes such as *Gata6* or *Hhex* (Morgani et al., 2013).

So, how do the conditions used to maintain ESCs influence the gene-expression state and populations contained
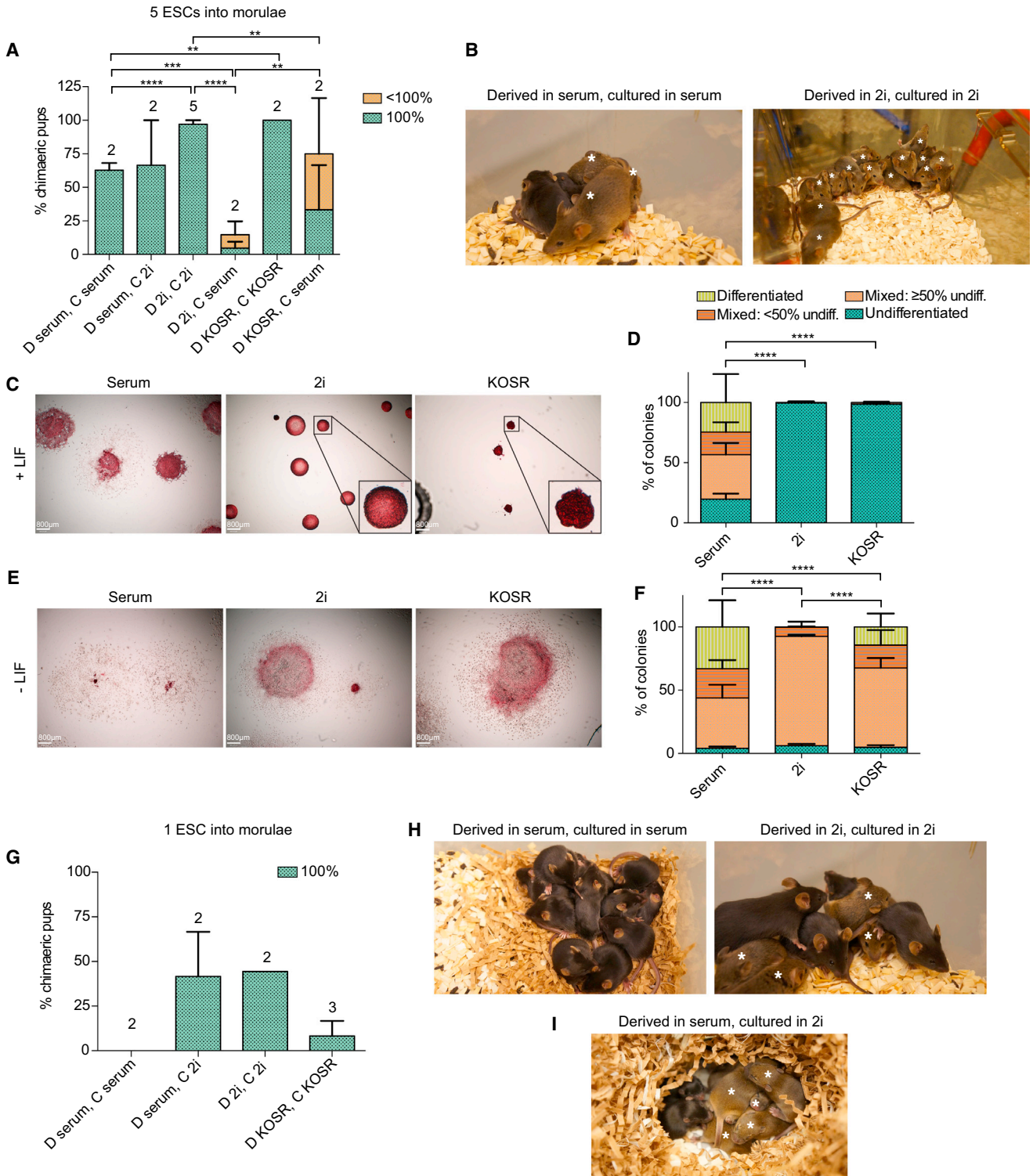
Figure 1. KOSR and 2i Culture Enhances ESC Single-Cell Potency

ESCs were derived (D) in and cultured (C) under various conditions; D in serum and C in serum, D in serum and C in 2i, D in 2i and C in 2i, and D in KOSR and C in KOSR.

(A and B) Multiple (5) ESCs were injected into morulae and their contribution to full-term chimeras assessed according to coat color.

(A) Graph showing the percentage of chimeras born out of the total number of pups and quantification of coat color chimerism. n Values are

(legend continued on next page)

within the culture? In this paper we explore this question by testing the impact of culture and derivation conditions on ESC populations, comparing ESC gene expression and heterogeneity, and the capacity of individual ESCs to contribute to full-term embryonic development. We found that ESCs maintained in standard serum culture conditions were comparable to populations of the late blastocyst (embryonic day 4.5 [E4.5]) ICM, at which point cells are specified and restricted in their functional potential. Conversely, ESCs cultured in 2i or KOSR showed a correlation with embryos from as early as the 2C stage, when cells are unrestricted and highly plastic. Consistent with expression data, we observed that single 2i and KOSR, but not serum, cultured ESCs could generate high-level chimeras when injected into either morulae or 2C embryos. This suggests that different ESC culture conditions support the expansion of populations reminiscent of different embryonic stages with distinct functional potentials. We found that populations induced during derivation could be "reprogrammed" by transferring ESCs to a different culture condition.

RESULTS

KOSR and 2i Culture Enhances ESC Single-Cell Potency

ESC lines have been derived and maintained in a number of different medium compositions (Smith et al., 1988; Ward et al., 2002; Williams et al., 1988; Ying et al., 2008). We sought to compare the functional potential of individual ESCs derived and/or maintained by different protocols. As ESCs of different genetic backgrounds generate chimeras with variable efficiencies (Auerbach et al., 2000; Brook and Gardner, 1997), we wished to start with high-quality ESC lines capable of generating chimeras from single cells. We

therefore utilized mice from an F1 hybrid genetic background, reported to generate chimeras with high efficiency based on hybrid vigor (Eggan et al., 2001). We derived F1 129S2;C57BL/6N hybrid ESC lines in conventional serum/LIF (serum) culture conditions, as well as 2i/LIF (2i) and KOSR/LIF (KOSR). We confirmed that resulting ESC lines were of high quality by standard injection of multiple ESCs into morulae. ESC lines generated 100% ESC-derived mice as judged by coat color and internal organs (Figures 1A, 1B, S1A, and S1B; Table 1) and contributed to the germline (n = 15/15, Table 2). However, ESCs derived in 2i, but switched to serum medium, showed a significant reduction in their capacity to generate chimeras in this assay (Figure 1A).

We also assessed the capacity of these ESC lines for clonal self-renewal in vitro. Single ESCs were expanded and stained for alkaline phosphatase activity, a marker of undifferentiated cell types (Figures 1C–1F and S1C). While the majority of 2i- and KOSR-cultured ESC colonies were entirely undifferentiated, a significant fraction of serum-cultured colonies exhibited some degree of differentiation (Figures 1C and 1D). When challenged to differentiate in the absence of the cytokine LIF, all ESCs efficiently differentiated, although fewer colonies were entirely differentiated when ESCs were previously cultured in 2i or KOSR (Figures 1E and 1F). This suggested that individual cells in these conditions retain some intrinsic capacity to self-renew despite exposure to differentiation-promoting signals. There was no difference in the proliferation rate of ESCs in each condition (Figure S1D).

After this initial validation of ESC lines, we wished characterize the impact of serum, 2i, and KOSR culture on the potency of individual ESCs. To this end we injected single ESCs into morulae. Single ESCs, derived and cultured in 2i, generated 100% ESC-derived mice, as judged by coat color (Figures 1G and 1H; Table 1). ESCs cultured in KOSR

provided above bars and refer to the number of independent ESC clones tested for each condition. Error bars indicate SEM. Results for 2i conditions are a combination of ESCs cultured with and without mouse embryonic fibroblasts (MEFs) to control for the effect that MEFs may have in serum and KOSR cultures. No difference was observed with or without MEFs. **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001, Student's unpaired t test. (B) Representative images of litters of mice. Asterisks indicate mice with ESC contribution.
(C and D) ESCs were cultured in serum, 2i, or KOSR and stained for alkaline phosphatase activity. (C) Representative images of ESC colonies. (D) Quantification of ESC colony categories as defined in Figure S1C. Error bars represent SEM of three biological replicates using independent ESC lines. ****p ≤ 0.0001, two-tailed chi-square test.
(E and F) ESCs were cultured in serum, 2i, or KOSR and then differentiated by removal of LIF for 5 days. Cells were stained for alkaline phosphatase activity. (E) Representative images of differentiated colonies. (F) Quantification of ESC colony categories as defined in Figure S1C. Error bars represent SEM of three biological replicates of independent ESC lines. ****p ≤ 0.0001, two-tailed chi-square test.
(G–I) Single ESCs were injected into morulae and their contribution to full-term chimeras assessed according to coat color. (G) Graph showing the percentage of chimeras born out of the total number of pups and quantification of coat color chimerism. n Values are provided above bars and refer to the number of independent ESC clones tested for each condition. Error bars indicate SEM. Results for 2i conditions are a combination of ESCs cultured with and without MEFs to control for the effect that MEFs may have in serum and KOSR cultures. No difference was observed with or without MEFs. (H and I) Representative images of litters of mice. Asterisks indicate mice with ESC contribution.
See also Figure S1.

