

Derivation and Maintenance of Murine Trophoblast Stem Cells under Defined Conditions

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SUMMARY

Trophoblast stem cells (TSCs) are in vitro equivalents to the precursor cells of the placenta. TSCs are cultured in serum-rich medium with fibroblast growth factor 4, heparin, and embryonic-fibroblast-conditioned medium. Here, we developed a simple medium consisting of ten chemically defined ingredients for culture of TSCs on Matrigel or synthetic substrates, named TX medium. Gene expression and DNA methylation profiling demonstrated the faithful propagation of expression profiles and epigenomic characteristics of TSCs cultured in TX. Further, TX medium supported the de novo derivation of TSC lines. Finally, TSCs cultured in TX differentiate into all derivatives of the trophoblast lineage in vitro, give rise to hemorrhagic lesions in nude mice, and chimerize the placenta, indicating that they retained all hallmarks of TSCs. TX media formulation no longer requires fetal bovine serum and conditioned medium, which facilitates and standardizes the culture of this extraembryonic lineage.

INTRODUCTION

Murine trophoblast stem cells (TSCs) can be derived from the polar trophoblast of preimplantation embryos (E3.5) and postimplantation from the extraembryonic/chorionic ectoderm up to E8.5 (Tanaka et al., 1998; Uy et al., 2002). They represent the stem cells of the trophoblast lineage and retain the capacity to differentiate into all trophoblast derivatives of the later placenta in vitro. They also give rise to transient hemorrhagic lesions mimicking their physiological role in the placenta when transplanted subcutaneously into nude mice (Kibschull et al., 2004; Kuckenberger et al., 2011). Finally, when injected into blastocysts, they chimerize the placental portion of the conceptus (Tanaka et al., 1998). TSCs are propagated under complex cell-culture conditions that are poorly defined due to presence of 20% fetal bovine serum (FBS), supplemented with fibroblast growth factor 4 (FGF4) and heparin. Further, they require growth-inactivated feeder cells or feeder-cell-conditioned medium (CM). In 2004, Erlebacher et al. (2004) identified transforming growth factor β (TGF- β) and the related factor activin as active components secreted by feeder cells to maintain TSC proliferation. Nevertheless, the remaining unknown factors secreted by feeder cells and the variations of growth factors present in serum (exacerbated by the substantial serum

content of the media) lead to ill-defined culture conditions. Quality of serum and feeder cells is highly variable, and the animal origin of these supplements is a frequent source of contamination. Because these fluctuations hamper the interpretation of effects of exogenous agents on growth and differentiation of TSCs, there is an urgent need for developing chemically defined and standardized media.

In recent years, this need for serum-free and animal-origin-free media led to the end of the FBS era for growing embryonic and other stem cell types (Silva and Smith, 2008; Ying et al., 2008). First attempts to culture human embryonic stem cells (hESCs) in defined media used complex and cost-intensive formulations (Ludwig et al., 2006a; 2006b). Chen et al. (2011) re-examined the individual components for hESC and induced pluripotent stem cell (iPSC) culture and formulated a cell culture system in which all protein reagents for liquid media are chemically defined. We reasoned that TSCs, like hESCs and murine epiblast stem cells are epithelial stem cell types with similar growth factor requirements, depending on FGF and activin signaling. TSCs are routinely supplemented with FGF4, whereas hESCs are grown with fibroblast growth factor 2 (FGF2). We therefore hypothesized that TSCs can also be propagated in defined media and, due to common growth factor requirements, we used the formulation of hESC synthetic media as a starting point.



Here, we demonstrate that TX medium supports long-term self-renewal (>40 passages) of three independent TSC lines (TS-enhanced GFP [EGFP], TS_{3.5}, and TS_{6.5}) derived from E3.5 and E6.5 embryos. TSCs can be maintained in the undifferentiated state and retain full differentiation potential *in vitro* and *in vivo*. Moreover, new TSC lines can be derived in TX media, indicating its suitability for promoting the undifferentiated state of TSCs. We determined that global gene expression and methylation patterns are extremely similar between TSCs derived and cultured in conventional compared to TX media, indicating that TX medium is capable of propagating the genuine TSC state.

RESULTS

Establishment of a Serum-Free Culture Medium for TSCs

In order to develop a serum-free-defined medium for the derivation and maintenance of TSCs, we reasoned that defined media for hESCs/iPSCs might be a starting point, because of common growth factor requirements. We decided to use the media formulation by [Chen et al. \(2011\)](#) for our analyses, due to its lack of serum albumin components. Our medium formulation uses Dulbecco's modified Eagle's medium (DMEM)/F12 as basal medium, supplemented with ten ingredients. Insulin, l-ascorbic acid, sodium bicarbonate, sodium selenite, transferrin, and TGF- β were added in final concentrations according to [Chen et al. \(2011\)](#). Instead of FGF2, we used FGF4 and adjusted the concentration to that used in standard TS media, which is 25 ng/ml. Further, the cofactor heparin, which is known to facilitate and stabilize the uptake of FGFs, was added ([Furue et al., 2008](#)). Furthermore, L-glutamine, present in standard TS media, was supplemented and penicillin and streptomycin were added to the medium to prevent bacterial contamination. Sodium pyruvate, an energy source used in standard TS media, was already present in the DMEM/F12 basal medium. Osmolality of TX medium was 325.3 ± 5.8 mmol/kg, compared to 301.8 ± 8.0 mmol/kg of TS medium.

Matrigel-Coated Surfaces Support Growth of TSCs in TX Media

In order to initiate the experiments, two well-characterized TSC lines that were established in our lab (TS_{3.5} and TS_{6.5}) and a TSC line derived by the Rossant lab (TS-EGFP; [Tanaka et al., 1998](#)) were grown in standard media as published. After passage 30 (TS_{3.5}), passage 11 (TS_{6.5}), and passage 8 (TS-EGFP), cells were left to adhere to the dish and form TSC colonies (after 1 to 2 days), representing late passage, intermediate, and early passage stem cell lines, respectively. From then on, cell lines were cultured side by side in

