—Original Article—

Altered Gene Expression Profiles in Mouse Tetraploid Blastocysts

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Abstract. In this study, it was demonstrated that tetraploid-derived blastocyst embryos had very few Oct4-positive cells at the mid-blastocyst stage and that the inner cell mass at biomarkers Oct4, Sox2 and Klf4 was expressed at less than 10% of the level observed in diploid blastocysts. In contrast, trophoctoderm-related gene transcripts showed an approximately 10 to 40% increase. Of 32,996 individual mouse genes evaluated by microarray, 50 genes were differentially expressed between tetraploid or diploid and parthenote embryos at the blastocyst stage ($P < 0.05$). Of these 50 genes, 28 were more highly expressed in tetraploid-derived blastocysts, whereas 22 were more highly downregulated. However, some genes involved in receptor activity, cell adhesion molecule, calcium ion binding, protein biosynthesis, redox processes, transport, and transcription showed a significant decrease or increase in gene expression in the tetraploid-derived blastocyst embryos. Thus, microarray analysis can be used as a tool to screen for underlying defects responsible for the development of tetraploid-derived embryos.

Key words: Gene expression, Mouse, Tetraploid embryo

(J. Reprod. Dev. 58: 344–352, 2012)

Tetraploid embryos are commonly used to rescue embryonic lethality as a result of defective extra-embryonic phenotypes in laboratory mouse strains, as well as a method for generating mice directly from embryonic stem (ES) cells [1]. Even though spontaneous embryonic tetraploidy is rare [2–4], there are several methods of inducing tetraploidy. The first method is to surgically add a diploid (2n) nucleus to a zygote [5, 6]. The second method is the use of chemicals or heat-shock to inhibit cleavage. Initially, cytochalasin B (CB), a microtubule destabilizer, was used as the inhibitor [7–9]. The third method is to fuse 2 diploid embryonic cells. Currently, the most commonly used method for producing tetraploid embryos is electrofusion by electrical stimulation, a technique first developed for the fusion of plant protoplasts [10]. The successful use of this technique was first demonstrated in mice and rats [11, 12]. While adequately demonstrating blastomere fusion, tetraploid mammalian embryos produced by electrofusion were not proven to be capable of subsequent *in vitro* development until work was performed later on rabbit embryos [13, 14].

Tetraploid embryos are not independently capable of completing normal development [2, 15], but when complemented by the introduction of ES cells, they develop into conceptuses in which embryonic lineages are derived entirely from the ES cells and

extraembryonic lineages arise largely from the 4n component [2, 16]. This method is known as 4n embryo complementation. Mice derived from ES cells show normal phenotype, growth and fertility, indicating that ES mice can be used as founders for establishing new lines of mice. Because all germ cells in ES mice are derived from ES cells, germline transmission of ES cells is easier in ES mice than in 2n chimeric mice [17].

Even though the trophoctoderm (TE) and inner cell mass (ICM) grow within the same environment in the blastocyst and have the same genome, they have very different developmental fates. The first cell lineage specification in mouse embryo development is the formation of the TE and ICM. The TE engages in implantation by directly interacting with the uterus and gives rise to tissues in the placenta. After implantation, 3 germ layers form from the ICM, which generates all the tissues in the animal body [18]. Although these modifications are caused by changes in Oct4/Cdx2 expression profiles, a major question is how the correct pluripotency-related genes kept active in the ICM are selectively inactivated upon switching to the tetraploid state through fusion at the 2-cell stage. In hatched tetraploid blastocysts, there were alterations in the metabolic processes and in protein localization and transport according to a cDNA microarray analysis [19]. To understand the mechanisms underlying early differentiation of tetraploid embryos during preimplantation development, we set out to compare the expression of multiple genes in tetraploid-, haploid- and diploid-derived blastocysts. In this study, many differentially expressed genes were identified in the comparison between tetraploid- and haploid- or diploid-derived blastocyst embryos.

