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Supplemental Information

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

Javier Martin Gonzalez, Sophie M. Morgani, Robert A. Bone, Kasper Bonderup, Sahar Abelchian, Cord Brakebusch, and Joshua M. Brickman

Figure S1
Martin-Gonzalez et al., 2015

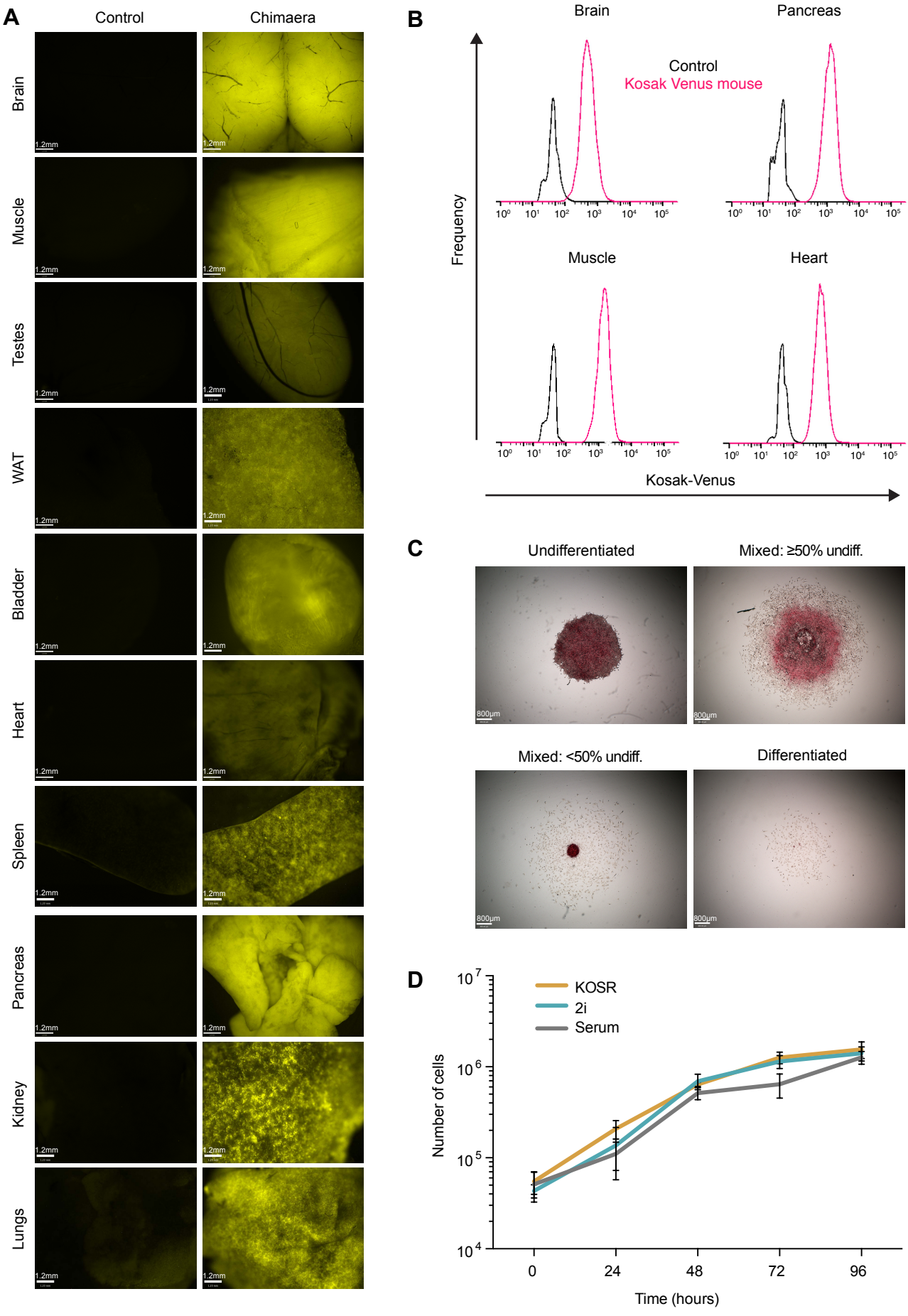


Figure S2
Martin-Gonzalez et al., 2015

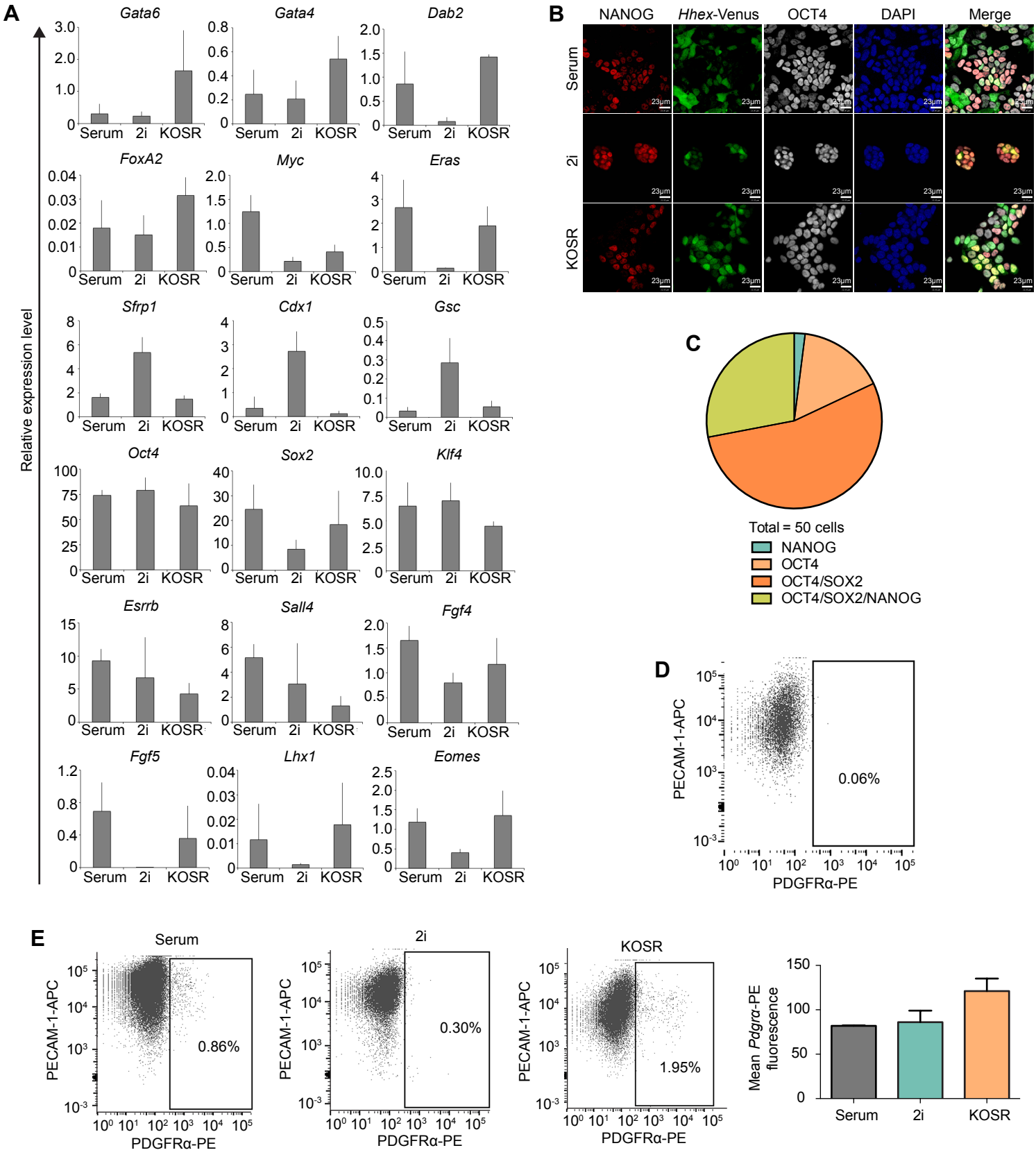
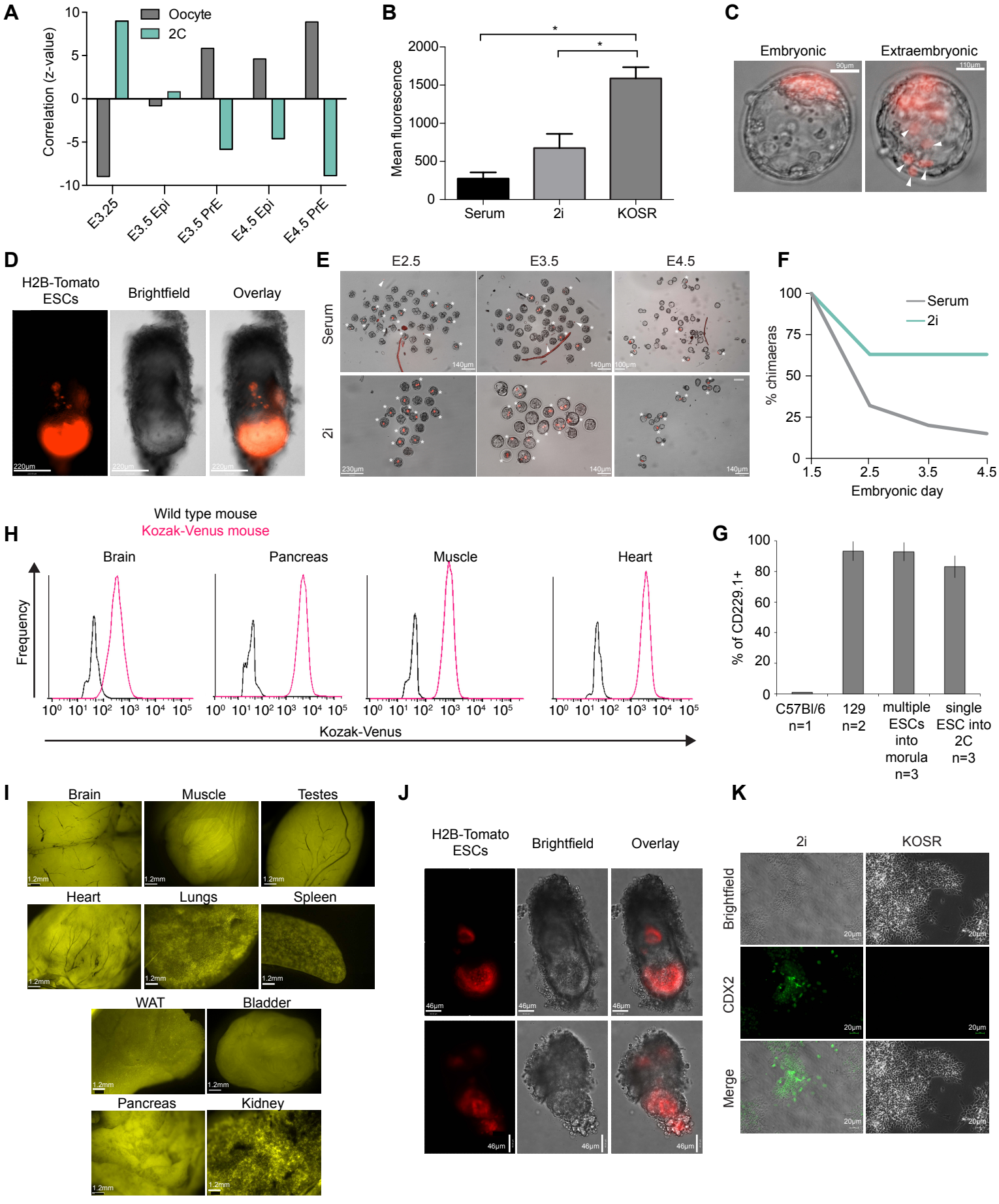


Figure S3
Martin-Gonzalez et al., 2015



Supplementary figures legends

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 $PDGFR\alpha^+$ 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. * $P \leq 0.05$. **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