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—Original Article—

Extracellular glucose levels in cultures of undifferentiated mouse trophoblast stem cells affect gene expression during subsequent differentiation with replicable cell line-dependent variation

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Abstract. Mouse trophoblast stem cells (TSCs) have been established and maintained using hyperglycemic conditions (11 mM glucose) for no apparent good reason. Because glucose metabolites are used as resources for cellular energy production, biosynthesis, and epigenetic modifications, differences in extracellular glucose levels may widely affect cellular function. Since the hyperglycemic culture conditions used for TSC culture have not been fully validated, the effect of extracellular glucose levels on the properties of TSCs remains unclear. To address this issue, we investigated the gene expression of stemness-related transcription factors in TSCs cultured in the undifferentiated state under various glucose concentrations. We also examined the expression of trophoblast subtype markers during differentiation, after returning the glucose concentration to the conventional culture concentration (11 mM). As a result, it appeared that the extracellular glucose conditions in the stem state not only affected the gene expression of stemness-related transcription factors before differentiation but also affected the expression of marker genes after differentiation, with some line-to-line variation. In the TS4 cell line, which showed the largest glucose concentration-dependent fluctuations in gene expression among all the lines examined, low glucose (1 mM glucose, LG) augmented H3K27me3 levels. An Ezh2 inhibitor prevented these LG-induced changes in gene expression, suggesting the possible involvement of H3K27me3 in the changes in gene expression seen in LG. These results collectively indicate that the response of the TSCs to the change in the extracellular glucose concentration is cell line-dependent and a part of which may be epigenetically memorized.

Key words: Epigenetics, Glucose, Trophoblast stem cells

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Mouse trophoblast stem cells (TSCs) are maintained in a proliferative, undifferentiated state in the presence of exogenous factors such as FGF4, heparin and activin A [1, 2]. Withdrawal of these factors attenuates cell growth and induces their spontaneous differentiation primarily into trophoblast giant cells, with a small population differentiating into spongio- or labyrinthine trophoblast cells [3]. Because of their differentiation potency, TSCs have been widely used for two decades as an *in vitro* model for the study of the molecular and gene expression changes that occur during placentation. TSCs were originally derived from embryonic day (E) 3.5 blastocysts and the extraembryonic ectoderm of E6.5 post-implantation embryos [1]. The conventional culture conditions have been based on RPMI1640 medium containing 11 mM glucose [1, 4], which is far greater than the physiological glucose concentrations in blood (5.5 mM), and the oviductal (1.1 mM) and uterine (0.6 mM) fluids in the mouse [5]. Although maternal hyperglycemia is speculated to cause placental abnormalities in humans due, at least in part, to trophoblast dysfunction [6, 7], the impact of extracellular glucose conditions on gene expression and differentiation in TSCs has not been addressed, and thus remains unclear. A recent report showed that the human trophoblast cell line, BeWo, has a different transcriptome and metabolome at low (1 mM) versus high (25 mM) glucose conditions [8]. It has been also reported that high glucose (25 mM) has a negative influence on the proliferation of mouse embryonic stem cells, and on their ability to differentiate into neural lineage cells and cardiomyocyte cells [9, 10]. These studies prompted us to hypothesize that changes in extracellular glucose levels may affect the properties of TSCs.

Glucose is used as an energy resource, and as a biosynthetic material through its metabolism, and so changes in the levels of extracellular glucose could possibly affect cell viability and proliferation, particularly in highly proliferative cells such as cancer cells and stem cells [11–14]. In addition, metabolites of glucose such as acetyl-CoA and UDP-GlcNAc are used as the substrates for epigenetic modifications. Therefore, changes in extracellular glucose levels may modify the cellular epigenetic status. In fact, several studies have shown that changes in extracellular glucose levels can indeed modulate the levels of epigenetic modifications, such as histone acetylation and DNA methylation, resulting in alterations in gene expression and changes in cell function [15–18]. Since epigenetic changes can be inherited in cells after mitosis as an epigenetic memory, change in the extracellular glucose levels, even for a short time, may affect

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cellular functions over the long term via these epigenetic changes [15]. Therefore, extracellular glucose levels are predicted to widely affect cellular properties such as cell proliferation, gene expression, and epigenetic status in TSCs.

Here, we aimed to understand the acute and delayed effects of extracellular glucose levels on TSCs. For this, we cultured TSCs in media containing various glucose levels, and investigated the effects on cell proliferation, gene expression, epigenetic status as acute effects, and phenotypes after differentiation as delayed effects. The results indicated that the glucose levels in the undifferentiated state affect gene expression even after differentiation, with some line-to-line variation.

Materials and Methods

Reagents

All reagents were purchased from Wako (Osaka, Japan) unless otherwise noted. All PCR primers were purchased from Sigma-Aldrich (Tokyo, Japan) or Eurofins Genomics (Tokyo, Japan). The primer sequences and antibodies used in this study are listed in Supplementary Tables 1 and 2 (online only), respectively.

Cell culture

Four TSC lines on a C57BL/6 background (TS2, TS4 established in our laboratory) [19], a CD1(ICR) background (TS3.5-GFP) [1] and a JF1 × C57BL/6 F1 hybrid (JBF1TS1) background [20] were used in this study. The cells were maintained in an undifferentiated state in TS medium supplemented with 25 ng/ml FGF4, 10 ng/ml activin A (R&D Systems, Minneapolis, USA) and 1 μg/ml heparin (Sigma-Aldrich) (stem conditions) [4]. Differentiation was induced by withdrawal of these supplements. Glucose-free RPMI1640 and D-(+)-glucose solution (45%) (Sigma-Aldrich) were used to prepare modified RPMI1640 media containing 1, 5, 11, or 25 mM glucose. Where mentioned, D-mannitol (Sigma-Aldrich) was also added, to adjust for osmolality effects. For the Ezh2 inhibitor analysis, the cells were cultured in media containing 0.5 μM DZNep.