Received: August 12, 2011

Accepted: February 2, 2012

Published online in J-STAGE: February 24, 2012

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Materials and Methods

Animal ethics

All animal experiments were approved and performed under the guidelines of the Konkuk University Animal Care and Experimentation Committee (IACUC approval number: KU11035).

Electrofusion of diploid fertilized embryos

Female ICR mice (aged, 8–10 weeks) were superovulated by the injection of 5 IU of pregnant mare's serum gonadotropin (PMSG; Sigma-Aldrich, St. Louis, MO, USA), followed 48 h later by the injection of 5 IU of hCG, and then mated with male ICR mice. Embryo collection was performed under a stereo microscope in Chatot, Ziomek, Bavister (CZB)-HEPES medium. The collected one-cell embryos were cultured in droplets of modified CZB medium under paraffin oil in plastic dishes at 37 °C in a 5% CO₂ incubator until reaching the 2-cell stage. To generate tetraploid embryos, the 2-cell stage embryos prepared by *in-vitro* culture were electrofused using an electro cell fusion generator (LF101; Nepa Gene, Chiba, Japan). The electrofusion solution was composed of 0.3 mM mannitol, 0.1 mM MgSO₄ and 0.05 mM CaCl₂ supplemented with 0.01% bovine serum albumin (BSA) (w/v). Each group of < 20 oocytes was transferred into 20 µl of the electrofusion solution and positioned in a chamber between two parallel stainlesssteel electrodes. Fusion of 2-cell embryos was induced by a single pulse of 20 volts (AC) and 100 volts (DC) for 40 µsec using an electro cell fusion generator (LF101; Nepa Gene, Chiba, Japan). For haploid embryos, one-cell embryos were collected from the oviducts of the female mice at 24 h after hCG injection and then activated using an electro cell fusion generator. Electrofused embryos were incubated for development in mCZB medium. The proportion of fused embryos was determined 2 h after the electrofusion procedure. The stages of tetraploid embryo development were evaluated under an inverted microscope at 24 h intervals.

Assessment of Oct4 and Cdx2 expression during the preimplantation development of tetraploid embryos

After washing twice with Ca²⁺- and Mg²⁺-free Dulbecco's phosphate-buffered saline containing 0.1% polyvinyl alcohol (PBS-PVA; Sigma-Aldrich, St. Louis, MO, USA), blastocyst-stage embryos were fixed for 30 min in PBS-PVA with 4% paraformaldehyde. The fixed embryos were then washed twice in PBS-PVA and stored overnight at 4 °C in PBS supplemented with 2% BSA (PBS-BSA; Sigma-Aldrich) and 0.1% Triton X-100 (Nacalai Tesque, Kyoto, Japan). To stain the TE and ICM, the samples were incubated for 90 min at room temperature in mouse anti-*Cdx2* IgG (1:100 dilution; BioGenex), a TE marker, and rabbit anti-OCT4 IgG (POU domain, class 5, transcription factor 1, also known as OCT3/4, 1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), as an ICM marker. After the samples were washed twice in PBS-BSA, they were incubated with Alexa Fluor 568-labeled goat anti-mouse IgG and Alexa Fluor 488-labeled chicken anti-rabbit IgG antibodies (1:100 dilution; Molecular Probes, Eugene, OR, USA). After 3 washes of 10 min each in PBS-BSA, the DNA was stained for 30 min with 2 µg/ml 4',6-diamidino-2-phenylindole dihydrochloride (Molecular Probes). After the samples were thoroughly washed, they

were mounted on slides using VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA, USA) and observed with an epifluorescence microscope. The processes of fixation and staining of samples were performed in 96-well culture dishes (STEMCELL Technologies, Vancouver, BC, Canada) and the samples were transferred for subsequent treatments by mouth pipette.