Table 1. Chimera Frequencies, Related to Figures 1 and 3

Condition	Line	Injected/Transferred	Chimeras/Pups
1 ESC into Morula			
D serum, C serum	3.4 + MEFS	25	0/11
	3.2 + MEFs	25	0/2
D serum, C 2i	3.4 + MEFs	31	6/9
	3.2 + MEFs	19	1/6
D 2i, C 2i	2.2H + MEFs	38	4/9
D KOSR, C KOSR	5.3 + MEFs	30	0/2
	5.45 + MEFs	i. 30 ii. 45	i. 0/5 ii. 2/8
5 ESCs into Morula			
D serum, C serum	3.4 + MEFs	i. 17 ii. 40	i. 3/5 ii. 1/2
	3.2 + MEFs	i. 24 ii. 30	i. 3/4 ii. 2/3
D serum, C 2i	3.4 + MEFs	12	4/4
	3.2 + MEFs	20	2/6
D 2i, C 2i	2.2H + MEFs	i. 25 ii. 56	i. 4/5 ii. 21/21
	2.2Ack1#5 + MEFs	13	1/1
	2.2Ack1#11 + MEFs	15	6/6
	2.2Ack1#28 + MEFs	15	6/6
	2.2Ack1#14 + MEFs	15	4/4
	2.2PTIP#8 + MEFs	42	4/5 (1 < 100%)
	2.2PTIP#9 + MEFs	30	4/4
D 2i, C serum	2.5	30	0/1
	2.2	30	1/7
D KOSR, C KOSR	5.3 + MEFs	30	14/14
	5.45 + MEFs	25	3/3
D KOSR, C serum	5.3 + MEFs	28	4/6
	5.45 + MEFs	25	5/6 (5 < 100%)
1 ESC into 2C Embryo			
D serum, C serum	3.4 + MEFs	24	0/6
	3.2 + MEFs	13	0/8
D serum, C 2i	3.4 + MEFs	24	5/6
	3.2 + MEFs	14	2/6
D 2i, C 2i	2.5	30	2/18
	2.5 + MEFs	30	0/7
	2.2	59	6/11
	2.2 + MEFs	30	3/8
	2.6	26	3/18 (1 < 100%)
	2.2H + MEFs	24	7/14 (2 < 100%)
	2.2V + MEFs	58	5/11 (1 < 100%)
D 2i, C serum	2.2	28	0/6
	2.5	17	0/5
D KOSR, C KOSR	5.3 + MEFs	13	2/5
	5.45 + MEFs	13	1/3
5 ESCs into 2C Embryo			
D serum, C serum + MEFs	3.4	48	0/3
D serum, C 2i + MEFs	3.4	40	6/7

ESCs were derived (D) in and cultured (C) under various conditions. Table shows the number of embryos injected and transferred and the resulting number of chimeras out of total pups born from each cell line under each derivation and culture condition. Individual injection experiments of the same clone are marked as (i) and (ii). Results are shown for single- and multiple-cell injection into morulae and single- and multiple-cell injection into 2-cell (2C) embryos.

also demonstrated this capacity, albeit to a lower extent (Figure 1G and Table 1). Conversely, ESCs derived and cultured in serum did not generate full-term chimeras when injected as single cells into morulae (Figures 1G and 1H; Table 1). We also found that ESCs derived in serum conditions were not inherently limited in their capacity to generate chimeras as, after transient transfer of these cells to 2i (three passages), single cells now contributed to embryos (Figures 1G and 1I; Table 1). This suggests that in chimera assays, as in vitro (Figures 1C and 1D), culture in 2i or KOSR increases the clonogenicity of ESCs compared with standard serum culture.

2i- and KOSR-Cultured ESCs Represent Early Stages of Embryonic Development

To determine the transcriptional landscape related to these functional differences, we performed microarray expression analysis on ESCs cultured in these conditions (GEO: GSE72445). When compared with serum cultures, 1,473

Table 2. Germline Contribution, Related to Figures 1 and 3

| Condition | Line | Experimental Setup Mice Were Generated from: | |
		1 ESC into 2C Embryo	5 ESCs into Morula
D serum, C serum	3.2	–	1/1
	3.4	–	1/1
D 2i, C 2i	2.2	3/3	3/3
	2.5	1/1	2/2
	2.6	1/1	1/1
D KOSR, C KOSR	5.3	–	2/2
	5.45	–	2/2

F1 129S6;C57BL/6N ESCs were derived (D) in and cultured (C) under various conditions. Chimeric mice were generated by different means (described in table headings) and then tested for ESC germline contribution. Results show the number of mice demonstrating germline transmission out of the number of mice tested per condition.

genes were upregulated and 2,249 genes were downregulated in 2i, and 546 genes were upregulated and 493 genes were downregulated in KOSR (Figure 2A, Tables S1–S4, FDR ≤0.05, fold change ≥2). Genes upregulated in 2i and KOSR showed a strong signature for lipid and glutathione metabolism (Tables S5 and S6), while genes upregulated in KOSR were also associated with meiosis and anterior-posterior patterning (Table S6). Downregulated genes in 2i were associated with extracellular matrix, differentiation, and the mitogen-activated protein kinase pathway (Table S7). Similarly, genes downregulated in KOSR were related to cell junctions, matrix adhesion, and fibroblast growth factor (FGF) signaling (Table S8). Consistent with previous reports (Marks et al., 2012; Morgani et al., 2013), we also observed that 2i cultures downregulated mesoderm and ectoderm markers while having little effect on endodermal gene expression (Figure 2B). However, KOSR culture increased the expression of endoderm markers but had little effect on pluripotent gene expression (Figure 2B). The coexpression of pluripotency and extraembryonic genes is an expression state previously associated with totipotency (Morgani et al., 2013).

As 2i- and KOSR-cultured ESCs both generated high-level chimeras from single cells, we asked whether they shared a common gene-expression signature. As serum-cultured ESCs did not demonstrate this property, we overlapped genes that were differentially expressed in 2i or KOSR compared with standard serum cultures (Tables S1–S4) and found a highly significant, non-random overlap (Figure 2C). In KOSR-cultured ESCs of annotated genes that were significantly changing in expression, 46% of upregu-

lated (representation factor 5.5, $p < 8.1 \times 10^{-84}$) and 62% of downregulated genes (representation factor 5.0, $p < 6.7 \times 10^{-101}$) overlapped with those up- or downregulated in 2i (Figure 2C and Table S9). Common upregulated genes were associated with germ cell development, e.g., *Dazl*, *Hormad1*, and *Sohlh2*, while downregulated genes were enriched for adhesion, migration, and differentiation markers, e.g., *Lamb3*, *Egfr*, and *Neurod1* (Table S10).

As the correlation between KOSR-cultured ESCs and PrE had not previously been reported, we investigated this in more detail. We confirmed by qPCR that the PrE markers *Gata4*, *Gata6*, *Dab2*, and *FoxA2* were upregulated in KOSR compared with either serum or 2i conditions (Figure S2A). KOSR culture also resulted in elevated expression of a *Hhex*-Venus (HV) transgene, which marks a PrE-primed ESC population (Canham et al., 2010) (Figure 2D). We observed that expression of the *Nanog*-GFP reporter (Chambers et al., 2007) was increased in KOSR cultures compared with serum cultures, although not to levels as high as in 2i (Figure 2E). In serum cultures, the PrE marker *Hhex* and the Epi marker *Nanog* are expressed mutually exclusively, whereas in 2i they are coexpressed (Canham et al., 2010; Morgani et al., 2013). Similar observations were made in KOSR, where the *Hhex*-expressing subpopulation contained a high number of *Nanog*-positive cells (Figure S2B). We also observed that KOSR cultures contained cells that coexpressed OCT4, SOX2, and GATA6 protein not observed in either serum or 2i cultures (Figures 2F and S2C). Similar observations were made by flow cytometry, as we observed a fraction of ESCs expressing the PrE marker platelet-derived growth factor receptor α (PDGFRα) in KOSR, but not serum or 2i (Figures S2D and S2E).

As there were marked transcriptional and functional differences between ESCs maintained in serum, 2i, or KOSR, we asked how these expression states compared with gene expression during embryonic development. We compared our microarray data with expression data from pre-implantation embryo populations (Ohnishi et al., 2014) using ExAtlas (FDR ≤0.05, fold change ≥2) (Sharov et al., 2015). We observed a correlation between ESCs cultured in 2i and KOSR and early embryos (E3.25) (Figure 2G and Table 3, z = 3.5 and 3.6, respectively), when ICM cells are unspecified and have a wide functional potency (Grabarek et al., 2012; Rossant and Lis, 1979). This is consistent with our previous observation that 2i cultures contain totipotent cells (Morgani et al., 2013). In addition, 2i-cultured ESCs showed a correlation with E3.5 early Epi (Figure 2G and Table 3, z = 7.4), which may reflect a second population of Epi-like *Nanog*-positive, *Hhex*-negative cells. As KOSR-cultured ESCs express endoderm markers, it is not surprising that gene expression in these conditions correlates with all stages of PrE development (Figure 2G and Table 3, E3.5 PrE z = 7.5, E4.5 PrE z = 9.4). Conversely,
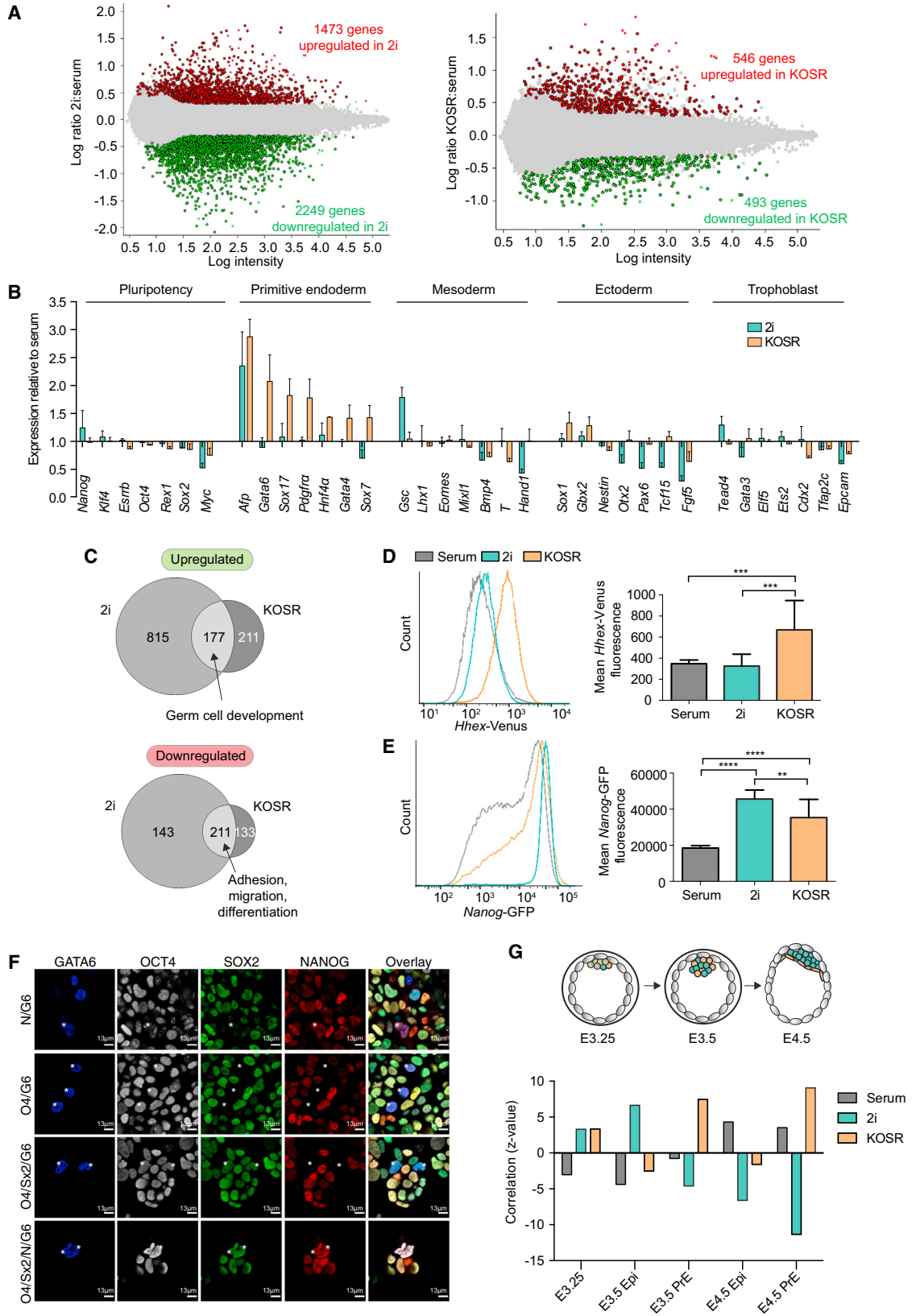
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gene expression in serum-cultured ESCs is correlated with later E4.5 embryos (Figure 2G and Table 3, E4.5 Epi z = 4.5, E4.5 PrE z = 3.3) when Epi and PrE are fully specified and functionally restricted (Grabarek et al., 2012). As ESCs cultured in serum undergo a degree of spontaneous differentiation into cell types reminiscent of the later embryo, these differentiated cells could also contribute to the increased correlation of the serum transcriptome to later developmental stages. This spontaneous differentiation is inhibited by culture in either 2i or KOSR.