For cell growth analysis, TS4 cells were seeded on 3.5 cm dishes at 2×10^4 cells per dish in triplicate at each different glucose concentration and cells number was determined at days 2 and 4 of culture.

The methods for all biochemical experiments and the statistical analyses are described in detail in the Supplementary Methods (online only).

Results

Each TSC line responds differently to changes in glucose levels

To evaluate the effect of changes in extracellular glucose levels on the gene expression profile in TSC lines, we cultured four different TSC lines (TS2, TS4, TS3.5-GFP, and JBF1TS1) in the presence of various concentrations of glucose (1, 5, 11, and 25 mM) for 4 days while maintaining the cells in the stem condition. Following this, the cells were induced to differentiate using the conventional glucose concentration (11 mM glucose, equivalent to that used in RPMI1640) for 10 days (Fig. 1A). The gene expression of stemness-related transcription factors (TFs) [21–25] (Fig. 1B), and several markers for differentiated trophoblast subtypes [26–29] (Fig. 1C) were investigated by RT-qPCR at days 0 and 10 of differentiation, respectively.

The expression of stemness-related TFs fluctuated in the different extracellular glucose conditions in all four TSC lines, but the extent and direction appeared to be line-dependent. At 1 mM glucose (the low glucose condition, LG), for example, *Cdx2* expression was decreased in TS2 and TS3.5-GFP cells, increased in TS4 cells, and not affected in JBF1TS1 cells (Fig. 1B). On the other hand, *Sox2* was decreased in LG in all four cell lines. Interestingly, even though the cells were cultured at various glucose concentrations only under the stem condition, and were differentiated using the conventional 11 mM glucose concentration, fluctuations in the expression of some of these marker genes were observed 10 days after the differentiation, albeit with some line-to-line variations (Fig. 1C), implying that the extracellular glucose conditions modify the differentiation potency of TSCs. In particular, the expression levels of *Prl3d1* and *Tpbpa* were elevated only in the TS4 line cultured in LG.

A principal component analysis of the RT-qPCR results confirmed a cell line-dependent bias (Fig. 1D). Interestingly, the two TSC lines cultured under the same glucose condition did not cluster together. Instead, with the exception of TS4, each TSC line cultured under different glucose conditions formed separate clusters from the other cell lines. The genes most affected by the differing glucose concentrations, as well as direction of the fluctuation also appeared to be cell line-dependent.

Analysis of the effect of LG on the TS4 cell line

Among the four TSC lines, the gene expression profile of TS4 cells was the most affected by changes in extracellular glucose concentrations (Fig. 1D). We therefore selected the TS4 cell line for further experiments to investigate the effect of changes in glucose concentrations on the phenotype of TSCs in more detail. First, we assayed the cell growth of TS4 under various glucose conditions during the stem state. Compared with conventional glucose levels, there was no difference in cell proliferation for 4 days in any of the conditions assayed (Fig. 2A). The cells were morphologically indistinguishable, formed epithelial cell colonies, and could be maintained for at least two passages in all the glucose conditions examined (Supplementary Fig. 1: online only), indicating that extracellular glucose levels did not affect cell proliferation, at least over a few passages.

In the TS4 line, LG conditions modulated the gene expression of both stemness-related TFs and trophoblast subtype markers (Fig. 1B and C). Because hyperosmotic conditions have been reported to increase the expression of TFs related to trophoblast development in TSCs [30], we assessed if the hypoosmolarity of the LG condition was responsible for fluctuations in the expression levels of stemness-related TFs in TS4. As a result, an increase in the expression levels of *Cdx2* and *Eomes*, and a decrease in the expression levels of *Sox2,* were observed even when the osmolality was compensated for by the addition of an appropriate concentration of mannitol (Fig. 2B), suggesting that hypoosmolality of 1 mM glucose condition *per se* does not account for the changes observed in *Cdx2*, *Eomes,* and *Sox2* expression. The increased expression of the Cdx2 protein under LG conditions was also demonstrated by immunofluorescence staining (Fig. 2C and 2D). Of note, the distribution of the nuclear Cdx2 intensity (Fig. 2E) indicated an increase in the proportion of