standard TS medium and TX medium, enabling direct comparison through culture in the same incubator. The TX-medium-supported growth of TSCs and colonies retained a tight epithelial morphology, displaying bright boundaries, when using phase-contrast optics. Of note, although all analyzed cell lines represent well-characterized TSC lines, they vary in morphology when cultured in TS medium. After adaptation to TX, morphological differences between cell lines were less pronounced ([Figure S1A](#) available online). Additionally, in contrast to cells in standard TS medium, cells grown in TX medium on Matrigel homogeneously maintained a tight, TS-like morphology ([Figure 1A](#)). Whereas initial growth of TSCs after switching to TX media was not affected, several attempts to passage TSCs in defined medium failed due to cells not attaching to the cell culture dishes. In standard medium, TSCs are routinely grown on uncoated plates. TSCs seeded in TX medium did not adhere to plastic surfaces of uncoated dishes (data not shown), indicating that, in the absence of serum, TSCs require extracellular matrix proteins. In order to examine the suitability of various coating substrates, tissue culture dishes were coated with either growth-factor-deprived Matrigel, gelatine, poly-L-lysine, fibronectin, as well as commercially available synthetic matrix-mimicking compounds (Synthemax and StemXVivo) according to manufacturer's recommendation. One thousand five hundred TS_{6.5} cells were plated, and the colonies were counted after 4 days to determine the plating efficiency. Matrigel-coated dishes showed the highest plating efficiency, whereas the remaining compounds showed poor performance ([Table 1](#); [Figure S1B](#)). Further culture for additional ten passages of TSCs grown on Matrigel or Synthemax revealed that expression of trophoblast markers on mRNA and protein level remained constant ([Figures S1C](#) and [S1D](#)). This indicates that both Matrigel and Synthemax coatings are suitable for the culture of TSCs in TX media. Because Matrigel displayed the best plating efficiency, it was used for all further experiments. Under these conditions, TSCs were propagated for several months (>40 passages) and maintained a morphologically undifferentiated appearance throughout, indicating unaffected self-renewal of cells (not shown). When analyzing cell cycle distribution in three TSC lines by propidium iodide (PI) staining and flow cytometry, there was no significant difference between standard and defined media (paired t test, two tailed; [Figure 1B](#); [Table 2](#)). However, when analyzing a single cell line, two out of the three cell lines showed more cells in S-phase in TS medium compared to TX medium ([Figure S2A](#)). This was analyzed in more detail for TS_{6.5} cells and confirmed by fewer cells in TX medium, as determined by counting cell numbers ([Figure S2B](#)) and a longer population-doubling time (22.49 ± 1.23 hr in TX versus 13.00 ± 1.56 hr in TS medium; [Figure S2C](#)). TSCs are routinely

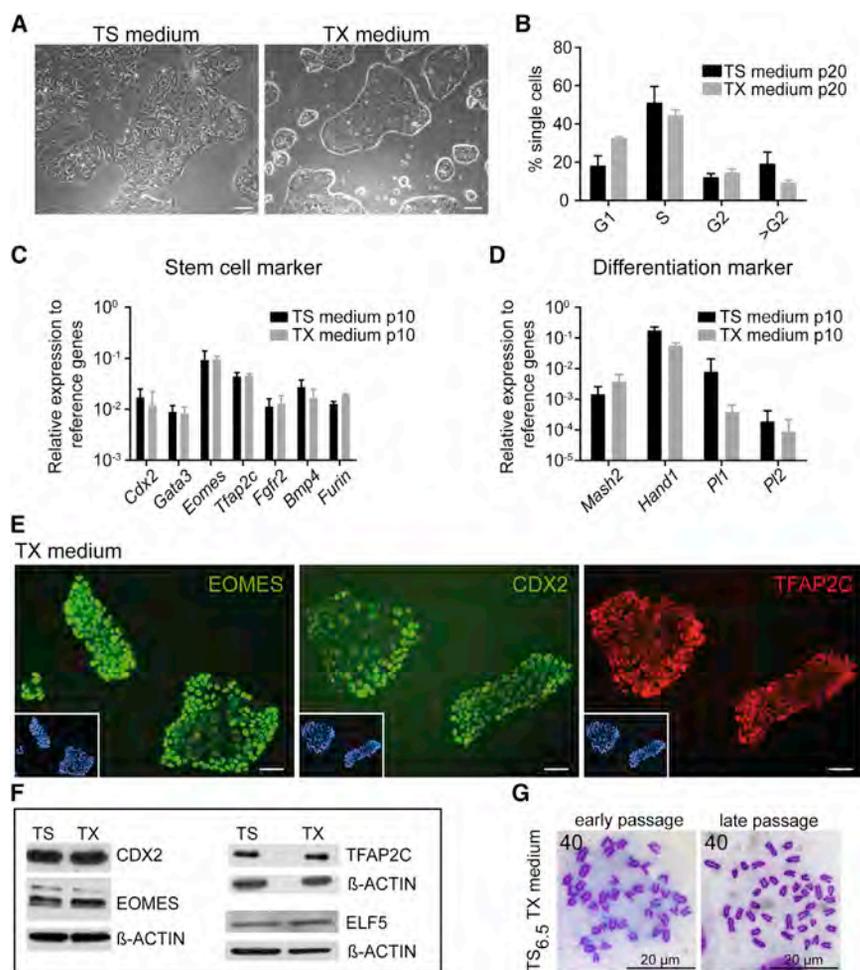


Figure 1. Maintenance of TSC Characteristics in TX Medium

(A) Photomicrographs of TS_{6.5} cells in TS and TX medium at passage 11+10. The scale bar indicates 100 μ m. (B) Cell-cycle analysis using propidium iodide for TS cells grown in TX medium (gray bars) and TS medium (black bars) for 20 passages. Data are presented as mean of three TSC lines with SD.

(C and D) Quantitative RT-PCR analysis of indicated marker genes of undifferentiated (C) and differentiated (D) trophoblast in TSCs cultured in TS (black bars) and TX media (gray bars) for ten passages. Bars represent mean values with SD of three different TSC lines (TS-EGFP, TS_{6.5}, and TS_{3.5}).

(E) Immunofluorescent staining against EOMES, CDX2, and TFAP2C of TSCs cultured for ten passages in TX medium. Smaller pictures display Hoechst staining. The scale bar represents 50 μ m.

(F) Immunoblot against CDX2, EOMES, TFAP2C, and ELF5 of whole-cell lysates from TSCs cultured for ten passages in TS and TX medium. β -ACTIN serves as a loading control.

(G) Metaphase spreads of TS_{6.5} cells at early (p11+12) and late (p11+40) passage in TX medium.

See also Figures S1–S3 and Table 2.

cryopreserved using FBS and DMSO. In order to avoid contact and potential influence of the cells by FBS during this procedure, we tested cryopreservation in KnockOut Serum Replacement supplemented with 10% DMSO. Here, the plating efficiency after thawing was comparable to standard procedures indicating that FBS could be omitted during cryopreservation (Figure S2D). It must be mentioned that cells in serum-free medium are more prone to damage by mechanical stress, due to the lack of albumin, which is being discussed to act as a protective agent against shear forces (Zhang et al., 1995). The apoptosis rate of TSCs in TX medium ($5.7\% \pm 0.7\%$ annexin-positive cells) was significantly increased in comparison to TS medium ($2.3\% \pm 1.1\%$; Figure S2E).