2i- and KOSR-Cultured ESCs Can Contribute to 2C Embryos

As gene-expression profiles of 2i and KOSR-cultured ESCs correlated with the early blastocyst (Figure 2G), in addition to containing subpopulations that coexpressed embryonic and extraembryonic determinants (Figure 2E) (Morgani et al., 2013), reminiscent of early developmental stages, we asked whether they showed a correlation to even earlier stages of development. We compared our microarray data with RNA-sequencing data on oocytes and 2C embryos (Macfarlan et al., 2012) (ExAtlas, FDR $\leq$0.05, fold change $\geq$2). Gene expression of ESCs cultured in 2i and KOSR was correlated with data from 2C embryos, but not oocytes (Figure 3A, z values of 5.1 and 2.4, respectively). However, these correlations were not as strong as those to later stages of development (Figure 2G). Conversely, serum-cultured ESCs showed a correlation with oocytes (z = 5.6) but not 2C embryos (z = −5.6) (Figure 3A). This may be a result of the correlation in gene expression between the late blastocyst and oocytes that we found when re-analyzing published datasets (Figure S3A). To further assess the significance of a potential 2C-like population, we asked whether culture in different conditions could induce the expression of a reporter for the 2C-specific marker (Falco et al., 2007; Hirata et al., 2012), *Zscan4*. An induction of *Zscan4*-Emerald was observed in both 2i and KOSR (Figures 3B and S3B).

Based on the capacity of 2i- and KOSR-cultured ESCs to stimulate extraembryonic and 2C gene expression, we asked whether they were capable of contributing to the earliest stages of embryonic development. We injected single 2i-cultured ESCs labeled with H2B-Tomato into 2C embryos (Figure 3C). In the majority of cases (n = 8/9), 100% of NANOG$^+$ Epi cells were generated from the single injected ESC (Figures 3D, S3C, and S3D). We also observed a contribution of ESC progeny to the extraembryonic trophoblast (Figure S3C). While 2i-cultured ESCs introduced at the 2C stage divided and populated the ICM, the majority of serum-cultured cells were excluded during pre-implantation development (Figures S3E and S3F).

Despite the high levels of chimerism observed in single-cell injections as late as mid-gestation (Figure S3D) (Morgani et al., 2013), no live-born chimeras were obtained from 129/Ola lines injected as single cells. In contrast, full-term ESC-derived mice were obtained from the injection of single F1 129S2;C57BL/6N hybrid 2i- or KOSR-cultured ESCs, but not from those cultured in serum (Figures 3G–3I and Table 1). As with morula injections, these mice showed a high, virtually homogeneous contribution to ectoderm, judged by coat color and internal organs (Figures S3G and S3I). All tested ESC-derived mice contributed to the germline (n = 5/5, Table 2). Similar to the single-cell morula injections (Figures 1G and 1I), transfer of serum-derived ESCs to 2i culture endowed these cell lines with the capacity to contribute to 2C embryos (Figures 3F and 3G; Table 1). In addition, when 2i-derived ESCs were switched to serum-containing medium, either directly or by gradual adaptation, these cells lost this capacity (Figure 3F).

We then asked whether the inability of serum-cultured ESCs to contribute to 2C embryos was due to their

Figure 2. KOSR, Serum, and 2i Support Unique Transcriptional States

(A and B) Microarrays were carried out on 129/Ola ESCs cultured in serum, 2i, and KOSR conditions. At least three biological replicates of independent ESC lines were analyzed per condition. (A) Pairwise comparisons (FDR <0.05, $\geq$2-fold expression levels) were performed between serum-cultured and 2i- or KOSR-cultured ESCs to reveal non-redundant, significant changes in gene expression. Red dots represent significantly upregulated genes, green dots represent significantly downregulated genes, and gray dots represent genes that are not significantly changing in expression. (B) Expression changes in lineage markers in 2i- and KOSR-cultured ESCs relative to the expression in standard serum cultures.

(C) Venn diagrams illustrating the overlap between genes up- and downregulated in 2i and KOSR compared with standard serum cultures (identified in A).

(D and E) Flow cytometry profiles and corresponding mean fluorescence values for PECAM-1$^+$ HV (D) or *Nanog*-GFP TNGB (E) ESCs cultured in serum, 2i, and KOSR. n = 3 independent experiments. Error bars indicate SD of the mean. **p $\leq$ 0.01, ***p $\leq$ 0.001, ****p $\leq$ 0.0001, Student's unpaired t test.

(F) Confocal optical section of ESCs cultured in serum, 2i, and KOSR immunostained for GATA6, OCT4, SOX2, and NANOG. Asterisks indicate coexpressing ESCs. The same observation was made in two independent cell lines.

(G) Graph showing the correlation of serum-, 2i-, and KOSR-cultured ESCs with published microarray data of different embryonic stages (Ohnishi et al., 2014).

See also Figure S2.

Table 3. Lists of Genes Correlated between 2i- and KOSR-Cultured ESCs and Different Stages of Embryonic Development, Related to Figure 2

Symbols	EPFP	Gene Title	RefSeq
E3.25 versus 2i (z Value, 3.508; Correlation, 0.1001)			
Bhmt2	0.0237	betaine-homocysteine methyltransferase 2	NM_022884
Spic	0.0283	Spi-C transcription factor (Spi-1/PU.1 related)	NM_011461
Jakmip1	0.1569	janus kinase and microtubule interacting protein 1	NM_178394
Ly6a	0.2471	lymphocyte antigen 6 complex, locus A	NM_010738
Gsc	0.2702	goosecoid homeobox	NM_010351
Rab38	0.2745	RAB38, member of RAS oncogene family	NM_028238
Khdc1a	0.2745	KH domain-containing 1A	NM_183322
Fxyd4	0.2859	FXYD domain-containing ion transport regulator 4	NM_033648
Bdh1	0.319	3-hydroxybutyrate dehydrogenase, type 1	NM_175177
Bcl2l14	0.3263	BCL2-like 14 (apoptosis facilitator)	NM_025778
Cdkn1a	0.4118	cyclin-dependent kinase inhibitor 1A (P21)	NM_007669
Gss	0.4612	glutathione synthetase	NM_008180
Pdp2	0.4887	pyruvate dehydrogenase phosphatase catalytic subunit 2	NM_001024606
E3.25 versus KOSR (z Value, 3.59; Correlation, 0.1024)			
Pdgfra	0.1396	platelet-derived growth factor receptor, α polypeptide	NM_011058
Cphx1	0.3658	cytoplasmic polyadenylated homeobox 1	NM_175342
Gata6	0.3658	GATA binding protein 6	NM_010258
Pnliprp2	0.3658	pancreatic lipase-related protein 2	NM_011128
E3.5 Epi versus 2i (z Value, 7.351; Correlation, 0.2063)			
Spic	0.0087	Spi-C transcription factor (Spi-1/PU.1 related)	NM_011461
Gsc	0.1293	goosecoid homeobox	NM_010351
Aspa	0.1293	aspartoacylase	NM_023113
Gsta2	0.1293	glutathione S-transferase, α 2 (Yc2)	NM_008182
Mal	0.3835	myelin and lymphocyte protein, T cell differentiation protein	NM_010762
Khdc1a	0.4185	KH domain-containing 1A	NM_183322
E3.5 PrE versus KOSR (z Value, 7.538; Correlation, 0.2113)			
Pdgfra	0.1232	platelet-derived growth factor receptor, α polypeptide	NM_011058
Cubn	0.1416	cubilin (intrinsic factor-cobalamin receptor)	NM_001081084
Tcf23	0.1416	transcription factor 23	NM_053085
Gata4	0.236	GATA binding protein 4	NM_008092
Cpn1	0.236	carboxypeptidase N, polypeptide 1	NM_030703
Gata6	0.2364	GATA binding protein 6	NM_010258
Gdpd5	0.2364	glycerophosphodiester phosphodiesterase domain-containing 5	NM_201352