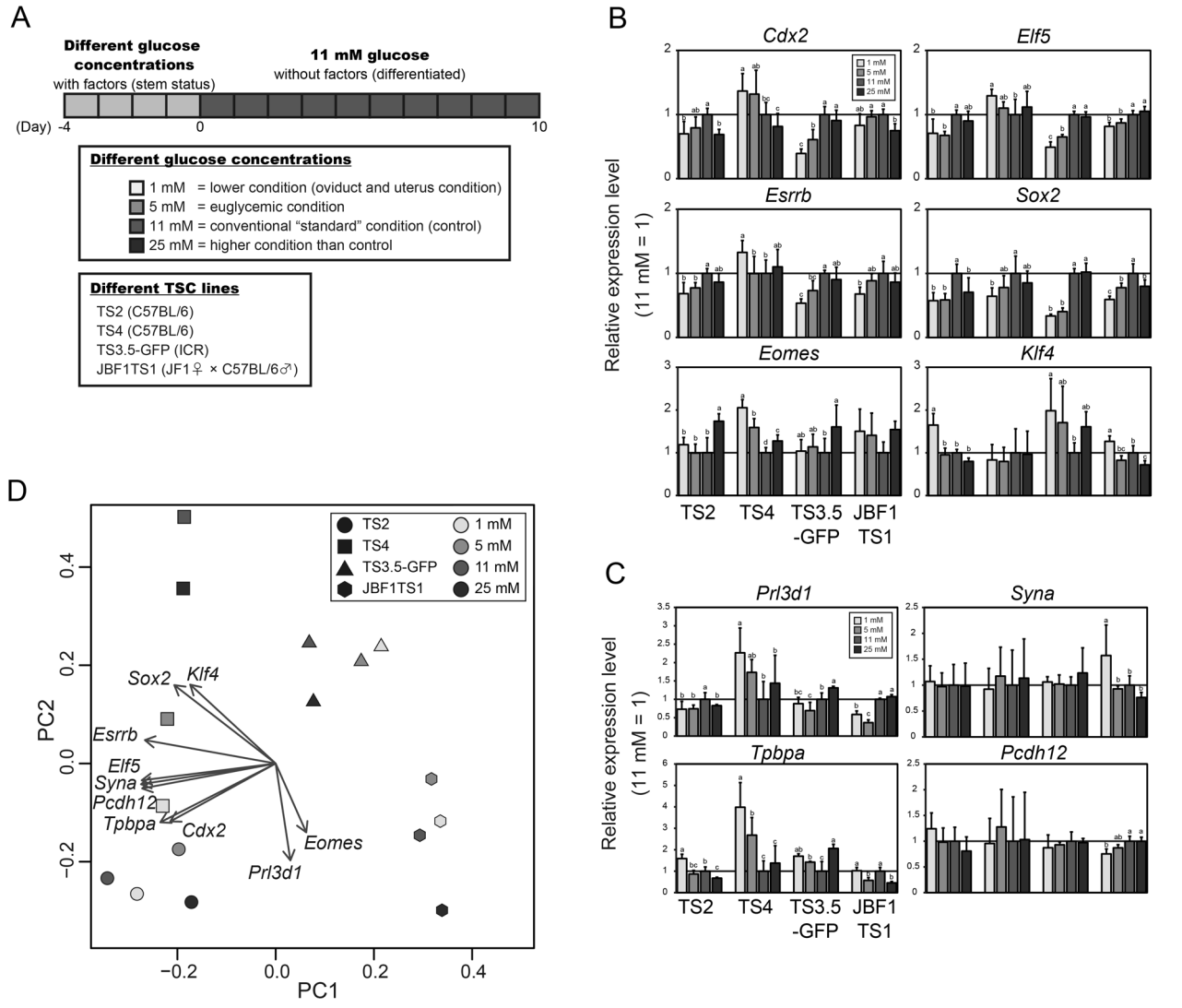
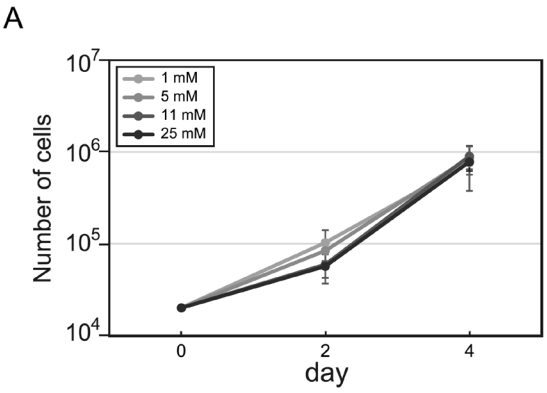
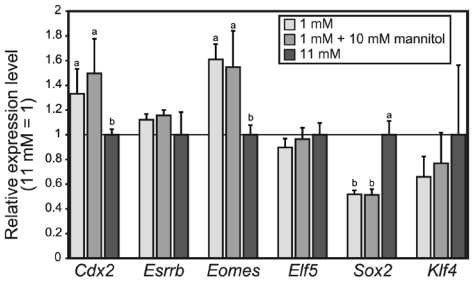


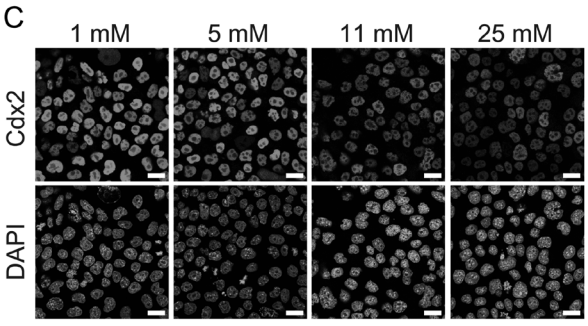
Fig. 1. Effect of extracellular glucose concentration on the gene expression profiles of TSCs before and after differentiation. (A) Experimental design. Four independent TSC lines in the stem state were cultured in the presence of different glucose levels for 4 days, after which the cells were differentiated for 10 days using the conventional 11 mM glucose concentration. (B, C) The relative mRNA levels of stemness-related transcription factors (B) and trophoblast subtype marker genes (C). The mean expression level (± SD) was normalized to the expression level of *Actb* at 11 mM glucose, which was arbitrarily set as 1. Means with the same letters were not significantly different (Tukey-Kramer test). (D) Principal component analysis based on the expression values of the genes investigated in (B) and (C). Each arrow with the gene names indicates the direction of the change in expression. Expression level becomes increasing toward the direction of an arrowhead.

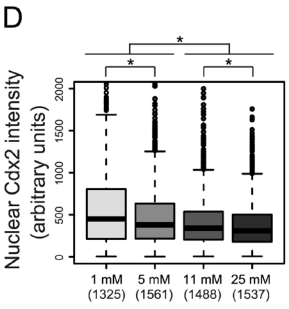
the TSCs with relatively higher Cdx2 levels (intensity > 750) and a decrease in those with lower Cdx2 levels (< 500). Moreover, the upregulation of the trophoblast subtype marker genes, *Prl3d1* and *Tpbpa*, which occurred following differentiation, was also observed under osmolality-adjusted LG conditions (Fig. 2F). Likewise, the increase in placental lactogen 1 (PL-1) protein (Fig. 2G and 2H), encoded by the *Prl3d1* gene, was confirmed by western blotting (WB) at day 10 of differentiation, and also in the 1 mM glucose + 10 mM mannitol condition (Fig. 2I and 2J).