TSCs Grown in TX Media Maintain Expression of Stem Cell Markers and a Stable Karyotype

To further validate that TSCs grown in defined media retain their stem cell characteristics, we isolated total RNA of TSCs of all three lines grown in standard or TX media after ten passages. Quantitative RT-PCR (qRT-PCR) analyses demon-

strate that *Cdx2*, the caudal-related homeobox 2 transcription factor required for TSC specification and maintenance (Strumpf et al., 2005), GATA-binding protein 3 (*Gata3*; Ralston et al., 2010), and transcription factors eomesodermin (*Eomes*; Russ et al., 2000) and AP-2 γ (*Tfap2c*; Kuckenberg et al., 2010) were similarly expressed in both media conditions (Figure 1C). Aside from the transcription factors indicative of undifferentiated TSCs, we analyzed the expression of fibroblast growth factor receptor 2 (*Fgf2*), *Bmp4*, and the endoprotease *Furin*, which are all highly expressed in TSCs (Donnison et al., 2005). Similarly, expression levels of these TSC factors were unaffected by TX medium (Figure 1C). To determine whether the cells are prone to spontaneous differentiation, markers of differentiated trophoblast giant cells (*Pl1* and *Pl2*) were analyzed (Figure 1D). Statistical analysis (two-tailed, paired t test) revealed only one significant difference of expression levels between both conditions, which was a higher *Furin* expression in TX medium ($p = 0.011$), suggesting that TX media culture did not induce a change in TSC identity. The qRT-PCR analysis was repeated at a higher passage (>40), with

**Table 1. Plating Efficiency in TX Medium**

Coating	Colonies on 12-Well Dish
Matrigel	233.33 ± 20.98
Synthemax	83.00 ± 13.00
StemXVivo	15.33 ± 5.86
Fibronectin	8.00 ± 3.61
Gelatine	1.00 ± 1.73
Poly-L-lysine	0.00

Number of colonies on 12-well dish after plating single cells on different coating substrates in TX medium. Values are mean values of triplicate wells ± SD.

no significant differences between both medium conditions (Figures S2F and S2G). Maintenance of TSC identity was further confirmed by immunofluorescent staining against TSC markers CDX2, EOMES, and TFAP2C (Figure 1E). Additionally, immunoblotting against CDX2, EOMES, TFAP2C, and ELF5 confirmed no changes in protein levels in TSCs cultured in both media conditions (Figure 1F). Cytogenetic analysis of TSCs cultured for 12 passages in standard and defined media revealed that they maintained normal karyotype with 40 chromosomes in 19 out of the 20 analyzed metaphases (Figures 1G and S3A). However, when we analyzed metaphase spreads of all three TSC lines at 40 passages in defined versus standard TS medium, almost all cell lines displayed a mild aneuploidy, irrespective of the culture medium, with 41 chromosomes being the predominant abnormality (Figures S3A–S3C). TS_{6.5} was analyzed after 12 and 40 passages in both media and maintained a stable (euploid) karyotype in TX medium, demonstrating that TX medium does not negatively affect chromosomal stability (Figure 1G). Nevertheless, the gain of an additional chromosome seems to be a common problem in TSC culture (Oda et al., 2009).

TSCs Retain Full Differentiation Potential in TX Media In Vitro

To assess the developmental potency of cells grown in defined media, we subjected TS_{6.5} grown for ten passages in TX or conventional TS medium to a standard in vitro dif-

ferentiation protocol (Kuckenberg et al., 2011). Cells were plated either in TS diff media (TS media without FGF4/heparin and CM) or TX diff media (TX media without FGF4/heparin and TGF-β1), and their differentiation potential was compared to cells of the same line, previously grown in standard TS media (Figure 2A). Cells from TX medium differentiated in TS diff media showed morphological differentiation indistinguishable from control cells, marked by enlargement and flattening of cells (Figure 2B) and decreased proliferation. The morphology of cells in TX diff media was different from differentiation in TS diff media, but nevertheless, cells previously grown in TX medium displayed a similar pattern of changes in gene expression after differentiation (Figure 2C). Downregulation of *Cdx2* occurred in all conditions, whereas indicators for intermediate differentiated trophoblast (*Hand1* and *Tpbpa*) as well as markers for terminally differentiated giant cells (*Pl1*, *Pl2*, *Ctsq*, and *Prl2c2*) were upregulated (Figure 2C). Of note, *Ctsq* was stronger upregulated when cells from serum medium were differentiated, whereas *Prl2c2* induction was more prominent in differentiated cells from TX medium. These data indicate that the TX culture system is able to support TSCs in the stem cell state without compromising their differentiation capability in vitro and that TSCs can be differentiated in the absence of serum.

TSCs Differentiate into Hemorrhagic Lesions and Chimerize the Placenta

Next, we asked whether the TX-cultured TSCs had retained their in vivo differentiation potential. In the developing placenta, the physiological role of trophoblast giant cells is to invade maternal blood vessels (Rossant and Cross, 2001). This can be mimicked by transplantation of cells subcutaneously into the flank of nude mice (Kibschull et al., 2004; Kuckenberg et al., 2011). TS_{3.5} cells cultured for more than ten passages in TX medium were transplanted. Seven days after injection, animals were analyzed (n = 3). The transplanted TSCs, which had invaded the blood vessels, gave rise to hemorrhagic lesions. Blood vessels were identified by hematoxylin and eosin (H&E) staining (Figure 2D) and immunohistochemistry (IHC) staining against the endothelial marker CD31. IHC staining detected TFAP2C-positive cells, suggesting that these cells indeed represent TS derivatives. Next, TS-EGFP cells

Table 2. Cell-Cycle Analyses

	G1 [%]		S [%]		G2 [%]		>G2 [%]	
	TS	TX	TS	TX	TS	TX	TS	TX
p20	17.76 ± 5.61	32.11 ± 1.18	50.79 ± 8.81	43.93 ± 3.59	11.69 ± 2.34	14.10 ± 2.36	18.75 ± 6.55	8.85 ± 1.62
p30	21.24 ± 5.93	26.44 ± 5.58	53.70 ± 8.07	43.44 ± 13.17	10.90 ± 1.41	17.45 ± 5.52	13.40 ± 4.23	11.96 ± 8.95

Summary of cell-cycle analyses using PI staining and flow cytometry. Values of three TSC lines are shown as mean ± SD. See also Figures 1 and S2.