(Continued on next page)

Table 3. Continued

Symbols	EPFP	Gene Title	RefSeq
Srgn	0.2364	serglycin	NM_011157
Meis1	0.2364	Meis homeobox 1	NM_010789
Sox17	0.2364	SRY (sex determining region Y)-box 17	NM_011441
Pth1r	0.2389	parathyroid hormone 1 receptor	NM_011199
Bmp6	0.247	bone morphogenetic protein 6	NM_007556
Aqp8	0.26	aquaporin 8	NM_007474
E4.5 PrE versus KOSR (z Value, 9.405; Correlation, 0.2604)			
Cubn	0.0421	cubilin (intrinsic factor-cobalamin receptor)	NM_001081084
Sox7	0.3368	SRY (sex determining region Y)-box 7	NM_011446
Srgn	0.3378	serglycin	NM_011157
Nostrin	0.3378	nitric oxide synthase trafficker	NM_181547
Pdgfra	0.3378	platelet-derived growth factor receptor, α polypeptide	NM_011058
Sox17	0.3439	SRY (sex determining region Y)-box 17	NM_011441
Gata4	0.3536	GATA binding protein 4	NM_008092
Pth1r	0.3953	parathyroid hormone 1 receptor	NM_011199
Ctsh	0.4678	cathepsin H	NM_007801

Pearson correlation of our microarray data of ESCs cultured in serum, 2i, and KOSR and microarrays on different stages of embryonic development (Ohnishi et al., 2014) was determined using ExAtlas online tool for gene expression meta-analysis. The list of correlated genes (FDR ≤0.1, fold change ≥1.5), z value, and correlation value is shown. EPFP, expected proportion of false positives.

previously observed reduced clonogenicity (Figure 1G). We injected five ESCs into 2C embryos but no full-term chimeras were obtained (Figure 3H and Table 1). When these serum-derived ESCs were switched to 2i culture, the injection of multiple ESCs into 2C embryos resulted in six out of seven 100% ESC-derived mice (Figure 3H and Table 1). This suggests that, as well as exhibiting a lower level of clonogenicity in morula injection, serum-cultured ESCs cannot contribute to early 2C embryos, even when injected as multiple cells.

We recently showed that a totipotent fraction of 2i-cultured cells is characterized by the expression of an HV transgene and the coexpression of embryonic and extra-embryonic determinants within individual cells (Morgani et al., 2013). These cells also demonstrated an enhanced capacity to differentiate toward the extraembryonic lineages. As KOSR-cultured ESCs coexpressed embryonic and extraembryonic markers and demonstrated a similar clonal capacity to contribute to early pre-implantation development, we asked whether KOSR-cultured cells could generate extraembryonic cell types, both trophoblast and extraembryonic endoderm (XEN). We have previously shown that 2i-cultured ESCs generate CDX2⁺ trophoblast-like cells when cultured in trophoblast stem cell (TSC) medium (Mor-

gani et al., 2013). ESCs cultured in either serum or KOSR did not have this capacity (Figure S3K). Serum-cultured ESCs died after two passages while KOSR-cultured cells survived but did not express CDX2. However, when ESCs were transferred to XEN cell media, in the absence of lineage-promoting cytokines, both KOSR and 2i-cultured ESCs displayed a XEN-like morphology, downregulated the pluripotency marker NANOG, and expressed the endoderm marker GATA6 (Figures 3I and 3J). While the majority of serum-cultured ESCs downregulated NANOG, they did not express GATA6. Preliminary data from injections of KOSR-cultured ESCs into morulae suggests that these cells may also to contribute to both embryonic and extraembryonic lineages in vivo (Figure S3J). Taken together, these data indicate that ESCs cultured in 2i or KOSR have the capacity to generate extraembryonic lineages and this correlates with their gene-expression profiles, in addition to their capacity to colonize 2C embryos.

DISCUSSION

In this paper we have shown that the culture conditions in which ESCs are grown can be used to re-program their
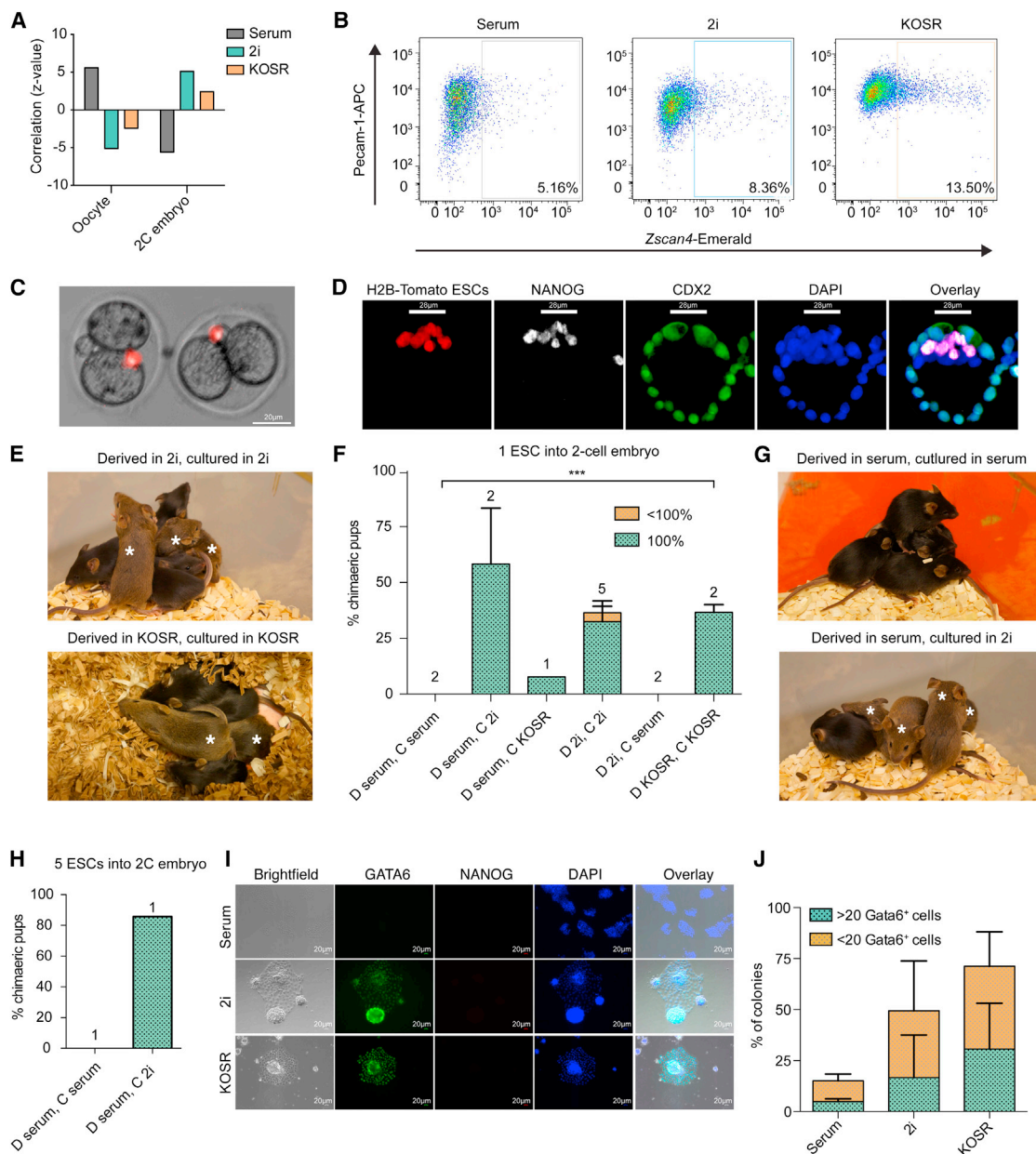
Figure 3. 2i and KOSR ESCs Can Form 100% ESC-Derived Mice When Injected into 2C Embryos

ESCs were derived (D) in and cultured (C) under various conditions; D and C in serum, D in serum and C in 2i, D and C in 2i, D in 2i and C in serum, and D and C in KOSR. Single ESCs from each of these conditions were injected into 2C embryos.

(A) Graph showing the correlation of serum-, 2i-, and KOSR-cultured ESCs with published microarray data of oocytes and 2C embryos (Macfarlan et al., 2012).

(B) Flow cytometry profiles for *Zscan4-Emerald* ESCs, immunostained for the marker of undifferentiated ESCs PECAM-1, cultured in serum, 2i, and KOSR.

(C) Representative 2C embryos immediately after injection with a single ESC expressing a constitutive H2B-Tomato marker.

(D) Confocal optical sections of an immunostained late blastocyst resulting from the injection of a single 2i-cultured ESC, expressing a constitutive H2B-Tomato marker, into a 2C embryo.

(E) Representative images of litters containing 100% ESC-derived agouti mice generated from the injection of a single 2i- or KOSR-cultured cell into a 2C embryo, along wild-type C57BL/6 littermates. Asterisks indicate mice with ESC contribution.

(F and G) Single ESCs were injected into 2C embryos and their contribution to full-term chimeras assessed according to coat color. (F) Graph showing the percentage of chimeras born out of the total number of pups and quantification of coat color chimerism. n Values are provided

(legend continued on next page)

potency irrespective of the protocol by which they were derived. Single ESCs cultured in 2i and KOSR culture conditions demonstrated the capacity to contribute effectively to embryonic development from both the morula and 2C stage. Moreover, this enhanced capacity to contribute to embryonic development correlated with the expression of extraembryonic-associated RNAs and 2C determinants. Thus, while pluripotency factors were expressed in all three culture conditions, ESCs showed distinct gene-expression profiles, and these profiles appear to be correlated with cell behavior in vivo.