Extraction and amplification of mRNA

The mRNA from pools of 2N and 4N blastocysts was extracted using a Dynabeads mRNA Direct Kit (Invitrogen Dynal As, Oslo, Norway) according to the manufacturer's instructions. Due to the low concentrations of the extracted mRNAs, the integrity and purity of the preparations could not be verified. The mRNA from 3 pools of blastocysts (1N, 2N and 4N) was subjected to 2 rounds of linear amplification, and *in vitro* transcription was performed using a RiboAmp HS RNA Amplification Kit (Arcturus, Sunnyvale, CA, USA) according to the manufacturer's instructions.

Microarray analysis

An Applied Biosystems Mouse Genome Survey Array (Applied Biosystems, Foster City, CA, USA) was used to analyze the transcriptional profiles of all samples according to the nucleic number of blastocysts. This array contains 32,996 60-mer oligonucleotide probes representing 32,381 individual mouse genes and >3,000 control probes. The microarray probes used were transcripts listed in the Celera Genomics Mouse Genome Database (<http://www.celera.com>, Celera Discovery System), RefSeq transcripts that were structurally created from the LocusLink public database (<http://ncbi.nlm.nih.gov/LocusLink/refseq.html>), high-quality cDNA sequences from the Mammalian Gene Collection (MGC; <http://mgc.ncbi.nih.gov>), and transcripts that had been experimentally validated at Applied Biosystems. Array hybridization, chemiluminescence detection, image acquisition and analysis were performed using an Applied Biosystems Chemiluminescence Detection Kit and an Applied Biosystems 1700 Chemiluminescent Microarray Analyzer, according to the manufacturer's protocol. The captured images were auto-gridded, and the chemiluminescent signals were quantified and corrected for background. Differences in microarray spot intensities were normalized. The Applied Biosystems Expression System software was used to extract the assay signal to noise ratio values from the microarray images. Bad spots flagged by the software (>100) were removed from the analysis. To select differentially expressed genes, the remaining genes were further filtered using the Standard Expression Array System signal to noise threshold (S/N > 3 in at least 3 samples). The filtered genes were then normalized by the Lowess Regression normalization method. Genes with significantly different expression were identified using the 2-sample *t*-test (*P* < 0.05) and the fold change method (Fold change ≥ 2). Fold changes (tetraploid-derived blastocyst vs. diploid/haploid derived blastocyst assay signal ratio) were calculated from the average intensities of each replicated sample. To minimize multiple testing problems, we performed Benjamini-Hochberg's FDR. Genes with significantly different expression were then categorized into particular functional categories using the Protein Analysis Through Evolutionary Relationships (Panther) classification system, which is a unique resource that classifies genes by