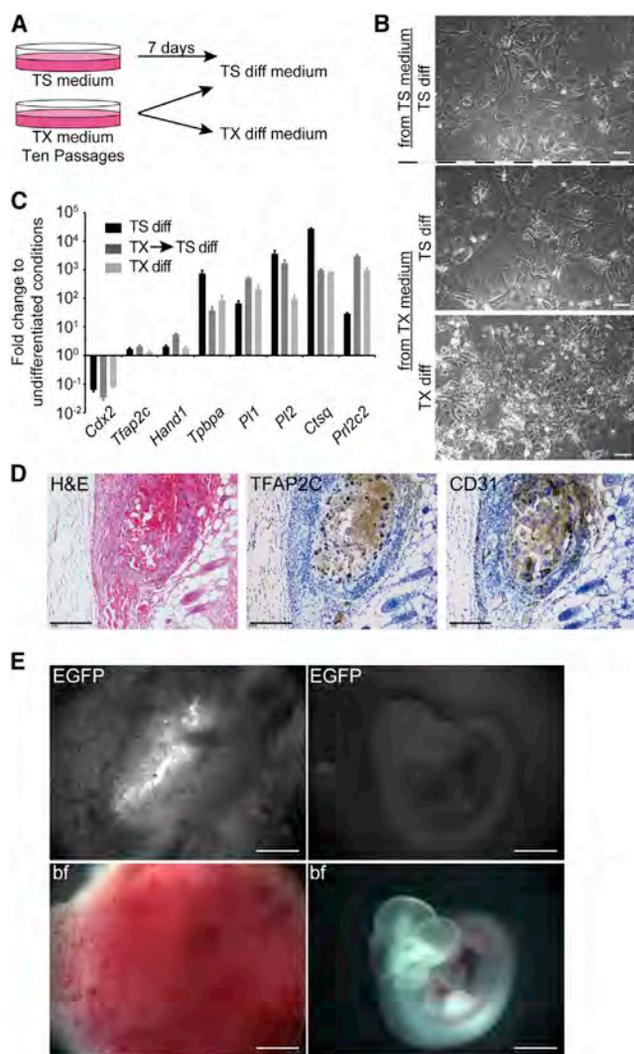


Figure 2. In Vitro and In Vivo Differentiation Potential of TSCs Cultured in TX Medium Compared to TS Medium

(A) Schematic of experimental approach. $TS_{6,5}$ cultured for ten passages in TS and TX media were switched to differentiation media.

(B) Photomicrographs of TSCs differentiated for 7 days, previously cultured in TS and TX media in the indicated differentiation conditions. The scale bar represents 100 μ m.

(C) Quantitative RT-PCR analyses of indicated marker genes in TS diff media (black bars), TX \rightarrow TS diff (dark gray bars), and TX diff (light gray bars) conditions. Bars represent mean values ($n = 3$) and SD of individual RNA preparations.

(D) Stained paraffin sections of hemorrhagic lesions 7 days after subcutaneous injection of $TS_{3,5}$ cells cultured in TS and TX media (p30+12). H&E, hematoxylin and eosin; immunohistochemical staining against TFAP2C and CD31. The scale bars represent 200 μ m.

(E) Photographs of a chimeric placenta (view of the embryo-facing side) following blastocyst injection of TS-EGFP cells cultured in TX medium and the corresponding embryo. TS-EGFP cells contributed to the placenta, but not to the embryo proper. EGFP, green channel; bf, bright field. The scale bar represents 1 mm.

cultured for 11 passages in TX medium were injected into blastocysts and implantations were analyzed on E10.5. EGFP-positive cells contributed to the placenta but were not found in the corresponding embryo (Figure 2E). Of note, the cells were cultured back in serum-containing medium for three passages prior to injection, in order to adjust for the presence of serum during the injection procedure in blastocyst medium, which contains 10% FBS (Longenecker and Kulkarni, 2009).

Derivation of TSCs in Defined Media from E6.5

Because we could demonstrate that established TSC lines can be adapted to grow in defined media, we asked whether the defined media also supported de novo derivation and maintenance of TSC lines. Therefore, the extraembryonic ectoderm (ExE) of E6.5 embryos were dissected, enzymatically digested, and plated on feeder cells on either uncoated or Matrigel-coated dishes. We were able to derive TSC lines in TX media in both conditions. In total, 5 out of 14 conceptuses gave rise to TS colonies. For comparison, in TS media in three out of six wells, TS colonies appeared (Table S1). These results indicate that the multipotent stem cells of the ExE can survive in serum-free medium and give rise to self-renewing TSC lines. Of note, we found that feeder cells can substitute for Matrigel coating. We hypothesize that this effect is most likely due to feeder cells secreting extracellular matrix proteins, which facilitate attachment of the cells. Due to poor attachment of the feeder cells on uncoated dishes in TX media, we had to preplate them using standard serum-containing medium. Thereafter, the feeder cell monolayer was rinsed with PBS and medium was changed to TX media. Newly generated TSC lines were cocultured on Matrigel-coated dishes with feeder cells until passage 5, thereafter TSC lines were plated on Matrigel-coated dishes and considered stable TSC lines from then on (Figure 3A).

Figure 3B displays typical TSC morphology of TSC line 1 (TS-L1) after one and ten passages. Two lines (TS-L1 and TS-L4; p10) were analyzed for the expression of trophoblast stem cell markers (*Cdx2*, *Eomes*, *Tfap2c*, *Gata3*, *Fgf2*, *Bmp4*, and *Furin*) and compared to cell lines, which were derived in standard conditions and later switched to defined media. Similar marker-gene expression confirmed that the derived cell lines are indeed true TSC lines (Figure 3C).