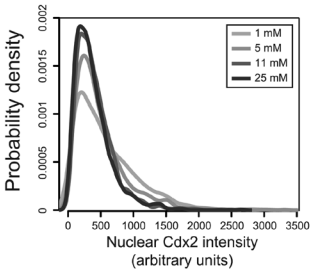
Among all the trophoblast subtypes, PL-1 is expressed specifically in trophoblast giant cells (TGCs), which have a large polyploid nucleus resulting from endoreduplication [31, 32]. Based on these data, it was hypothesized LG conditions promote the differentiation of TSCs into TGCs. To assess whether this was the case, we measured the size and DNA content of the nuclei at day 10 of differentiation, after exposing the TSCs to various glucose conditions only during the stem state, as shown in Fig. 1A (Fig. 2K, 2L, and 2M). Under control conditions, the nuclear sizes at day 10 of differentiation were significantly larger than those at day 1, suggesting that the nuclear size can be used as an index of TGC differentiation (Fig. 2L). For the TSCs incubated with the differing levels of glucose, there were no significant differences in the distribution of nuclear

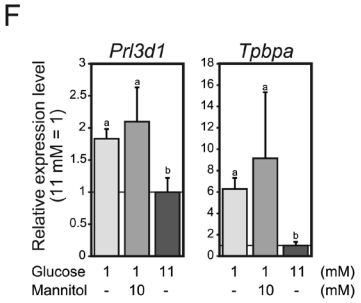

A
Number of cells
day
1 mM
5 mM
11 mM
25 mM

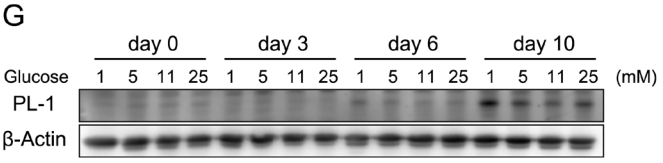

B
Relative expression level (11 mM = 1)
1 mM
1 mM + 10 mM mannitol
11 mM
Cdx2
Esrrb
Eomes
Elf5
Sox2
Klf4

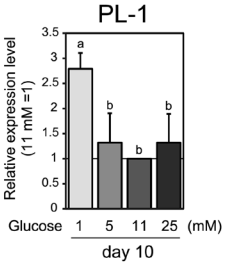

C
1 mM
5 mM
11 mM
25 mM
Cdx2
DAPI

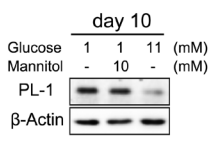

D
Nuclear Cdx2 intensity (arbitrary units)
1 mM (1325)
5 mM (1561)
11 mM (1488)
25 mM (1537)

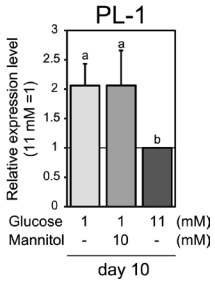

E
Probability density
Nuclear Cdx2 intensity (arbitrary units)
1 mM
5 mM
11 mM
25 mM

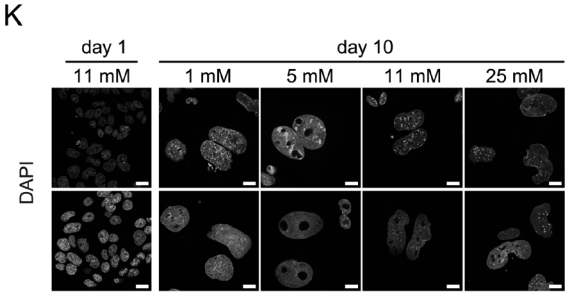

F
Prl3d1
Tpbpa
Relative expression level (11 mM = 1)
Glucose
Mannitol
(mM)

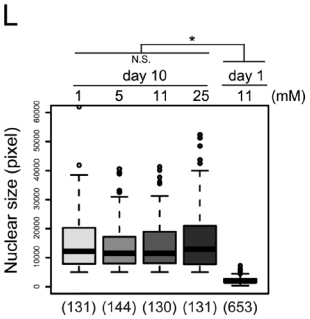

G
day 0
day 3
day 6
day 10
Glucose
(mM)
PL-1
β-Actin

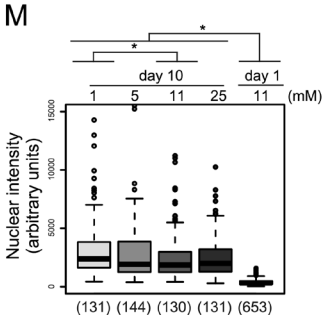

H
PL-1
Relative expression level (11 mM = 1)
Glucose
(mM)
day 10


I
day 10
Glucose
Mannitol
(mM)
PL-1
β-Actin


J
PL-1
Relative expression level (11 mM = 1)
Glucose
Mannitol
(mM)
day 10


K
day 1
day 10
11 mM
1 mM
5 mM
11 mM
25 mM
DAPI


L
N.S.
day 10
day 1
(mM)
Nuclear size (pixel)
(131) (144) (130) (131) (653)


M
day 10
day 1
(mM)
Nuclear intensity (arbitrary units)
(131) (144) (130) (131) (653)

size at day 10 among all the glucose conditions examined. These data indicate that differentiation into TGCs takes place equally under all the conditions examined. Quantification of the intensity of DAPI fluorescence also supported this notion. The intensities of nuclear DAPI staining measured at day 10 were significantly higher than those at day 1, with only the 1 mM glucose condition showing a slightly, but significantly, higher staining intensity than the control (Fig. 2M). Thus, TSCs appear to undergo endoreduplication, regardless of the glucose level during the stem state, and exposure to LG might enhance endoreduplication.