Canonical ESC derivation protocols yield variable efficiencies depending on the genetic background of the mice used (Auerbach et al., 2000; Brook and Gardner, 1997). Using improved ESC derivation protocols, it is now possible to generate ESCs from a variety of backgrounds (Nichols et al., 2009a; Nichols and Ying, 2006; Yang et al., 2009; Ying et al., 2008). Although we analyzed inbred (129) and outbred (F1) genetic backgrounds in this study, to compare different derivation approaches we started with embryos of an F1 genetic background known to generate high-quality ESC lines based on hybrid vigor (Eggan et al., 2001; Rideout et al., 2000). The ability of single ESCs to contribute to 2C or morula stages was independent of background, but the capacity to generate live-born entirely ESC-derived mice appeared to be linked to an F1 genetic background. However, as the 129 ESCs used in this study have been in culture for longer periods, cultural adaptation may contribute to this reduced efficiency. Nevertheless, previous data show that F1 hybrid ESC lines produce viable offspring with increased efficiency and suggest that genetic heterozygosity is a requirement to support post-natal development of entirely ESC-derived mice (Eggan et al., 2001; Kirchain et al., 2008).

Previous studies suggest that approximately one out of ten serum-cultured ESCs contribute to embryonic development (Wang and Jaenisch, 2004). If 2i and KOSR cultures generated more single-cell chimeras by increasing the proportion of functionally potent ESCs, we would also expect to observe some chimeras from serum-cultured ESCs (n = 87 injected embryos). As a result, it is not merely that serum-cultured ESCs contain fewer "pluripotent" cells,

but that their clonogenic capacity to self-renew is limited. The increased functional potential of individual 2i-cultured (Morgani et al., 2013) and KOSR-cultured ESCs to generate embryonic and extraembryonic lineages could be a feature of their increased clonogenicity. It has previously been shown that ESCs cultured in the presence of the 2i inhibitors as well as an FGF receptor inhibitor (3i) exhibit increased clonogenicity compared with serum-cultured cells in vitro (Ying et al., 2008), and single 2i-cultured cells show an increased capacity to generate multiple cell types in differentiation assays (Morgani et al., 2013). If 2i- and KOSR-cultured ESCs undergo increased numbers of self-renewing cell divisions, even in the presence of signals that promote lineage restriction, they may generate a critical mass to effectively contribute to both Epi and extraembryonic lineages. In support of this hypothesis, ESC clones exhibiting a greater rate of proliferation generate a higher number of chimeras than those with slower proliferation rates (Wang and Jaenisch, 2004).

Although individual clonogenic ESCs can appear totipotent, the fraction of these cells in different culture conditions may vary, and this could explain why aggregate gene-expression data indicate that 2i cells represent distinct developmental stages (Boroviak et al., 2014). Here we find two clear signatures in the 2i dataset, early pre-implantation development and Epi, perhaps representing this heterogeneity.

We also observed that ESC culture in KOSR enhances their functional potency. At a transcriptional and functional level, these cells are biased toward endoderm. This is distinct from cells cultured in 2i that can differentiate into both trophoblast and extraembryonic endoderm in vitro and in vivo (Morgani et al., 2013). However, ESCs grown and derived in either condition exhibit enhanced potency on a single-cell level. Thus ESC culture conditions may enhance potency by promoting competence for extraembryonic lineages. Moreover, a number of recent reports on ESC totipotency use KOSR in their culture medium (Abad et al., 2013; Macfarlan et al., 2012). As a result, it is intriguing to speculate that this media condition can enhance the reprogramming of normal ESCs to a totipotent, clonogenic state.

above bars and refer to the number of independent ESC clones that were tested for each condition. Error bars indicate SEM. (G) Representative images of full-term mice generated from the injection of single-cell ESCs, D and C in serum (upper panel), or D and C in 2i (lower panel), into 2C embryos. Asterisks indicate mice with ESC contribution.

(H) Graph showing the percentage of chimeras born out of the total number of pups and quantification of coat color chimerism. n Values are provided above bars and refer to the number of independent ESC clones tested for each condition.

(I) ESCs were pre-cultured in serum, 2i, or KOSR for three passages and then switched to XEN cell medium for two passages. Images show representative colonies.

(J) Quantification of the average proportion of colonies under each condition expressing GATA6. A distinction was made between colonies with >20 GATA6+ cells and those with <20 GATA6+ cells. Error bars indicate SD of the mean of five randomly selected fields of view. See also Figure S3.

EXPERIMENTAL PROCEDURES

Mouse Line Maintenance

Mouse lines were maintained under 12/12-hr light/dark cycles in the designated facilities at the University of Copenhagen. Mice were checked for copulation plugs each morning and embryos were considered embryonic day 0.5 (E0.5) on the day of plug detection. All animal work was carried in accordance with European legislation. All work was authorized by and carried out under Project License 2012-15-2934-00743 issued by the Danish Regulatory Authority.

ESC Line Derivation

ESCs derived in this study were F1 hybrid 129S2;C57BL/6N, and 129S6;C57BL/6N lines. Embryos were obtained from mice supplied by commercial vendors with their corresponding certificates (129S6, Taconic; 129S2, Charles River; C57BL/6, Harlan). Females were superovulated at 4 (C57BL/6) or 7 (129) weeks old and mated with fertile males (8–24 weeks old). For derivation in 2i, E2.5 embryos were flushed from oviducts in M2 medium and cultured in KSOM (Millipore) for approximately 8 hr until the evening where they were transferred to KSOM + 2i (1 μM PD0325901, Stemgent; 3 μM CHIR99021, Biovision). Embryos were cultured overnight until E3.5 blastocysts, then transferred to individual wells of gelatinized 96-well plates containing 2i/LIF ESC culture medium (2i inhibitors in N2B27 medium). Embryos were cultured for 5–8 days at 37°C, 5% CO_2, and 90% humidity, until a cluster of cells generated from the ICM emerged. ICM outgrowths were picked and dissociated into smaller clusters in Accutase (Sigma) for 3–5 min at 37°C before being plated into fresh medium for expansion. The same protocol was used to derive ESCs in serum and KOSR conditions but with pre-culture of embryos in KSOM alone and then transfer to 96-well plates containing either serum/LIF or KOSR/LIF. Derivation of ESC lines in serum/LIF and KOSR/LIF was only possible on mouse embryonic fibroblasts (MEFs), although in 2i/LIF conditions ESCs could be derived on gelatin.

ESC Culture

ESC lines were maintained in serum/LIF (Canham et al., 2010), 2i/LIF (Ying et al., 2008) as previously described or KOSR/LIF medium (80% Knockout DMEM [Gibco], 20% Knockout Serum Replacement [Gibco], non-essential amino acids [Gibco], glutamine and sodium pyruvate [Gibco], 0.1 mM β-mercaptoethanol [Sigma]). H2B-Tomato ESCs were generated using E14 ESCs from the 129/Ola background (Morgani et al., 2013). Newly derived ESC lines described are F1 hybrid 129S6;C57BL/6N lines. F1 hybrid lines are named as follows. 2i-derived lines: TCF2.2, TCF2.5, TCF2.6; serum-derived lines: TCF3.4, TCF3.5; KOSR-derived lines: TCF5.30, TCF5.45. ESC lines were switched from their original derivation medium to alternative medium conditions. In the case of serum-cultured cells, ESCs were maintained in the serum for a minimum of 12 passages before being switched to 2i for three passages prior to injection. F1 ESC lines were also electroporated with a constitutive pCAG Kozak-Venus marker (a kind gift from Dr. Heiko Lickert). *Zscan4*-Emerald ESCs were obtained from the Ko laboratory (Zalzman et al., 2010).

XEN and TSC Differentiation Assays

For XEN cell differentiation, ESCs were cultured for three passages in serum, 2i, or KOSR and transferred to XEN cell medium (RPMI [Sigma], 15% fetal bovine serum, glutamine and sodium pyruvate, 0.1 mM β-mercaptoethanol) for two passages (6 days total). Cells were immunostained for GATA6 and NANOG and the average proportion of GATA6^{+} colonies was quantified using five random fields of view per condition. As there was variation in the number of GATA6^{+} cells/colony, we distinguished between colonies with <20 GATA6^{+} cells or >20 GATA6^{+} cells. Overall colony size did not significantly differ. For TSC differentiation, ESCs were cultured for three passages in serum, 2i, or KOSR, then transferred to TSC medium for three passages (total 6 days) (Tanaka et al., 1998). Resultant cultures were stained for CDX2.

Alkaline Phosphatase Staining

ESCs were plated at clonal density and cultured for 9–10 days. Alkaline phosphatase staining was carried out as per manufacturer's instructions. Colonies were imaged using a Nikon AZ-100 microscope.

Chimera Generation and Embryo Culture

Embryos were obtained as for ESC derivation. Single or multiple (5) ESCs were injected into 2C or 8-cell embryos. Inbred cell lines were sorted by flow cytometry prior to injection (Figure S3F) as previously described (Morgani et al., 2013). For 2C embryo injection, E1.5 wild-type C57BL/6N embryos were flushed from oviducts. Embryos were de-compacted in PB1 medium without calcium and magnesium for 20 min at room temperature and a single cell introduced by microinjection. The same process was used for injection of E2.5 morula. Resultant embryos were cultured ex vivo in KSOM microdrops covered with mineral oil (Sigma) or transferred to pseudopregnant CD1 females (8–13 weeks old) and allowed to develop to term.

Microarray Analysis

Unsorted ESCs were maintained in serum/LIF, 2i/LIF, or KOSR/LIF. Samples were collected from three independent ESC clones from the 129/Ola background: E14, HVJu5.1, and HVJu9.3 (Morgani et al., 2013). For 2i/LIF, five samples were collected with two samples each for HVJu5.1 and HVJu9.3 cell lines; for serum/LIF and KOSR/LIF conditions, one sample was collected for each cell line. Each condition is represented at least as biological triplicates. RNA isolation was performed using Qiagen RNeasy kits according to the manufacturer's instructions. RNA pellets were re-suspended in Nuclease Free Water (Ambion) and RNA concentration was measured using the Nanodrop system. 260/280 values for all samples were in the range of 1.8–2.0. RNA quality was additionally assessed using the Agilent 2100 BioAnalyzer with all samples having a RIN of ≥ 9.7. Transcriptome analysis was carried out in-house with one-color 8 × 60K Mouse Gene Expression Microarrays (Agilent Technologies, G4852A) as per manufacturer's instructions. In brief, 150 ng of total RNA was labeled using the Low Input Quick Amp Labeling Kit (Agilent Technologies, 5190-2305). Labeled samples were hybridized overnight, then washed and scanned using the high-sensitivity protocol (AgilentG3_HiSen_GX_1color) on a SureScan microarray scanner (Agilent

Technologies). Probe intensities were obtained by taking the gProcessedSignal from the output of Agilent feature extraction software using default settings. Probe intensities were analyzed using the NIA Array Analysis tool (http://lgsun.grc.nia.nih.gov/ANOVA/).