Genome-wide Transcriptional and DNA Methylation Profiles of TSCs in TS versus TX Media Are Highly Similar

To address the question whether growth in TX media has an effect on global gene expression, we generated comparative gene expression data by microarray analysis. RNA from three TS cell lines (TS-EGFP, $TS_{6,5}$, and $TS_{3,5}$) grown in TX and TS media was analyzed using Affymetrix

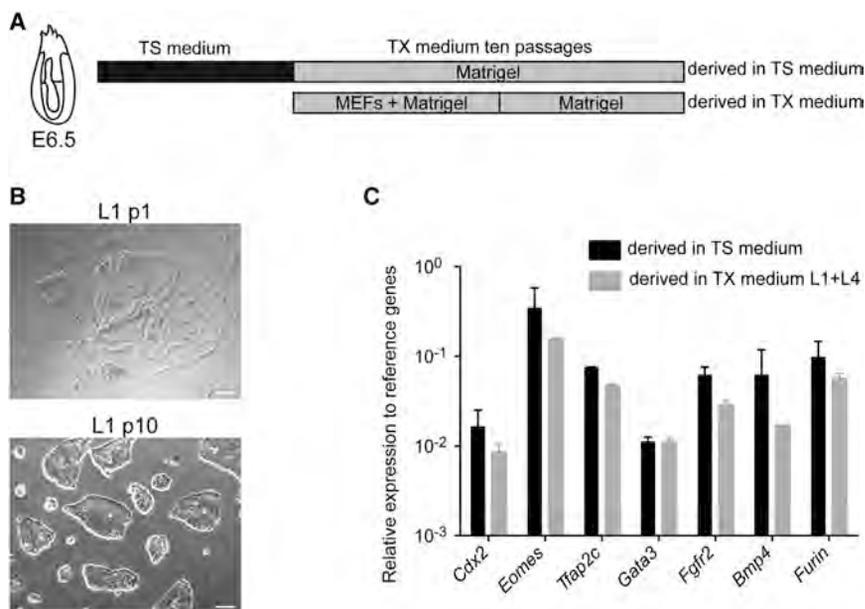


Figure 3. Characterization of De-Novo-Derived TSC Lines in TX Medium

(A) Schematic representation of culture conditions of newly derived TSC lines.

(B) Photomicrographs of TSC line 1 at p1 and p10 derived in TX media displaying typical TSC morphology. The scale bar represents 100 μm.

(C) Quantitative RT-PCR analysis of indicated TS marker genes of TSC lines derived in TX medium compared to TSC lines derived in standard conditions and thereafter cultured for ten passages in TX medium. Each bar represents mean values and SD of two individual TSC lines.

See also Table S1.

GeneChip Mouse Genome 430 2.0 Arrays. Hierarchical clustering revealed that samples clustered together according to their cell line of origin and not to the medium condition they were cultured in (TS_{6.5} and TS_{3.5}), except for TS-EGFP, where the sample grown in TS medium clusters most distinct from the other samples (Figure 4A). An exemplary scatterplot depicted in Figure 4B shows a very high degree of similarity in the expression profiles in both media conditions with 99.5% of all analyzed transcripts lying within the boundaries of the 2-fold change (Log₂ scale) in gene expression. For the other two cell lines analyzed, the percentages of similarity are 99.7% and 99.6%, respectively (Table S2). More importantly, key TSC transcription factors (*Cdx2*, *Gata3*, *Elf5*, *Eomes*, and *Tfap2c*) are found close to the midline of the scatterplot, indicating that their expression levels are identical under both conditions. Additionally, there is no transcript common to all three cell lines with a more than 2-fold decrease in TX versus TS medium (Figure 4C). Only nine genes were commonly upregulated in TX versus TS medium (Figure 4D; Table S3). This upregulation of transcripts in TX media was verified by qRT-PCR. All analyzed transcripts (*Dazl*, *Syce1*, *Ripk3*, and *Itih5*) displayed higher expression in TX media, thus confirming microarray results (Figure S4).

Next, we analyzed the epigenetic characteristics of cells grown in TX and compared them to standard TSC culture. To that end, two TSC lines cultured in parallel in TS and TX media (TS_{6.5} and TS-EGFP) and two that were derived in TX medium (TS-L1 and TS-L4) were chosen for immunoprecipitation of methylated DNA with an antibody against 5-methylcytosine followed by high-throughput Illumina sequencing (meDIP-Seq) (Senner et al., 2012; Weber et al.,

2005). Initially, we analyzed the methylation status of key stem cell genes under tight epigenetic control, notably the *Elf5* and *Nanog* promoter regions. *Elf5* hypomethylation is specifically observed in the trophoblast lineage and is required for its expression, whereas *Elf5* is tightly methylated and repressed in embryonic-lineage derivatives of the early conceptus (Ng et al., 2008). Vice versa *Nanog* is methylated and silenced in TSCs but hypomethylated and active in ESCs (Kuckenberg et al., 2011). The trophoblast lineage-characteristic methylation patterns at *Elf5* and *Nanog* were retained, indicating that, in the analyzed TSC lines, TX media did not have an effect on the epigenetic regulation of these key stem cell genes (Figures 5A, 5B, 5SA, and 5SB). On a global level (5 kb in-silico-designed probes spaced 20 kb apart) all TSC lines (including those that were derived in TX medium) exhibited an overall very similar distribution of DNA methylation throughout the genome, with R values of 0.939 (TS-EGFP) and 0.951 (TS_{6.5}; Figures 5C, 5D, 5SC, and 5SD). Only when specifically searching for methylation differences across different genomic features, few differences were observed between cells grown in TX and standard TS conditions that predominantly affected CpG islands (CGIs) with a similar, less striking effect at promoters (Figure S5E). Overall, TSC samples from TX media were relatively hypomethylated at CGIs compared to TSC samples from TS media (Figure 5E). Consistent with this, when using CGIs for closest neighbor analysis, the TSCs grown in TX media clustered together (Figure S5F). This was not observed when looking at promoters or contiguous 2 kb probes over the whole genome (not shown). When filtering for those CGIs with at least 8-fold more reads in TS versus TX, a set of