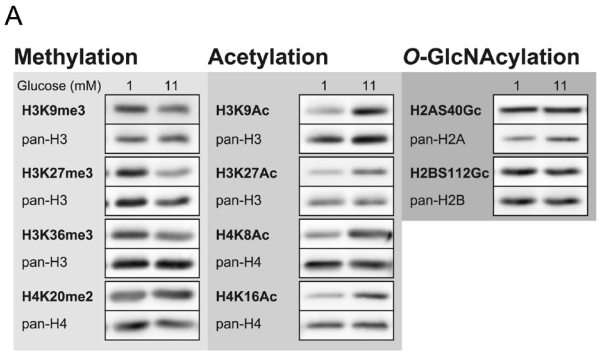
Histone methylation underlies the effect of LG condition

The TS4 line exposed to the LG condition showed higher expression levels of *Cdx2*/Cdx2 and *Prl3d1*/PL-1 before and after the differentiation, respectively, compared with the cells in the control condition. To examine the possible link between the deregulation of these two genes, we overexpressed HA-fused Cdx2 in the TS4 cell line cultured in 11 mM glucose (Supplementary Fig. 2A: online only). The data showed that while overexpression of Cdx2 was found before differentiation (Supplementary Fig. 2B and 2C), the increase in *Prl3d1*/PL-1 expression after differentiation did not occur (Supplementary Fig. 2D and 2E). Instead, in some Cdx2-overexpression experiments, the expression of *Prl3d1* was even lower than the control, although this was not consistently observed. These data indicate that the increase in Cdx2 levels under LG conditions is not a causal event for the higher expression of *Prl3d1*/PL-1 after differentiation.

As the differentiation of TSCs was carried out in the presence of 11 mM glucose in all experiments, it seemed reasonable to suspect that the effects of LG were epigenetically memorized before differentiation, which later resulted in an increase in the expression of *Prl3d1* and *Tpbpa* after differentiation of the TS4 line. Accordingly, we next compared the epigenetic status of TSCs exposed to either 1 mM or 11 mM glucose. WB using a variety of anti-methylated, anti-acetylated or anti-*O*-GlcNAcylated histone antibodies revealed that the acetylation levels of histone 3 lysine 9 and histone 3 lysine 27 (H3K9Ac and H3K27Ac, respectively) were decreased, and the levels of tri-methylation of histone 3 lysine 27 (H3K27me3) were increased, in cells cultured in 1 mM glucose compared to those in cells cultured in 11 mM glucose (Fig. 3A and 3B). Consistent with these results, a nucleosome repeat length assay suggested genome wide chromatin condensation occurred in cells cultured in 1 mM glucose (Supplementary Fig. 3A: online only). Whole DNA methylation levels and the amount of histones, on the other hand, were not different between the two glucose conditions (Supplementary Fig. 3B–E). We then investigated the levels of H3K27Ac and H3K27me3 at the following genomic regions; around the transcriptional start sites (TSS) for *Cdx2* and *Prl3d1*, upstream of *Cdx2* (UP), the regulatory regions of *Cdx2* effective in blastocysts (TEE) [33], and in TSCs (#1 and #3) [34], the putative binding sites for Hand1 (TSS (HAND1)), AP1 (AP1) and Gata2 (GATA) in the regulatory region of rodent *Prl3d1* [35–37], and a control region (TSS of *Actb*) (Fig. 3C and 3D). Compared to the control region, all regions exhibited lower H3K27Ac levels, and higher H3K27me3 levels, in each of the two culture conditions. Moreover, the H3K27Ac levels were lower in 1 mM glucose than in 11 mM glucose at all the regions examined, except for TSS (HAND1) and AP1, which were found to be barely acetylated in both conditions. Conversely, the H3K27me3 levels were higher in 1 mM glucose compared to the control at all regions, with the exception of *Actb* TSS. These data support the WB data, described earlier (Fig. 3A and 3B). The increase in the H3K27me3 levels in 1 mM glucose was not due to augmented expression of the H3K27me3 methylase, *Ezh2*, nor due to a reduction in the expression levels of the demethylases, *Kdm6a* and *Kdm6b* (Supplementary Fig. 3G). Consistent with the augmented H3K27me3 level, the DNA methylation rate around the TSS of *Prl3d1* was also higher in 1 mM glucose than in 11 mM; however, this change in DNA methylation showed a negative correlation with *Prl3d1* expression (Supplementary Fig. 3F).

To determine if the increase in H3K27me3 level underlies the observed effects of 1 mM glucose on TS4, we added an Ezh2 inhibitor (DZNep) to cells in the stem state cultured in 1 mM glucose. The H3K27me3 levels increased in 1 mM glucose, as seen previously, and this effect was abolished by treatment with the inhibitor for 4 days (Fig. 3E and 3F). As a result of the inhibitor treatment, the increase in *Cdx2* expression in the presence of 1 mM glucose was prevented (Fig. 3G). After differentiation, the expression of *Prl3d1*/PL-1 and *Tpbpa* in DZNep-treated TSCs was not upregulated even though the cells had been cultured in 1 mM glucose during the undifferentiated