Table 1. Primers and conditions used for real-time RT-PCR

Primer name	Primer sequence	PCR conditions	Size (bp)	GenBank
<i>β-actin</i>	F: AGTGTGACGTTGACATCCGTA R: GCCAGAGCAGTAATCTCCTTCT	58 C	112	NM007393
<i>Oct4</i>	F: CTCCCTACAGCAGATCACTCACA R: AACCATACTCGAACCACATCCT	63 C	220	NM013633
<i>Sox2</i>	F: CACAACCTCGGAGATCAGCAA R: CTCCGGGAAGCGTGACTTA	60 C	190	NM011443
<i>Klf4</i>	F: CTGAACAGCAGGGACTGTCA R: GTGTGGGTGGCTGTTCTTTT	58 C	218	NM010637
<i>Cdx2</i>	F: AGACAAATACCGGTGGTGTA R: CCAGCTCACTTTTCTCCTGA	60 C	153	NM007673
<i>Eomes</i>	F: GAGCTTCAACATAAACGGACTCAA R: CGGCCAGAACCCTTCCA	60 C	210	NM01013
<i>Krt8</i>	F: ATCGAGATCACCACCTACCG R: TGAAGCCAGGGCTAGTGAGT	63 C	151	NM03117
<i>Vdac</i>	F: AATGACGGGACAGAGTTTGG R: GGCTGACAACGTCAGTTTGA	58 C	231	NM011694
<i>Wfdc2</i>	F: TGCACTTTGGACAAGGACTG R: ACACCTAAGCCTTCCTGGT	60 C	230	NM02632
<i>Tuft1</i>	F: TCAGCGAGAAGCAGAACAGA R: AAACCCAGCCACTTCCTTTT	58 C	241	NM011656
<i>Lgals1</i>	F: CATCACAGAGGTGTGCATC R: AGGCCACGCACTTAATCTTG	57 C	150	NM008495
<i>Gsta4</i>	F: GCCAAGTACCCTTGGTTGAA R: TTTTCCTTGGGGGTTTT	55 C	196	NM01
<i>Cyp2s1</i>	F: CCTCACCCACATAAGGTGCT R: CCTGTCTCACACCTGGAAT	60 C	238	AK054324
<i>Chd8</i>	F: ACAAATGACCCCTGAGTTGC R: GGAGAGTCCCCATCTTCTCC	60 C	228	AK080266
<i>Gzmn</i>	F: GCCAGTAGGAGATGGAGCAG R: CCCCAGTGTGACTGTCATTG	61 C	193	NM153052.1
<i>Lpp</i>	F: TACGGCATCCACATCTTCAA R: ACCAACACAGGTTGGAGGAG	58 C	178	AK029335
<i>Alx3</i>	F: AAACCCACTACCCTGACGTG R: AGCTGAGGATGGCTGTCAGT	60 C	205	NM007441.2

their function. In this system, published scientific experimental evidence and evolutionary relationships are used to predict function even in the absence of direct experimental evidence (<https://parther.appliedbiosystems.com>).

Real-time RT-PCR analysis

Quantitative real-time RT-PCR reactions were performed using a kit from Bio-Rad (Munich, Germany) and the PCR reactions were conducted according to the manufacturer's instructions. The PCR involved an initial denaturation program (95 C for 10 min), followed by 35 cycles of amplification and quantification (95 C for 10 sec, 55 C /63 C for 30 sec and 72 C for 30 sec, with a single fluorescence measurement). The primer sets for each gene amplified in the RT-PCR are shown in Table 1.

Statistical analysis

Each experiment was repeated at least three times to obtain

40–100 embryos per treatment. The data were subjected to arcsine transformation for each replication. The transformed values were analyzed using one-way ANOVA. $P < 0.05$ was defined as indicating statistical significance.

Results

Comparison of ICM- and TE-biomarker mRNA expression between tetraploid- and diploid-derived blastocysts

The relative abundance of ICM- and TE-related transcripts (*Oct4*, *Sox2* and *Klf4* vs. *Cdx2*, *Eomes* and *Krt8*) was measured by real-time RT-PCR. Independent experiments ($n = 6$) using 10 embryos per group were carried out to yield quantitative data (Fig. 1). To normalize the RT-PCR reaction efficiency and to quantify ICM- and TE-related mRNA, β -actin was used as an internal standard. After normalization with β -actin mRNA, the mRNA expression of ICM- and TE-related genes exhibited a distinct developmental

Fig. 1. Relative abundance of transcripts from 3 different inner cell mass-related genes, Oct4 (A), Sox2 (B) and Klf4 (C) and 3 different trophoblast-related genes, Cdx2 (D), Eomes (E) and Krt8 (F), in mouse blastocysts derived from diploid (black) and tetraploid (white) embryos. mRNA from pools of 10 blastocyst-stage embryos was reverse transcribed and subjected to real-time quantitative PCR using transcript-specific primers (Table 1). All reactions were conducted in triplicate and normalized for β -actin mRNA expression. Data are presented as means $\pm$ SEM (bars) from 3 determinations. Different superscripts (a, b) indicate values with significant ($P < 0.05$) differences in relative transcript abundance between diploid and tetraploid embryos at the blastocyst stage.

pattern: The expression levels of ICM markers such as Oct4, Sox2 and Klf4 in 4n blastocysts were significantly lower than in 2n blastocysts. In contrast, the induction of tetraploidy did not affect Krt8 mRNA expression but increased the expressions of Cdx2 and Eomes at the blastocyst stage ($P < 0.05$). This observation suggested that it is possible that the reduced number of ICM cells reflects the attenuated expressions of Oct4, Klf4 and Sox2.