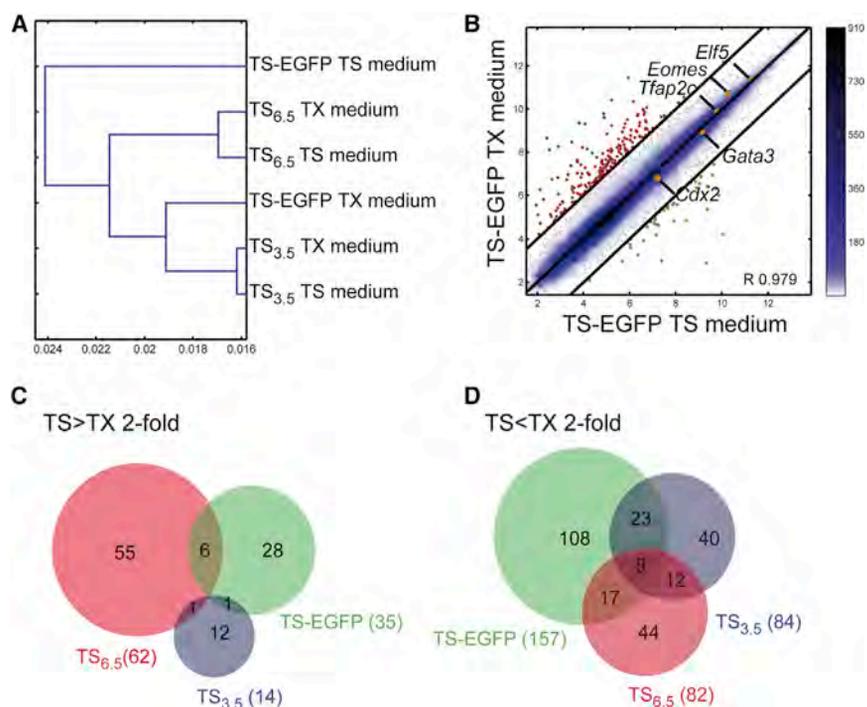


Figure 4. Global Gene Expression Analyses of TSCs Grown in TX Compared to Standard TS Medium

(A) Hierarchical clustering of TS lines (TS-EGFP, TS_{6.5}, and TS_{3.5}) in TS and TX media. (B) Pairwise scatterplot of global gene expression of TS-EGFP line in TS medium versus TX medium. Black lines indicate boundaries of 2-fold change in Log₂ scale. Genes downregulated in TX medium are represented as green dots; genes upregulated are represented as red dots. Color bar displays the scattering density: the darker the color, the higher the density. R, Fisher's correlation coefficient.

(C) Venn diagram comparing 2-fold down-regulated (in Log₂) transcripts in TX medium between three TSC lines (TS-EGFP, TS_{6.5}, and TS_{3.5}).

(D) Venn diagram comparing 2-fold up-regulated (in Log₂) transcripts in TX medium between three TSC lines (TS-EGFP, TS_{6.5}, and TS_{3.5}).

See also [Figure S4](#) and [Tables S2](#) and [S3](#).

58 hypomethylated CGIs was revealed. Of note, these 58 CGIs also appeared to have reduced methylation in E7.5 trophoblast, indicating that, at least in this respect, TSCs grown in TX medium are more similar to their *in vivo* counterparts ([Figure S5G](#)). These data strongly suggest that the epigenomic characteristics of bona fide TSCs are fully supported by culture in TX.

DISCUSSION

In the field of stem cell research, the demand for standardized media is rising, allowing for better reproducibility of results and thereby deepening our knowledge of the pathways regulating their maintenance and differentiation. Here, we set out to determine the minimum requirements for TSCs. Our results indicate that TSCs can be grown without FBS in media supplemented with insulin, transferrin, and low levels of the cytokines FGF4 and TGF- β 1 without losing their stem cell characteristics, self-renewal, and differentiation capability. Further, global gene expression and methylation analyses revealed a high degree of similarity of cells cultured in TX media when compared with standard TSC culture. We believe that TX medium now offers a tool for further studies on molecular pathways that govern cell-fate decisions, because of its well-defined extrinsic stimuli. One example for the importance of defined culture systems for the analysis of stem cell behavior was the bone-morphogenetic-protein-4-driven

induction of trophoblast from mouse embryonic stem cells, which is inhibited by serum or leukemia inhibitory factor, usually present in standard ESC culture ([Hayashi et al., 2010](#)).

A study from 2010 comparing different defined media formulations for hESCs used the criteria attachment, death, differentiated morphology, cell growth, and maintenance of stem cell surface marker expression on cells grown for ten passages in eight different media ([International Stem Cell Initiative Consortium et al., 2010](#)). According to this standard, our media formulation passed the test for all of the above-mentioned criteria. We could demonstrate that TX media in combination with Matrigel coating could support attachment, cell growth, and maintenance of stem-cell-marker expression without morphological appearance of differentiation in long-term culture. Nevertheless, we were not able to culture TSCs on a more defined substrate than Matrigel. Although we used a growth-factor-reduced Matrigel formulation, possible influences of Matrigel-derived factors cannot be excluded. Despite this limitation, it is striking that TSCs can cope with the absence of FBS, even after derivation in serum-containing medium and adaptation to standard culture conditions for more than 30 passages (i.e., TS_{3.5} cells). For some TSC lines, growth rates in TX media were lower when compared to TS media. This is most likely due to a combined effect of decreased proliferation rates and the increase of cell death. A possible explanation might be that, in TX medium, inhibition of the proapoptotic factor BIM by FGF4 alone is not

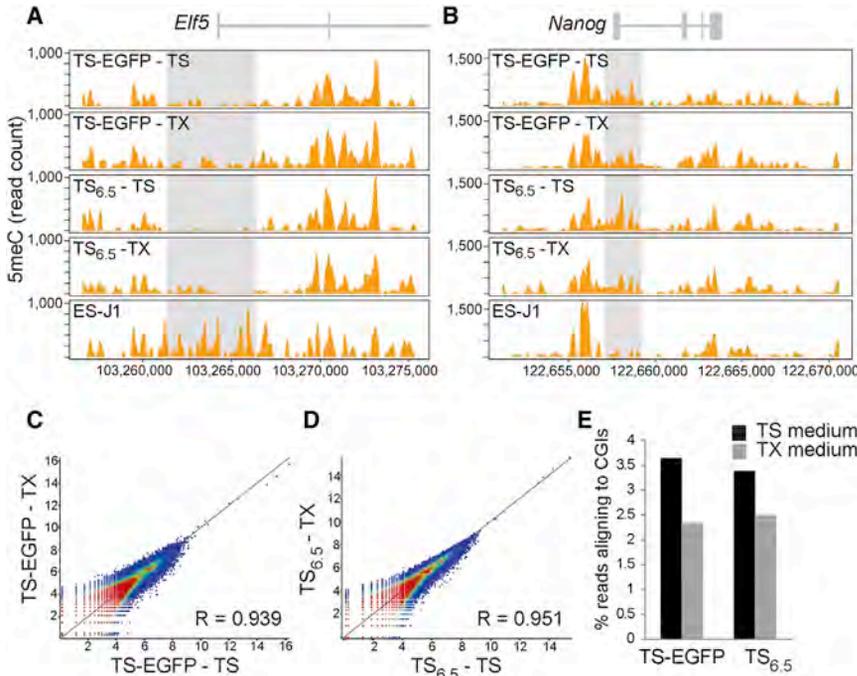


Figure 5. Global DNA Methylation Profiles of TSCs Grown in TX Compared to Standard TS Medium

(A and B) DNA methylation patterns determined by meDIP-Seq at the *Elf5* locus, which is hypomethylated in TSCs but methylated in ESCs, and *Nanog*, which exhibits the opposite methylation profile. The distribution and enrichment of methylation at these key transcription factors remains unchanged in TX medium.