Analysis of microarray data was carried out using ExAtlas online tool for meta-analysis of gene-expression data developed by the NIA (http://lgsun.grc.nia.nih.gov/exatlas/) (Sharov et al., 2015). Pearson correlation between datasets was measured using the following parameters: false discovery rate (FDR) ≤0.05, fold change ≥2, angle 0.8, expected proportion of false positives (EPFP) 0.5. To obtain correlating gene lists (Table S10), the following parameters were used: FDR 0.1, fold change ≥1.5, angle 0.8, EPFP 0.5. Gene ontology analysis was performed using the Gene Ontology Consortium online tool (http://geneontology.org).

Immunostaining of ESCs

ESCs were plated in ibiTreat 15 μ-Slide 8-well dishes (Ibidi). ESCs were immunostained as described by Canham et al. (2010). Primary antibodies were used at the following concentrations: Gata6 (R&D, AF1700 [Morgani et al., 2013]) 1:100, Gata6 D61 × 10^4 XP (Cell Signaling, 5851 [Lim et al., 2014]) 1:200, Oct4 (C-10 Santa Cruz Biotechnology, sc5279 [Hall et al., 2009]) 1:100, Sox2 (Santa Cruz, sc17320 [Huang et al., 2012]) 1:100, Nanog (eBioscience, 14-5761 [Morgani et al., 2013]) 1:200, Cdx2 (MU392A, Biogenex [Nichols et al., 2009b]) 1:200. Alexa Fluor secondary antibodies (Invitrogen) were diluted 1:500. When immunostaining for HV, a conjugated anti-GFP-Alexa 488 antibody (1:200, Molecular Probes, A21311 [Morgani et al., 2013]) was added at the same time as the secondary antibodies. Cells were imaged on a Leica TCS SP8.

Immunostaining of Embryos

Immunostaining of embryos was carried out as described by Nichols et al. (2009b). The primary antibodies were used at the following dilutions: Nanog (eBioscience, 14-5761 [Le Bin et al., 2014]) 1:200, Cdx2 (Biogenex, MU392A-UC [Nichols et al., 2009b]) 1:200. Alexa Fluor secondary antibodies were used at 1:500. Embryos were imaged in an Attofluor chamber (Life Technologies) on a 25-mm glass coverslip using a Leica TCS SP8 confocal.

Organ Disaggregation and Flow Cytometry

Internal organs were dissected in PB1 medium and imaged using a Nikon AZ100. A section of organ was manually disaggregated with scissors in PBS. Dissociated tissue was disaggregated in trypsin with 20 μL/mL DNase (Qiagen) while shaking at 37°C for 15 min. Disaggregated Kozak-Venus chimeric organs were re-suspended in PBS with 100 ng/mL DAPI and analyzed. The disaggregated spleen of chimeric mice generated from unlabeled 129 ESCs was re-suspended in PBS containing 1:50 anti-CD229.1-APC 30C7 conjugated antibody (560188, BD Pharmingen [Tawara et al., 2012]). Cells were incubated on ice for 15 min, then washed three times with PBS before being re-suspended in PBS with 100 ng/mL DAPI. Data were collected using a BD LSR Fortessa. Flow cytometry analysis was carried out using FlowJo software (Tree Star) first by gating on forward and side scatter to identify a cell population and eliminate debris, then to gate DAPI-negative, viable cells before assessing the level of Venus or APC. PDGFRα flow cytometry was carried out as described by Rugg-Gunn et al. (2012).

Antibody Staining and Flow Cytometry of ESCs

ESCs were collected by trypsinization, and antibody staining for PECAM-1 (BD Pharmingen, 551262 [Morgani et al., 2013]) was carried out as described by Canham et al. (2010). Data were collected using a BD LSR Fortessa. Flow cytometry analysis was carried out using FlowJo software (Tree Star) first by gating on forward and side scatter to identify a cell population and eliminate debris, then to gate DAPI-negative, viable cells and PECAM-1-APC-positive cells, before assessing the level of Venus or GFP. For *Zscan4*-Emerald analysis, gating was based on E14 wild-type ESCs.

qRT-PCR

Three independent ESC lines (E14, HVJu5.1, and HVJu9.3) were cultured in serum/LIF, 2i/LIF, or KOSR/LIF for three passages. RNA was isolated from cells (RNeasy, Qiagen). cDNA was synthesized from 1 μg of RNA using Superscript III according to the manufacturer's guidelines (Invitrogen). qRT-PCR was carried out on a Lightcycler480 (Roche) using UPL technology (Roche) utilizing primer sequences specified in Supplemental Experimental Procedures. Primers were used at a concentration of 1 μM. The geometric mean of the housekeeping genes *Tbp*, *Gapdh*, and *Pgk1* was used for normalization of Ct values detected for each sample.

Statistical Analysis

Statistical analysis was performed using the QuickCalc GraphPad (http://www.graphpad.com/quickcalcs/index.cfm). For non-parametric data, two-tailed chi-square tests were performed. For parametric data comparing two samples, an unpaired Student's t test was used. A p value is shown when the difference between compared groups is significant. The statistical significance of the overlap of gene lists was assessed using http://nemates.org/MA/progs/overlap_stats.cgi, using a genome size of 25,000. The representation factor is the number of overlapping genes divided by the expected number of overlapping genes drawn from two random, independent groups. A representation factor >1 indicates a greater overlap than expected by chance.

ACCESSION NUMBERS

The microarray data have been deposited under accession number GEO: GSE72445.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and ten tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.07.009.

AUTHOR CONTRIBUTIONS

J.M.G. conceived of and executed experiments. S.M.M. conceived of and executed experiments and wrote the paper with J.M.B. R.A.B., K.B., and S.A. executed experiments. C.B. designed and

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Supplemental Information

Embryonic Stem Cell Culture Conditions Support Distinct States Associated with Different Developmental Stages and Potency