Allocation of inner cell mass (ICM) versus trophoblast (TE) cells in tetraploid-derived blastocysts

Three different types of blastocyst embryos were derived from diploid embryos: diploid embryos derived from normal fertilization with spermatozoa, haploid embryos derived by parthenogenetic activation of oocytes and tetraploid embryos produced by electrofusion. These blastocysts, produced by normal fertilization, parthenogenetic activation and electrofusion, were individually stained by a differential staining method and then evaluated for total cell number. The total cell numbers in tetraploid embryos (29.3 ± 4.41 , $n = 37$) were significantly lower than those in haploid-derived embryos (127.6 ± 3.15 , $n = 11$) and in parthenogenetically-activated embryos (89.8 ± 1.74 , $n = 12$). Furthermore, the tetraploid-derived blastocysts had fewer inner cell mass cells (1.08 ± 0.37) compared

with haploid- (17.42 ± 0.56) and diploid-derived (35.82 ± 1.58) embryos ($P < 0.05$; Table 2). Representative fluorescence micrographs of mouse-expanded blastocysts (Day 4) derived from diploid- and parthenote-derived embryos after differential staining are shown in Figs. 2A and 2B, respectively. Tetraploid-derived blastocysts differentially stained for ICM and TE nuclei were individually classified into 3 groups (group I, II and III : 0%, ~2–6% and ~6–10%, respectively) according to the number of Oct4-positive cells and these embryos were compared with haploid- and diploid-derived embryos. In the tetraploid-derived blastocysts, 70.27% (26/37) were allocated to group I whereas 21.62% (8/37) and 2.7% (1/37) were allocated to groups II and III, respectively (Figs. 2C-I, 2C-II and 2C-III).

Genes differentially expressed between tetraploid- and diploid-derived blastocysts

To evaluate the gene expression status at the blastocyst stage, we performed oligo microarray analysis of 300 blastocysts, including those derived from tetraploid ($n = 100$), parthenote ($n = 100$) and diploid ($n = 100$) blastocysts. This array contained 32,996 60-mer oligonucleotide probes representing 32,381 individual mouse genes and more than 3,000 control probes. Due to the low concentrations

Table 2. Number of cells in tetraploid-, diploid- and haploid-derived blastocysts

Ploidy of the embryos	No. of blastocysts	No. of total cells (Mean $\pm$ SE)	No. of ICM cells (Mean $\pm$ SE)	No. of TE cells (Mean $\pm$ SE)
1N	12	89.8 $\pm$ 1.74 ^b	17.42 $\pm$ 0.56 ^b	72.42 $\pm$ 1.37 ^b
2N	11	127.6 $\pm$ 3.15 ^a	35.82 $\pm$ 1.58 ^a	91.73 $\pm$ 2.39 ^a
4N	37	29.3 $\pm$ 0.73 ^c	1.08 $\pm$ 0.37 ^c	28.22 $\pm$ 0.65 ^c

^{a, b, c} $P < 0.05$; The means are significantly different. ICM, inner cell mass; TE, trophectoderm.