Fig. 2. Phenotype analyses of TS4 cells cultured in different concentrations of glucose. (A) Cell growth in different concentrations of glucose. The mean values (± SD) of triplicates of four biological replicates are shown. (B) Relative mRNA levels of stemness-related TFs. Where indicated, 10 mM D-mannitol was added to adjust the osmolality. The mean (± SD) values normalized to *Actb* are indicated relative to values at 11 mM glucose, which was arbitrarily set as 1. Means with the same letters were not significantly different (Tukey-Kramer test). (C) Confocal microscopy images of the immunofluorescence staining for Cdx2. The cells were stained using an anti-Cdx2 antibody and counterstained with DAPI. Scale bars, 20 μm. The images were used to calculate the pixel intensity values using the CellProfiler software for further analyses (D and E). (D, E) Box plots (D) and histograms (E) showing the distribution of the Cdx2 signal intensity in each nucleus and the frequency distribution of the nuclear Cdx2 intensities found at each glucose concentration, respectively. The *P*-value was calculated using a Wilcoxon rank-sum test. The number of nuclei analyzed is shown in parentheses (D). (F) Relative mRNA levels of trophoblast subtype marker genes with, or without, osmolality adjustment by the addition of D-mannitol. Values are indicated as in (B). (G) Western blotting (WB) of PL-I at days 0, 3, 6, and 10 of differentiation. β-Actin was used as the loading control. A representative result of three biological replicates is shown. (H) Relative expression level of PL-1 on day 10 of differentiation. WB band intensities were calculated using ImageJ software. Mean (± SD) values, normalized to β-Actin, are indicated relative to the values for 11 mM glucose, which were arbitrarily set at 1. Means with the same letters were not significantly different (Tukey-Kramer test) (I) WB of PL-1 with, or without, osmolality adjustment. A representative result from three biological replicates is shown. (J) Relative expression level of PL-1, with or without the osmolality adjustment. WB band intensities were calculated using ImageJ software. Values are indicated as in (H). (K) Nuclear images of differentiated TSCs cells cultured in different glucose concentrations. Scale bars, 20 μm. The pixel intensity values were determined from the images using the CellProfiler software for further analyses (L and M). (L, M) The box plots indicate the distribution of nuclear sizes (L) and DAPI intensities (M), as determined using the CellProfiler software. The number of nuclei analyzed is shown in parentheses. The *P*-value was calculated using the Wilcoxon rank-sum test.


A
Methylation
Acetylation
O-GlcNAcylation
Glucose (mM) 1 11
H3K9me3
pan-H3
H3K27me3
pan-H3
H3K36me3
pan-H3
H4K20me2
pan-H4
H3K9Ac
pan-H3
H3K27Ac
pan-H3
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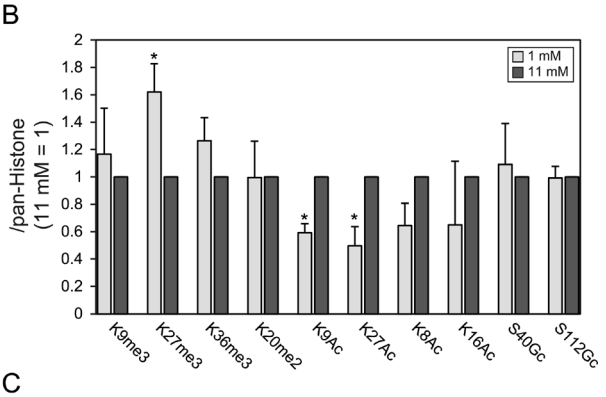
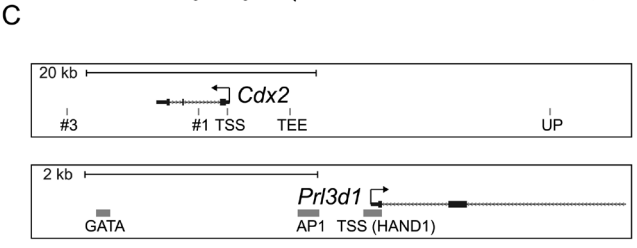
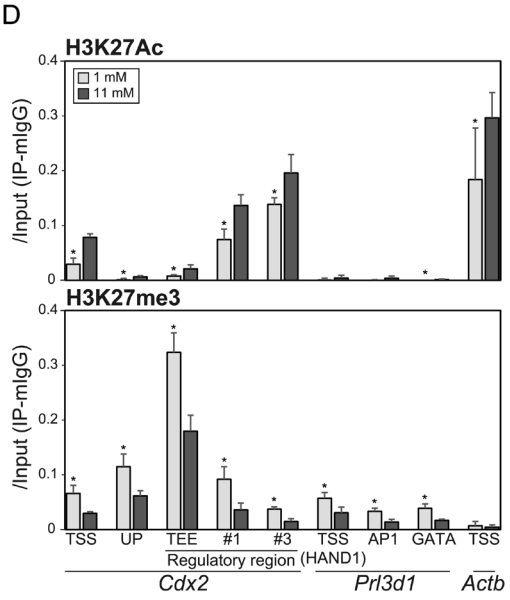
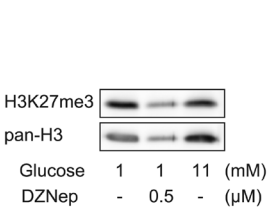
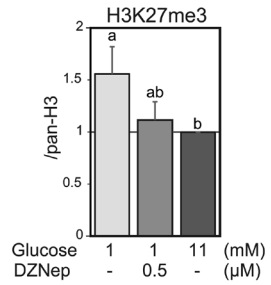
pan-H2A
H2BS112Gc
pan-H2B

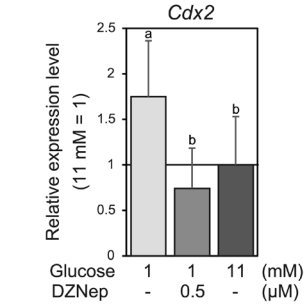

B
/pan-Histone (11 mM = 1)
1 mM
11 mM
K9me3
K27me3
K36me3
K20me2
K9Ac
K27Ac
K8Ac
K16Ac
S40Gc
S112Gc

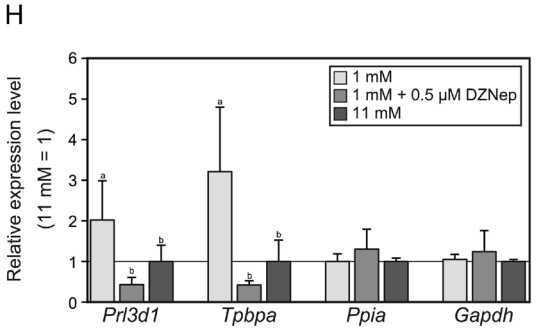

C
20 kb
Cdx2
#3
#1 TSS
TEE
UP
2 kb
Prl3d1
GATA
AP1
TSS (HAND1)