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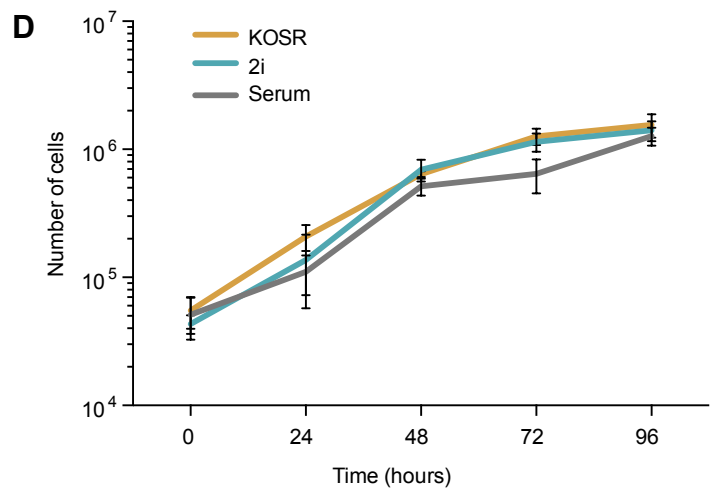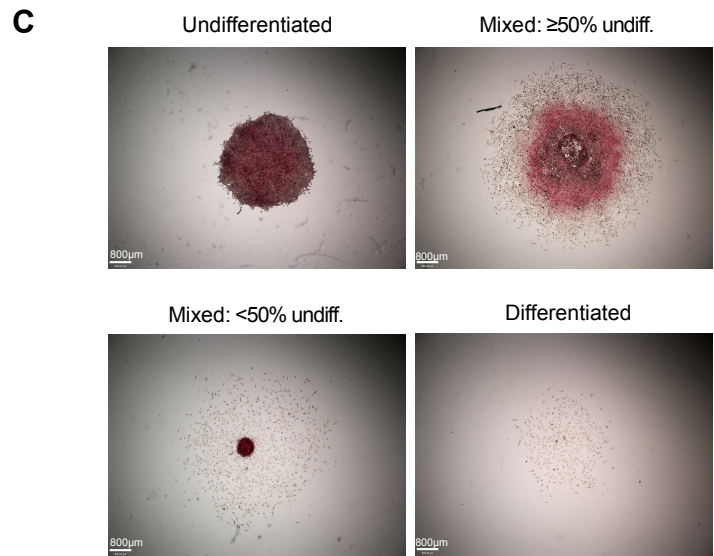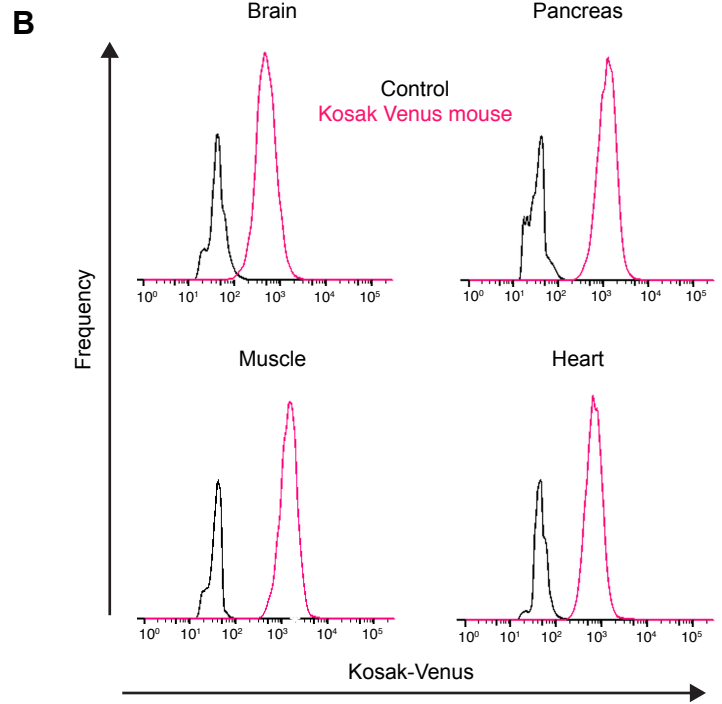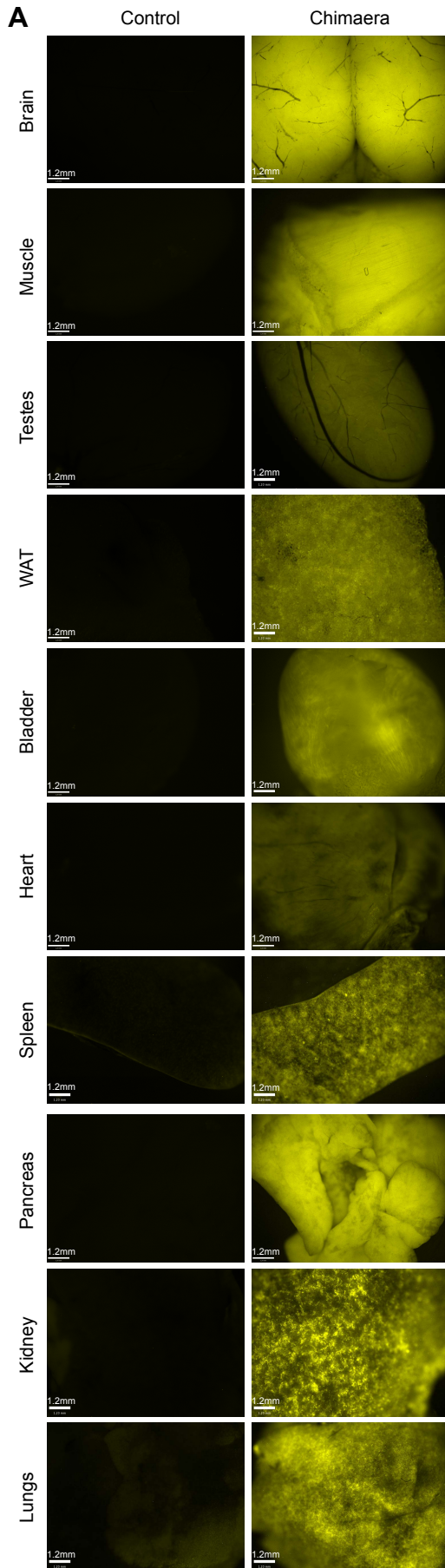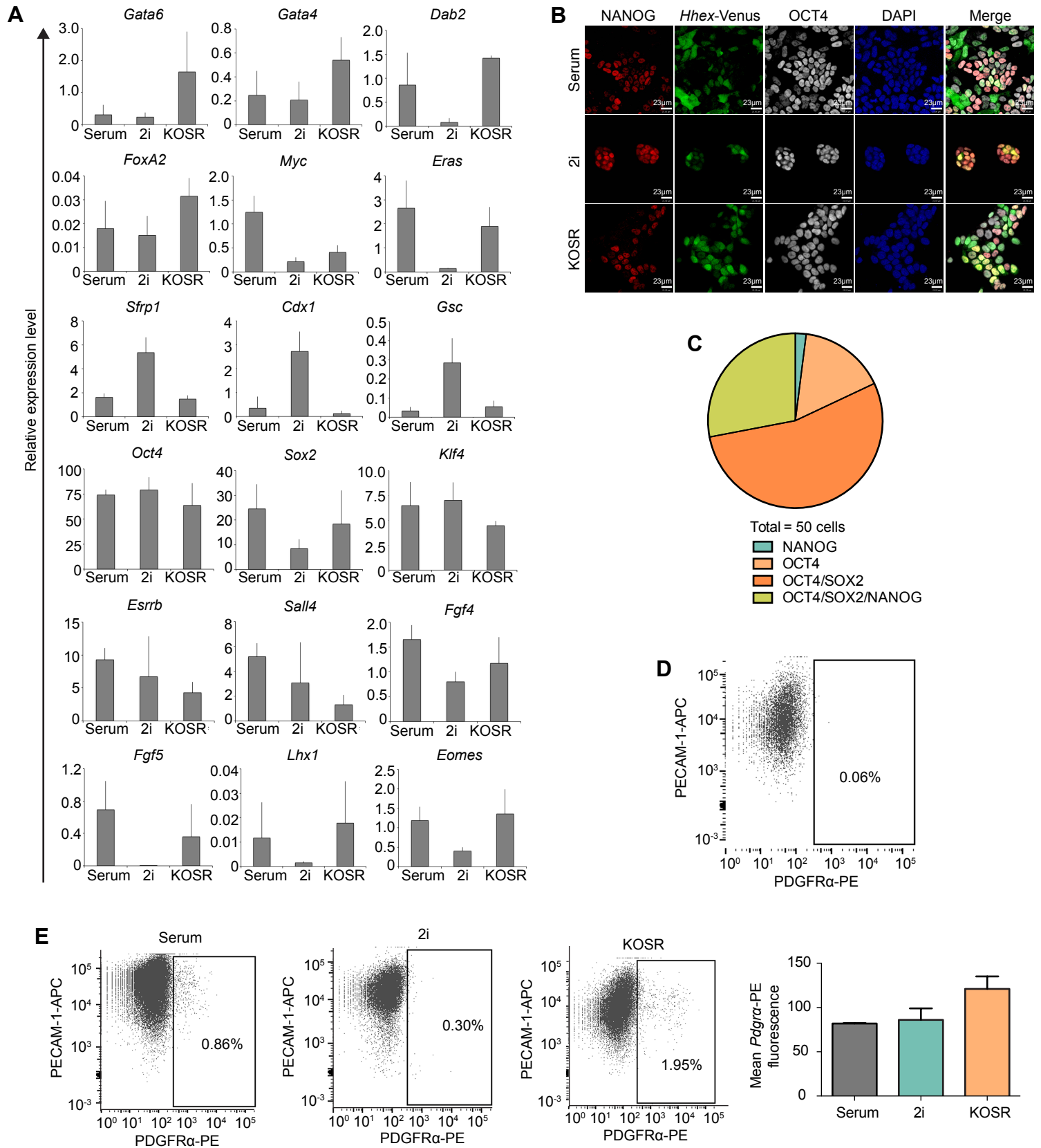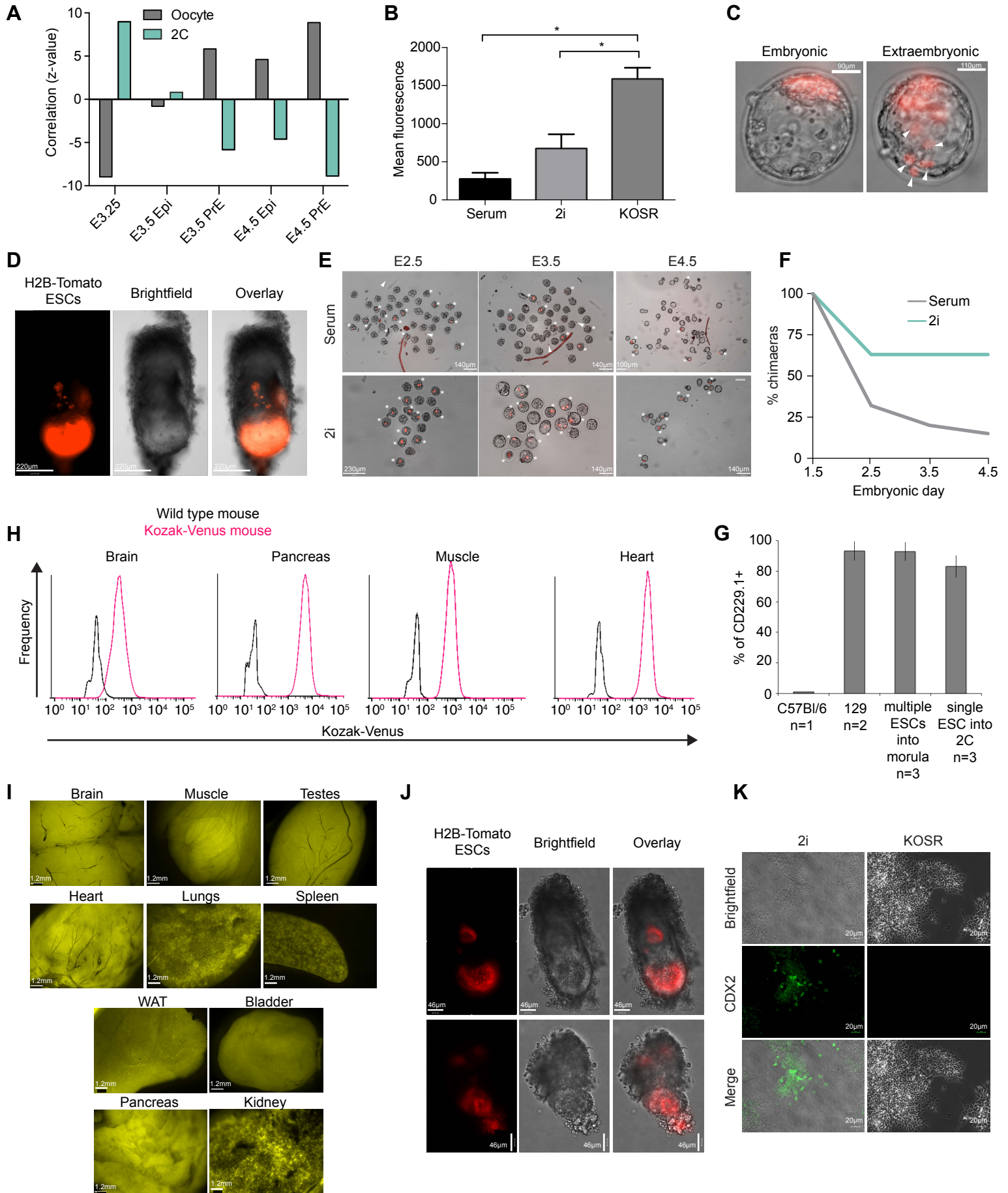**Figure S1. The injection of multiple ESCs into morulae generates 100% ESC-derived mice from all conditions, related to Fig 1.** ESCs, were derived (D) in and cultured (C) under various conditions; D in serum and C in serum, D in serum and C in 2i, D in 2i and C in 2i, D in 2i, C in serum and D and C in KOSR and D in KOSR and C in serum. **A.** Representative images of internal organs from mice generated by injection of multiple (5) Kozak-Venus ESCs into 8-cell morulae, alongside control wild type organs not expressing Kozak-Venus. WAT = white adipose tissue. **B.** Flow cytometry of internal organs from control wild type mice and mice generated by the injection of 2i-cultured Kozak-Venus ESCs. **C.** Representative images of Alkaline Phosphatase staining categories used for quantification in Fig. 1C-F. Colonies were scored as 100% differentiated or undifferentiated or as mixed colonies containing both undifferentiated and differentiated cells. **D.** Graph showing the proliferation rates of ESCs cultured in serum, 2i or KOSR over 96 hours of culture. n = 3. Error bars represent s.e.m.