(C and D) Scatter plots displaying Pearson's correlation of global methylation between cells grown in TS versus TX medium. Each point corresponds to a 5 kb in silico probe spaced 20 kb apart. Values were normalized for total read count and converted to a Log_2 scale. R values very close to one indicate that the methylation profiles of TSCs grown in TX and TS media are extremely similar to each other on a global scale.

(E) CpG islands (CGIs) displayed the most striking methylation differences between the two culture conditions with less reads mapping to CGIs in TX medium.

See also Figure S5.

sufficient and that there are other factors present in serum which additionally inhibit this factor (Yang et al., 2006).

In this study, our first goal was to validate the analyzed medium and to fully demonstrate the ability to maintain stem cell characteristics in defined medium. We did not try to exclude further factors from the original E8 formulation by Chen et al. (2011), albeit ascorbic acid is one of the supplements that may well be dispensable for TSC culture. Unlike hESCs, it is not required by mouse ESCs (mESCs), probably because of the inability of humans in contrast to mice to synthesize this vitamin (Furue et al., 2008). Our medium formulation can be considered a starting point for the defined culture of murine TSCs. We speculate that this medium might enable the derivation of human TSC lines, because the establishment of defined media for murine and human ESCs facilitated the derivation of ESC lines from additional species, such as rats (Nichols and Smith, 2009). Additionally, Furue et al. (2005, 2008) first established a defined medium for mESCs, which was then adjusted for the use of hESC culture.

Interestingly, mESCs grown in serum-containing medium display greater morphological heterogeneity when compared with so-called naive mESCs cultured in 2i, a defined medium that inhibits mitogen-activated protein kinase/extracellular-signal-regulated kinase kinase and glycogen synthase kinase 3 kinases (Marks et al., 2012). Because we also observed a similar effect on the morphology of the three different TSC lines when cultured

in TX media, it seems that lack of serum rather than inhibition of specific signaling pathways is responsible for the more-homogenous phenotype of different stem cell lines in defined media. This is also reflected by the fact that the TS-EGFP line cultured in TS medium clusters most distinct from the other two analyzed TSC lines in the microarray expression analysis, whereas after culture in TX media, the sample clusters closer to the other samples.

DNA methylation profiling revealed that lineage restriction by hypo- and hypermethylation of key regulators of trophoblast fate (*Elf5*) and ESC fate (*Nanog*) is not affected in TX media. Thus, trophoblast lineage identity remains fixed.

Recent reports demonstrate that culture of mESCs in 2i conditions causes global hypomethylation but spares key regulatory elements, such as imprinted differentially methylated regions (Leitch et al., 2013; Yamaji et al., 2013). Further, gene-expression-array-based hierarchical clustering revealed that the pluripotent cells (mESCs and embryonic germ cells) cluster according to the media they are grown in and not to their embryonic origin (Leitch et al., 2013). In our case, growth in TX medium did not cause hypomethylation on a global level but seemed to specifically result in lower methylation of CGIs. Accordingly, only the methylation levels of CGIs, but not of contiguous sequences or promoters, allowed a distinct clustering between the two media. This might be due to inherent differences in the biology of TSCs and mESCs or might reflect



the fact that the regular media used for TS culture already mirror the *in vivo* requirements to a very high extent. However, in TX media, the methylation levels of a set of CGIs more closely resembled the levels of *in vivo* E7.5 trophoblast cells, suggesting that TX conditions might reflect the *in vivo* situation even better than the regular media. Hypomethylation of CGIs is unlikely attributed to downregulation of the *de novo* methyltransferases *Dnmt3a*, *Dnmt3b*, and *Dnmt3l*, because they are not deregulated according to our microarray gene expression analysis.

Additionally, a surprising similarity between the work by [Leitch et al. \(2013\)](#) and our analysis is the fact that among the nine upregulated transcripts in TX media were two factors (*Dazl* and *Syce1*) whose expression is also induced by culture of mESCs in 2i. Taken together, we have established a platform for the standardized routine culture of murine TSCs under defined conditions, which might open the possibility for derivation of TSCs from other mammalian species.

EXPERIMENTAL PROCEDURES

Animal Studies

All experiments were conducted according to the German law of animal protection and in agreement with the approval of the local institutional animal care committees (Landesamt für Natur, Umwelt und Verbraucherschutz, North Rhine-Westphalia [approval ID number: 8.87-50.10.31.08.238]).

Cell Culture

Cells were grown on tissue culture plastic (TPP) at 37°C in humidified incubators containing 7.5% CO₂ (Heraeus, Thermo Fisher Scientific) to ensure a pH of 7.4 for both media conditions.

For conventional stem cell culture, cells were grown in TS medium containing serum and supplemented with 70% CM, 25 ng/ml human recombinant FGF4 (Reliatech) and 1 µg/ml heparin (Sigma-Aldrich) as published before ([Kuckenberg et al., 2010](#)). For differentiation experiments, either TS medium without CM, FGF4, and heparin (TS diff) or TX medium without FGF4, heparin, and TGF-β1 (TX diff) was used. For stem cell culture in defined medium, cells were cultured on Matrigel-coated dishes (Growth Factor Reduced Matrigel [BD Biosciences] diluted 1:30 in ice-cold DMEM/F12) in TX medium. TX medium formulation was DMEM/F12 without HEPES and L-glutamine (Life Technologies), 64 mg/l l-ascorbic acid-2-phosphate magnesium, 14 µg/l sodium selenite, 19.4 mg/l insulin, 543 mg/l NaHCO₃, 10.7 mg/l holo-transferrin (all Sigma-Aldrich), 25 ng/ml human recombinant FGF4 (Reliatech), 2 ng/ml human recombinant TGF-β1 (PeproTech), 1 µg/ml heparin (Sigma-Aldrich), 2 mM L-glutamine, 1% penicillin, and streptomycin (all PAN-biotech). Medium was prepared without growth factors; stored at 4°C; and FGF4, heparin, and TGF-β1 were added prior to use. Medium was changed every other day. Cells were passaged when subconfluent by incubation in trypsin-EDTA (PAN-biotech) for 4 min at 37°C. The enzyme was inactivated by addition of trypsin inhib-

itor (Life Technologies) in defined culture and by serum-containing medium in case of conventional culture. For cryopreservation, cell pellets were resuspended either in FBS/10% DMSO (standard conditions) or in KnockOut Serum Replacement/10% DMSO (Life Technologies). Passage numbers are represented as $x + y$, where x represents the passage number at which cells were transferred to TX medium and y being the passage number in TX.