Fig. 2. Differential expression of Oct4 and Cdx2 in haploid (A), diploid (B) and induced tetraploid (C) embryos at the blastocyst stage. Tetraploid-derived blastocysts differentially stained for inner cell mass and trophectoderm nuclei were individually classified into 3 groups according to the number of Oct4-positive cells (C-I, C-II and C-III: 0%, ~2–6% and ~6–10%, respectively) and these embryos were compared with parthenote- and diploid-derived blastocysts.

of the extracted mRNAs, the integrity and purity of the preparations could not be verified. At the $P < 0.05$ level of significance, microarray analysis revealed that 50 genes were differentially expressed at the blastocyst stage in tetraploid vs. diploid or parthenote embryos. Of these 50 genes, 28 were more highly expressed in tetraploid-derived blastocysts, whereas 22 were more highly expressed in diploid-derived blastocysts. Some of the genes were uncharacterized (unknown); we focused our attention on the known genes. Tables 3 and 4 show a list of the 50 genes with major variations observed upon comparison of the gene transcription in tetraploid blastocysts with that in diploid blastocysts. Even though data analysis using standard algorithms revealed no significant differences between the 2 groups, analyses of the common processes and gene pathways revealed that, overall, tetraploid embryos displayed upregulation

of receptor activity, cell adhesion molecule activity and calcium ion binding activity, whereas protein biosynthesis, redox activity, transporter activity and transcription activity were significantly downregulated.

Our most interesting finding was that granzyme N, heat shock protein 8, aristaless-like homeobox 3, cytochrome P450 family 2 and cathepsin D were all significantly upregulated, whereas voltage-dependent anion channel 1 and glutathione S-transferase alpha 4 were significantly downregulated (Table 3), indicating that tetraploid-derived blastocysts were experiencing progressive cell death. Also, we observed the upregulation of jumonji C domain-containing histone demethylase 1, chromodomain helicase DNA-binding protein, and LIM domain-containing preferred translocation partner genes, which are involved in chromatin remodeling and the

Table 3. Differentially expressed genes (DEGs) in the tetraploid blastocyst (4N) compared with haploid (1N) or diploid (2N) blastocysts (P<0.05)

Gene symbol	Gene description	RefSeq	GO molecular function	Fold (4N/2N)	Fold (4N/1N)
Genes with increased expression (>1.5-fold)					
1 <i>Jhdm1d</i>	Jumonji C domain-containing histone demethylase 1	NM_001033430	Metal ion binding	2.32	1.67
2 <i>Aclp7</i>	Microtubule-actin crosslinking factor 1	AK089453	Actin binding	1.92	1.51
3 <i>Frmd4a</i>	FERM domain containing 4A	NM_172475	Binding	1.60	1.55
4 <i>Dsg3</i>	Desmoglein 3	AK029018	Calcium ion binding	1.79	1.68
5 <i>Spock1</i>	SPARC/osteonectin, CWCV and kazal-like domains proteoglycan 1	AK049019	Calcium ion binding	1.63	1.61
6 <i>Ccnd2</i>	Cyclin D2	AK007904	Cell cycle	1.55	1.62
7 <i>Sema4a</i>	Sema domain, immunoglobulin domain (Ig)	NM_013658.2	Cell differentiation	1.51	1.51
8 <i>Chd8</i>	Chromodomain helicase DNA binding protein 8	AK080266	Development	1.85	1.63
9 <i>Dpp4</i>	Dipeptidylpeptidase 4	AK040409	Peptidase activity	1.97	1.63
10 <i>Gzmn</i>	Granzyme N	NM_153052.1	Peptidase activity	1.71	1.52
11 <i>Ctsd</i>	Cathepsin D	NM_009983.2	Peptidase activity	1.53	1.54
12 <i>Hspb8</i>	Heat shock protein 8 (Hspb8)	NM_030704.1	Protein binding	1.91	1.53
13 <i>Lpp</i>	LIM domain containing preferred translocation partner	AK029335	Protein binding	1.69	1.59
14 <i>Slc20a1</i>	Solute carrier family 20, member 1	AK084159	Receptor activity	1.83	1.62
15 <i>Ndufs1</i>	NADH dehydrogenase (ubiquinone) Fe-S protein 1	AK078590	Redox activity	1.92	1.58
16 <i>Cyp2s1</i>	Cytochrome P450, family 2, subfamily s, polypeptide 1	AK054324	Redox activity	1.59	1.57
17 <i>Fusip1</i>	FUS interacting protein (serine-arginine rich) 1	NM_010178.2	RNA binding	1.55	1.59
18 <i>Mbtd1</i>	MBT domain containing 1	AK049518	Transcription activity	1.76	1.68
19 <i>Alx3</i>	Aristaless-like homeobox 3	NM_007441	Transcription activity	1.75	1.62
20 <i>Flvcr2</i>	Feline leukemia virus subgroup C cellular receptor family	NM_145447.2	Transporter activity	1.62	2.14
21 <i>Hecw2</i>	HECT, C2 and WW domain-containing E3 ubiquitin ligase 2	AK047602	Ubiquitin-ligase activity	1.79	1.57
22 <i>5330440M16Rik</i>	Hypothetical protein	AK030640	Unclassified	2.16	1.83
23 <i>H2-T18</i>	Histocompatibility 2, T region locus 18	AK039341	Unclassified	1.97	1.66
24 <i>E030002K04Rik</i>	Hypothetical protein	AK086813	Unclassified	1.87	1.66
25 <i>B830005I23Rik</i>	Hypothetical protein	AK046784	Unclassified	1.84	1.65
26 <i>D930024N12Rik</i>	Hypothetical protein	AK086384	Unclassified	1.84	1.51
27 <i>Jakmip2</i>	Janus kinase and microtubule interacting protein 2	AK076652	Unclassified	1.76	1.73
28 <i>B930088G14Rik</i>	Hypothetical protein	AK047561	Unclassified	1.64	1.66