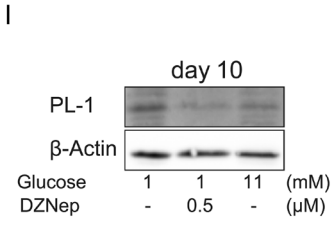

D
H3K27Ac
1 mM
11 mM
/Input (IP-mIgG)
H3K27me3
TSS
UP
TEE
#1
#3
TSS
AP1
GATA
TSS
Regulatory region (HAND1)
Cdx2
Prl3d1
Actb

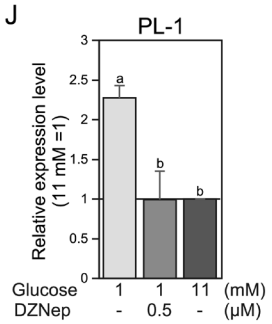

E
H3K27me3
pan-H3
Glucose 1 1 11 (mM)
DZNep - 0.5 - (μM)

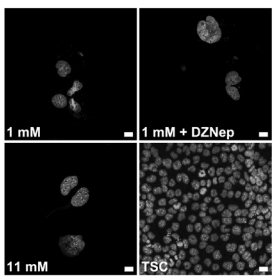

F
H3K27me3
/pan-H3
a
ab
b
Glucose 1 1 11 (mM)
DZNep - 0.5 - (μM)

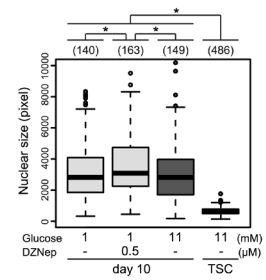

G
Cdx2
Relative expression level (11 mM = 1)
Glucose 1 1 11 (mM)
DZNep - 0.5 - (μM)


H
Relative expression level (11 mM = 1)
1 mM
1 mM + 0.5 μM DZNep
11 mM
Prl3d1
Tpbpa
Ppia
Gapdh


I
day 10
PL-1
β-Actin
Glucose 1 1 11 (mM)
DZNep - 0.5 - (μM)


J
PL-1
Relative expression level (11 mM =1)
Glucose 1 1 11 (mM)
DZNep - 0.5 - (μM)


K
1 mM
1 mM + DZNep
11 mM
TSC


L
(140)
(163)
(149)
(486)
Nuclear size (pixel)
Glucose 1 1 11 11 (mM)
DZNep - 0.5 - - (μM)
day 10
TSC

state (Fig. 3H–J). The expression of the housekeeping genes, *Ppia* and *Gapdh*, was not affected by the different glucose concentrations, or by treatment with the inhibitor (Fig. 3H). In addition, nuclei larger than the TSCs emerged after differentiation, regardless of the presence or absence of DZNep during the stem state (Fig. 3K and 3L), indicating that treatment with DZNep before differentiation specifically affects the expression of *Prl3d1* and *Tpbpa*, but not the differentiation ability of the TSCs. Taken together, the increase in H3K27me3 levels was likely to mediate the effect of LG on *Prl3d1*/PL-1 and *Tpbpa* expression after differentiation in TS4 cells.

Discussion

As gestational hyperglycemia is one of the risk factors for placental abnormalities, resulting, in some cases, in an impaired pregnancy and birth defects [6, 7], the effects of glucose levels on the differentiation and function of trophoblast cells has been of interest. It has been shown, for example, that exposure to high glucose levels during the differentiation of the choriocarcinoma-derived rat Rcho-1 trophoblast cell line represses the production of progesterone [38]. High-glucose concentrations also hindered the invasiveness of the HTR-8/SVneo trophoblast cells derived from the chorionic villi explants of human first-trimester placenta [39]. Another study demonstrated that upregulation of invasion-related genes, and also of invasive trophoblast marker genes, following high glucose treatment of the human BeWo choriocarcinoma cell line [40], suggesting there is a cell line-dependent difference in the response to hyperglycemic conditions. Unlike these previous studies, in which cells were continuously exposed to various glucose conditions during differentiation, here we focused on the effect of different extracellular glucose levels on stem cells of the trophoblast lineage using mouse TSCs. Different extracellular glucose levels not only affected the gene expression patterns of TSCs in the stem state, but also altered gene expression, with line-to-line variations, even after differentiation, despite the fact that differentiation *per se* was carried out using the conventional control glucose concentration of 11 mM glucose.

Besides its major use as an energy resource, glucose is also used as a material for biosynthesis through its metabolism. Particular types of cells, such as cancer cells and stem cells, which show high cell growth rates, exhibit a glycolysis-dominant metabolism to satisfy the demand for biomaterials to support rapid cell proliferation [41, 42]. Accordingly, the proliferative activity of these cells has been reported to decrease under low glucose conditions [11–13]. Conversely, in embryonic stem cells (ESCs), hyperglycemic culture conditions have a negative effect on cell growth via an inhibition of the cell cycle [14]. These studies indicate that both hyper- and hypo-glucose conditions can modulate cellular proliferative capability. TSCs also have a glycolysis-dominant metabolism during their proliferative, undifferentiated state [43]. Because of the similarity of their glucose metabolism profile to cancer cells and other types of stem cells, we expected that the proliferation of TSCs would be affected by different extracellular glucose levels. However, the growth of TSCs did not appear to be affected at any of the glucose levels evaluated in this study, indicating a high adaptability of TSCs to various glucose levels in terms of their cellular proliferation. Mammalian cells are surrounded by diverse nutrients and exhibit metabolic homeostasis to supplement the lack of one nutrient with alternative nutrients using nutrient sensor pathways such as the mechanistic target of rapamycin complex 1 (mTORC1) pathway [44]. In fact, maternal caloric restriction results in the downregulation of glucose transporter genes and the upregulation of amino acid transporter genes in mouse placenta [45, 46]. These studies suggest that trophoblast cells are highly adaptive to the availability of nutrients at the maternal-fetal interface, and thus TSCs might have the ability to maintain their cell growth by flexibly switching the source of biomaterials depending on the availability of glucose. A metabolome analysis of TSCs cultured under different glucose concentrations might allow us to reveal how TSCs maintain their cell growth rate under different glucose conditions.