Figure S2. Characterisation of KOSR-cultured ESCs, related to Fig. 2. A. qRT-PCR was carried out on ESCs cultured in serum, 2i or KOSR for 3 passages. Data represents the mean of n = 3 biological repeats from independent cell lines. Error bars indicate s.d. of the mean. Data is shown relative to the geometric mean of 3 housekeeping genes, *Gapdh*, *Tbp* and *Pgk1*. Consistent with previous observations (Marks et al., 2012; Morgani et al., 2013), genes including *Sfrp1*, *Cdx1* and *Gsc* were upregulated specifically in response to 2i culture, and the serum-dependent genes *Myc* and *Eras* were reduced to varying degrees in both serum-free conditions. **B.** Confocal optical sections of *Hhex*-Venus ESCs cultured in serum, 2i and KOSR immunostained for NANOG and OCT4. **C.** Quantification of immunostaining shown in Fig. 2F. 25 fields of view (n = 50 GATA6^{+} cells) were randomly chosen to manually quantify the coexpression of GATA6 in the ESC compartment with 3 pluripotency-associated markers, NANOG, OCT4 and SOX2. **D.** Flow cytometry profile of ESCs in the absence of PDGFRα staining, used as a control to set gates for PDGFRα staining in Fig. S2E. **E.** Flow cytometry profiles of ESCs cultured in serum, 2i or KOSR for 3 passages before being immunostained for the PrE marker PDGFRα and the ESC marker PECAM-1. Gates are based on ESCs unstained for PDGFRα (Fig. S2D). Percentages of PDGFRα^{+} cells are shown on the plots. Bar chart shows quantification of mean fluorescence in each condition. n = 2. Error bars indicate s.d. of the mean.

Figure S3. 100% ESC-derived mice are generated from the injection of single 2i or KOSR-cultured ESCs into 2C embryos, related to Fig. 3. A. Graph showing the correlation of published expression data from oocytes and 2C embryos (Macfarlan et al., 2012) with various stages of blastocyst development (Hiiragi et al., 2014). **B.** Mean fluorescence measured by flow cytometry of *Zscan4-Emerald* ESCs cultured in serum, 2i and KOSR (flow

cytometry profiles in Fig. 3B). y n = 2. Error bars indicate s.d. of the mean. * $P \le 0.05$. **C.** Representative images of chimaeric blastocysts generated by the injection of single ESCs into 2C embryos, showing ESC contribution (marked by H2B-Tomato) to the embryonic epiblast alone or both the embryonic and extraembryonic lineages. Arrowheads indicate ESCs contributing to the extraembryonic regions. **D.** 129 ESCs expressing a constitutive H2B-Tomato marker were cultured in 2i for at least 3 passages before single HV[+] ESCs were sorted by FACS and injected into morulae. Embryos were then dissected and imaged at E6.5. **E.** ESCs constitutively expressing H2B-Tomato were pre-cultured in 2i or serum. Single ESCs were injected into 2C embryos. Time course showing the loss of serum-cultured ESCs from embryos. Asterisks mark chimaeric embryos and arrowheads show eliminated cells. **F.** Quantification of the percentage of chimaeric embryos at each stage in E. **G.** Flow cytometry of disaggregated spleens from control C57BL/6 and 129 mice as well as from chimaeric mice generated using C57BL/6 host embryos injected with 129 ESCs. Data are shown for 2i-cultured ESCs. Single ESCs injected into 2C embryos compared to multiple ESCs injected into morula. Disaggregated cells were immunostained for the 129 cell surface antigen CD229.1. n values indicate the number of mice analysed. **H.** Flow cytometry of internal organs from control and Kozak-Venus mice from Fig. S3G. **I.** Representative images of internal organs from mice generated by injection of a single Kozak-Venus ESC into a 2C embryo. WAT = white adipose tissue. **J.** E6.5 chimaeric embryos generated by the injection of multiple (5) KOSR-cultured ESCs, expressing a constitutive H2B-Tomato marker, into morulae. n = 8/18 chimaeras (44%) from KOSR-cultured ESCs demonstrated contribution to both embryonic and extraembryonic lineages. **K.** ESCs were pre-cultured in serum, 2i or KOSR for 3 passages then switched to TS cell medium for 3 passages. Images show representative colonies. ESCs that were pre-cultured in serum did not survive after passage 2.

Table S1. List of genes significantly upregulated in 2i compared to serum ESC cultures, related to Fig. 2. Pair-wise comparisons (FDR ≤0.05, fold-change ≥2) were performed between serum and 2i ESC cultures to reveal significant changes in gene expression. Table shows 1445 genes significantly upregulated in 2i.

Table S2. List of genes significantly downregulated in 2i compared to serum ESC cultures, related to Fig. 2. Pair-wise comparisons (FDR ≤0.05, fold-change ≥2) were performed between serum and 2i ESC cultures to reveal significant changes in gene expression. Table shows 2248 genes significantly downregulated in 2i.

Table S3. List of genes significantly upregulated in KOSR compared to serum ESC cultures, related to Fig. 2. Pair-wise comparisons (FDR ≤0.05, fold-change ≥2) were performed between serum and KOSR ESC cultures to reveal significant changes in gene expression. Table shows 536 genes significantly upregulated in KOSR.

Table S4. List of genes significantly downregulated in KOSR compared to serum ESC cultures, related to Fig. 2. Pair-wise comparisons (FDR ≤0.05, fold-change ≥2) were performed between serum and 2i ESC cultures to reveal significant changes in gene expression. Table shows 482 genes significantly downregulated in KOSR.

Table S5. Gene ontology analysis of genes upregulated in 2i compared to serum ESC cultures, related to Fig. 2. Gene ontology analysis was performed using the Gene Ontology Consortium online tool. The set of genes was identified using criteria: FDR ≤0.05, fold-change ≥2.

Table S6. Gene ontology analysis of genes downregulated in 2i compared to serum ESC cultures, related to Fig. 2. Gene ontology analysis was performed using the Gene Ontology Consortium online tool. The set of genes was identified using criteria: FDR ≤0.05, fold-change ≥2.

Table S7. Gene ontology analysis of genes upregulated in KOSR compared to serum ESC cultures, related to Fig. 2. Gene ontology analysis was performed using the Gene Ontology Consortium online tool. The set of genes was identified using criteria: FDR ≤0.05, fold-change ≥2.

Table S8. Gene ontology analysis of genes downregulated in KOSR compared to serum ESC cultures, related to Fig. 2. Gene ontology analysis was performed using the Gene Ontology Consortium online tool. The set of genes was identified using criteria: FDR ≤0.05, fold-change ≥2.

Table S9. List of significantly changing genes that are overlapping between 2i and KOSR ESC cultures, related to Fig. 2. Pair-wise comparisons (FDR ≤0.05, fold-change ≥2) were performed between both serum and 2i and serum and KOSR ESC cultures and significantly up and downregulated genes were identified. From these 2 comparisons, the overlapping genes were identified.

Table S10. Gene ontology analysis of common up and downregulated genes in 2i and KOSR compared to standard serum cultures, related to Fig. 2. Gene ontology analysis was performed using the Gene Ontology Consortium online tool. The set of genes was identified using criteria: FDR ≤0.05, fold-change ≥2.

Extended Experimental Procedures

qRT-PCR primer details, related to Experimental Procedures. Primer sequences and the corresponding UPL probe used in this study.

Gene	Forward Primer	Reverse Primer	UPL Probe
Cdx1	ACGCCCTACGAATGGATG	TGTCCTTGGTTCGGGTCTTA	70
Dab2	GCAGTCGAACTTTCTGGATCTC	GGTGTTACTGGGACCGTACCT	51
Eomes	ACCGGCACCAAACTGAGA	AAGCTCAAGAAAGGAAACATGC	9
Eras	GCCCCTCATCAGACTGCTAC	GCAGCTCAAGGAAGAGGTGT	99
Esrrb	CAGACAGGGGACTGTCAG	ACATTCGGTTCAGCAGCA	25
Fgf4	GCAAGCTCTTCGGTGTGC	CGTAGGATTCGTAGGCGTTG	29
Fgf5	CATCACATTCCCGAATTAAGC	CATCACATTCCCGAATTAAGC	29
FoxA2	GAGCAGCAACATCACCACAG	CGTAGGCCTTGAGGTCCAT	77
Gata4	TTCGCTGTTTCTCCCTCAAG	CAATGTTAACGGGTTGTGGA	60
Gata6	GGTCTCTACAGCAAGATGAATGG	TGGCACAGGACAGTCCAAG	40
Gsc	GGAGACGAAGTACCCAGACG	CGGCGGTTCTTAAACCAG	32
Klf4	CGGGAAGGGAGAAGACACT	GAGTTCCTCACGCCAACG	62
Lhx1	CAGGAGACTGGCCTCAACAT	GTTTCATCCTTCGCTCCTTG	85
Myc	CCTAGTGCTGCATGAGGAGA	TCTTCCTCATCTTCTTGCTCTTC	77
Nanog	CCTCCAGCAGATGCAAGAA	GCTTGCACTTCATCCTTTGG	25
Oct4	GTTGGAGAAGGTGGAACCAA	CTCCTTCTGCAGGGCTTTC	95
Sall4	TCTCCCGAGGGTCACAAG	TGTGCTCGGATAAATGTTGG	75
Serpine2	TTGGGTCAAAAATGAGACCAG	CCTTGAAATACACTGCATTAACGA	64
Sfrp1	AGGCTGCCCTCTTAATACACC	CCACAGCACATGCACAGAG	97
Sox2	GGCAGAGAAGAGAGTGTTTGC	TCTTCTTTCTCCCAGCCCTA	34