modulation of histone deacetylation in tetraploid embryos (Table 4).

Validation of microarray data

To verify the expression of the 10 genes identified from the microarray, we performed real-time RT-PCR using diploid- and tetraploid-derived blastocysts. Even though gene expression was slightly variable, particularly in the tetraploid embryos, five genes (VDAC, WFDC2, TUFT1, LGALS1 and GSTA4) were identified by the array data as being downregulated. As shown in Fig. 3A, the expression of these genes was reduced by approximately 20 to 40%. In contrast, the expression levels of 5 other genes, CYP2S1, CHD8, GZMN, LPP and ALX3, were upregulated in the DNA chip array data; these genes were expressed at 1.2- to 1.8-fold higher levels in tetraploid blastocysts than in the controls (Fig. 3B).

Discussion

The present experiment was conducted to examine how many tetraploid-derived blastocyst embryos have Oct4-positive cells, which indicate inner cell mass status. At the early blastocyst stage,

Oct4 staining began to decrease in a few TE cells (peripheral cell nuclei) but was still detectable in both TE and ICM cells. However, at the expanded blastocyst stage, Oct4 was localized only in the ICM nuclei and was largely absent from the nuclei of the TE. In tetraploid embryos, Cdx2-positive nuclei were first detected at the late compaction stage, with greatly increased Cdx2 expression at the blastocyst and expanded blastocyst stages, whereas Oct4 expression had disappeared by the time tetraploid embryos reached the expanded blastocyst stage [20].

During the fourth or fifth cleavage division of mouse embryos, preimplantation embryos form the ICM from inner apolar cells and the TE from outer polar cells. Prospective ICM and TE cells express Oct4 and Cdx2, respectively [21]. In mid-blastocyst stages, Oct4-positive cells randomly appear within the ICM, whereas Cdx2-positive cells are localized to the outer surface of the ICM. As we previously reported [20], Oct4 expression from the 2-cell to the morula stage was similar in tetraploid and diploid embryos. Interestingly, we discovered that at the expanded blastocyst stage, Oct4 expression had disappeared in most of the tetraploid-derived embryonic cells, though some embryonic cells at the ICM site

Table 4. Differentially expressed genes (DEGs) in the tetraploid blastocyst (4N) compared with haploid (1N) or diploid (2N) blastocysts (P<0.05)