The overexpression of Cdx2 (Cdx2-OE) affected the expression of other stemness-related TFs in the TS4 cell line, although the changes in gene expression pattern observed in cells cultured in the presence of 1 mM glucose could not be recapitulated by Cdx2-OE. Previous studies, such as a genome-wide distribution analysis by ChIP-seq and ES-to-TS trans-differentiation experiments through the induction

Fig. 3. Effect of low glucose level on the epigenetic status of TS4 cells. (A) WB of histone modifications. WB using antibodies for each core histone were used as loading controls. The representative results of three biological replicates are shown. (B) Relative levels of the histone modifications. WB band intensities were calculated using ImageJ software. The mean (± SD) values, normalized to the pan-histones, are indicated relative to the values for 11 mM glucose, which were arbitrarily set at 1. The *P*-value was calculated relative to the 11 mM glucose concentration (two-tailed Student's *t*-test). (C) *Cdx2* and *Prl3d1* gene loci. Each gray box shows the genomic regions amplified by the ChIP primers. (D) Quantitative ChIP analyses of H3K27Ac and H3K27me3 levels at the *Cdx2* and *Prl3d1* loci. Values of technical triplicates of biological duplicates were normalized to that of the input DNA. The mean (± SD) of the IP subtracted by that of mouse IgG as a negative control are shown. The *P*-value was calculated relative to the 11 mM glucose concentration (two-tailed Student's *t*-test). (E) WB of H3K27me3 levels after treatment with DZNep. A representative result of three biological replicates is shown. Pan-H3 was used as the loading control. (F) Relative H3K27me3 level. The band intensities were determined using ImageJ software. The mean (± SD) levels were normalized to the intensity of the pan-H3 levels, and are indicated relative to the 11 mM glucose concentration, which was arbitrarily set as 1. Means with the same letters were not significantly different (Tukey-Kramer test). (G, H) Relative mRNA level of *Cdx2* before differentiation (G) and trophoblast subtype markers at day 10 of differentiation (H) after DZNep treatment during the stem state. The mean (± SD) levels were normalized to the expression level of *Actb,* and are indicated relative to the 11 mM glucose concentrations, which was arbitrarily set as 1. Means with the same letters were not significantly different (Tukey-Kramer test). (I) WB of PL-1 after treatment with DZNep. β-Actin was used as the loading control. A representative result from three biological replicates is shown. (J) Relative expression level of PL-1 with or without DZNep. WB band intensities were calculated using ImageJ software. Values are indicated as in (F). (K) Nuclear images of differentiated TSCs after DZNep treatment, and TSCs. Scale bars, 20 μm. Pixel intensity values were determined from the images using the CellProfiler software for further analyses (L). (L) The box plots indicate the distribution of the nuclear sizes, as determined using the CellProfiler software. The number of nuclei analyzed is shown in parentheses. The *P*-value was calculated using the Wilcoxon rank-sum test.

of Cdx2, revealed that Cdx2 is a core factor in the transcription factor network, acting along with Eomes and Elf5 to sustain the stem state in TSCs [47–50]. Moreover, from a ChIP analyses of TSCs and trans-differentiated ESCs, Cdx2 has been shown to bind to promoter region of the core TF genes, Eomes and Elf5, to regulate their expression [48, 51]. Indeed, in this study, Cdx2-OE resulted in a significant increase in *Eomes* and *Elf5* expression. In contrast, *Esrrb* expression was down-regulated by Cdx2-OE, consistent with previous studies [49, 52]. It should be noted, however, that the increase in *Eomes* and *Elf5* expression by Cdx2-OE in TSCs appeared to be modest compared to that in ESCs [49, 53]. This may be because endogenous Cdx2 already occupied the promoter regions of *Eomes* and *Elf5* genes so that exogenously expressed Cdx2 could not cause further drastic activation of *Eomes* and *Elf5* transcription. Regardless, here, the Cdx2-OE experiment confirmed that the regulatory network of core TFs does function in TSCs.

A positive role of H3K27me3, a well-known repressive epigenetic mark [54–56], in the increase of *Cdx2* and *Prl3d1* expression under low glucose conditions is paradoxical. The formation of heterochromatin through the accumulation of H3K27me3 contributes to X chromosome inactivation [57]. Although re-activation of some genes containing H3K27me3 on the inactive X chromosome by the co-localization of active histone marks, such as H4 acetylation and H3K4me2 [58, 59], is shown to occur more frequently in undifferentiated and differentiated TSCs than in somatic cells [58, 60–62], such an "escape" mechanism cannot explain the requirement for H3K27me3 revealed using the Ezh2 inhibitor. We assume that the increase in genome-wide H3K27me3 levels down-regulates the expression of transcription factors which negatively regulate *Cdx2* and *Prl3d1* genes, resulting in the up-regulation of *Cdx2* and *Prl3d1* genes overcoming the increased repressive histone marks at their regulatory regions. Further analyses of genome-wide chromatin structure, histone modification occupancy, and gene expression profiling are required to solve this paradoxical problem.

In summary, we have revealed that the extracellular glucose levels are one factor that can affect the properties of TSCs. The TSC lines unexpectedly exhibited different responses to different glucose levels, even though two of them had the same genetic background. Changes in the extracellular glucose levels only in the undifferentiated state affected gene expression pattern after differentiation, likely through epigenetic memory, indicating there are acute and delayed effects of extracellular glucose on stem cells of the trophoblast lineage.

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