Cell Lines

The TSC line TS-EGFP has been described previously ([Tanaka et al., 1998](#)). TSC lines TS_{3,5} and TS_{6,5} have been derived in our lab according to standard protocols from blastocysts and postimplantation conceptuses, respectively ([Himeno et al., 2008](#)). TS-L1 and TS-L4 were derived directly in defined medium. In brief, whole conceptuses were incubated in 40 µl trypsin-EDTA (PAN-biotech) for 15 min in the incubator and transferred to a 24-well plate on preplated irradiated mouse embryonic fibroblasts in either TS medium with 1.5× FGF4 and heparin concentration or TX medium with 1.5× FGF4 and heparin.

Immunofluorescent Staining, Protein Analysis

Before staining, cells were washed with PBS once and fixed for 10 min with 4% paraformaldehyde (Sigma-Aldrich). Permeabilization of cells was performed with 0.5% (v/v) Triton X-100 (Sigma-Aldrich) in PBS for 10 min and subsequent incubation for 30 min in blocking buffer (2% bovine serum albumin [Sigma-Aldrich], 0.1% [v/v] Triton X-100 in PBS). Samples were incubated overnight at 4°C with primary antibodies ([Table S1](#)). After three washing steps, detection was performed with appropriate Alexa Fluor-conjugated antibodies (Life Technologies) at a 1:500 dilution in blocking buffer for 1 hr at room temperature in the dark. After secondary antibody incubation, nuclei were stained with Hoechst (Sigma-Aldrich). Cells stained without primary antibodies served as controls. For western blot analysis, 23 µg of protein from cell extract were separated as published before ([Kuckenberg et al., 2010](#)). For antibody dilutions, see [Table S4](#).

RNA Isolation and Analysis

RNA was isolated from cells using the RNeasy Minikit (QIAGEN) according to the manufacturer's protocol. Five hundred nanograms total RNA was used for cDNA synthesis by RevertAidPremium (Fermentas, Thermo Scientific). Quantitative real-time PCR was performed on ViiA 7 (Applied Biosystems, Life Technologies) using the Maxima SYBR Green Master Mix (Fermentas, Thermo Scientific). For primer sequences, see [Table S5](#). Expression of target genes was normalized to the mean value of actin and glyceraldehyde-3-phosphate dehydrogenase. Each reaction was performed in triplicate. Microarray analysis using Mouse Genome 430 2.0 GeneChip expression arrays (Affymetrix) was performed with 100 ng total RNA as published before ([Kuckenberg et al., 2011](#)). The normalization was calculated with the robust multiarray analysis algorithm ([Irizarry et al., 2003](#)). Data postprocessing and graphics were performed with in-house developed functions in Matlab. Hierarchical clustering of genes and samples was performed with one minus correlation metric and



the unweighted average distance (unweighted pair group method with arithmetic mean; also known as group average) linkage method.

Cell Cycle Analysis

TSCs grown in TS and TX media were dissociated by incubation (10 min) in Accutase (PAA, GE Healthcare) and fixed with 70% ice-cold ethanol. Cells were stained in propidium iodide (PI) staining solution (50 $\mu\text{g}/\text{ml}$ PI [BD]; 0.1% [v/v] Triton X-100 [Sigma-Aldrich]; 0.2 mg/ml RNaseA [Sigma-Aldrich] in PBS) for 30 min at room temperature. Flow cytometric analysis was performed on a FACSCanto (BD Biosciences) and analyzed using FlowJo Software (Tree Star).

Metaphase Spreads

To prepare metaphase spreads, TSCs cultured in defined and standard medium were treated with 0.2 $\mu\text{g}/\text{ml}$ colcemid (Sigma-Aldrich) for 80 min at 37°C. After colcemid treatment, cells were harvested, suspended in 6 ml 0.56% KCl, and incubated at room temperature for 10 min. Ice-cold fixative (75% methanol and 25% acetic acid) was added dropwise to the cell suspension and incubated for 20 min on ice for three times. Cells were resuspended in 200 μl fixative and dropped onto microscope slides, stained for 10 min in 5% Giemsa solution (Sigma-Aldrich), and analyzed on a Leica DM LB microscope using a 100 \times lens. For every cell line and passage number, 20 metaphases were counted.

TSC Transplantation

A total of 5×10^6 TSCs were resuspended in 200 μl CM containing FGF4 and were injected subcutaneously into male nude mice. After 7 days, lesions were dissected, fixed in 4% paraformaldehyde overnight, embedded in paraffin, and sectioned (4 μm). Sections were stained with hematoxylin and eosin or subjected to routine immunohistochemical staining protocols, followed by incubation with primary α -Tfap2c antibody (1:500; 6E4/4; Santa Cruz Biotechnology) or α -CD31 (1:25; Dianova) for 30 min at room temperature. Signal detection was performed semiautomatically in the Autostainer 480 S (Medac) using the Bright Vision+ polymer detection system (Medac).

Blastocyst Injection

D2B6F2 blastocysts were injected with 12–20 TS-EGFP cells grown for 11–37 passages in TX medium and switched back to TS medium for at least three passages. Blastocysts were transferred into pseudo-pregnant females (CB6F1).

DNA Methylation Profiling

Genome-wide DNA methylation profiles of TSCs derived and/or grown in control and TX medium were determined by mDIP-Seq as described previously (Senner et al., 2012). Newly derived TSC lines (L1 and L4) were analyzed at passage 10, and established TSC lines were analyzed 11 and 12 passages after switch to TX media. Reads were mapped to the mouse genome build NCBIM37, and final data analysis was performed using SeqMonk software (<http://www.bioinformatics.ac.uk/projects/>).

ACCESSION NUMBERS

Microarray data determined in the course of this work were deposited in National Center for Biotechnology Information's Gene Expression Omnibus (GEO) database (Edgar et al., 2002) and are accessible through GEO Series accession number GSE47719.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and five tables and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2013.12.013>.

AUTHOR CONTRIBUTIONS

C.K., C.S., N.S., and A.B. collected and/or assembled data; C.K., C.S., M.J.A.-B., M.H., and H.S. analyzed and interpreted data; C.K., C.S., M.H., and H.S. wrote the manuscript; P.K., M.P., and H.S. provided concept and design; A.Z. and O.B. provided resources; and H.S. provided financial support and final approval of the manuscript.

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