Gene symbol	Gene description	RefSeq	GO molecular function	Fold (4N/2N)	Fold (4N/1N)
Genes with decreased expression (< -1.5-fold)					
1 <i>Dbi</i>	Diazepam binding inhibitor, transcript variant 2	NM_007830.3	Acyl-CoA binding	-1.62	-1.59
2 <i>Atp1a1</i>	ATPase, Na ⁺ /K ⁺ transporting, alpha 1 polypeptide	NM_144900.1	ATPase activity	-1.55	-1.69
3 <i>Vdac1</i>	Voltage-dependent anion channel 1	NM_011694.3	Ion channel	-1.80	-1.59
4 <i>Tpi1</i>	Triosephosphate isomerase 1	NM_009415.1	Isomerase activity	-1.52	-2.00
5 <i>Fabp3</i>	Fatty acid binding protein 3, muscle and heart	NM_010174.1	Lipid binding	-1.75	-1.51
6 <i>Bat1a</i>	HLA-B-associated transcript 1A	NM_019693.2	Nucleic acid binding	-1.76	-1.56
7 <i>Xpnpep1</i>	X-prolyl aminopeptidase (aminopeptidase P) 1	NM_133216.2	Peptidase activity	-1.97	-1.57
8 <i>Wfdc2</i>	WAP four-disulfide core domain 2	NM_026323.2	Protease inhibitor	-2.23	-1.71
9 <i>Spint1</i>	Serine protease inhibitor, Kunitz type 1	NM_016907.3	Protease inhibitor	-1.75	-1.58
10 <i>Snx17</i>	Sorting nexin 17	NM_153680.2	Protein binding	-1.76	-1.54
11 <i>Tuft1</i>	Tuftelin 1	NM_011656.2	Protein binding	-2.08	-1.53
12 <i>Zyx</i>	Zyxin	NM_011777.2	Protein binding	-1.86	-1.79
13 <i>EG668829</i>	Similar to ribosomal protein L24	XM_001005405.1	Ribosomal protein	-2.14	-1.56
14 <i>Mrpl17</i>	Mitochondrial ribosomal protein L17	NM_025301.2	Ribosomal protein	-1.96	-1.53
15 <i>EG622236</i>	Predicted gene, EG622236, transcript variant 1	XM_917756.3	Ribosomal protein	-1.86	-1.78
16 <i>Rpl23</i>	Ribosomal protein L23	NM_022891.2	Ribosomal protein	-1.91	-1.62
17 <i>Rpl32</i>	Ribosomal protein L32	NM_172086.2	Ribosomal protein	-1.70	-1.52
18 <i>LOC100044425</i>	Similar to ribosomal protein L36a	XM_001472147.1	Ribosomal protein	-1.95	-1.58
19 <i>Lgals1</i>	Lectin, galactose binding, soluble 1	NM_008495.2	Sugar binding	-2.48	-2.18
20 <i>Klf5</i>	Krüppel-like factor 5	NM_009769.4	Transcription factor	-1.69	-1.50
21 <i>Gsta4</i>	Glutathione S-transferase, alpha 4	NM_010357.1	Antioxidant activity	-1.71	-1.74
22 <i>Gars</i>	Glycyl-tRNA synthetase	NM_180678.3	tRNA ligase activity	-1.59	-1.95

did remain Oct4-positive. Previously, we reported that 83% of the tetraploid-derived blastocysts had 80 chromosomes, and 17% exhibited mixploidy [20], indicating a close relationship between the numbers of tetraploid-derived blastocysts with Oct4-positive cells and aberrant chromosome numbers. We also demonstrated that the transcription levels of ICM-related genes such as Oct4, Sox2 and Klf4 were 0.1-fold lower in tetraploid-derived blastocysts compared with diploid blastocysts (P<0.05). Therefore, the question becomes one of how the ICM can inhibit transcription and translation of these genes (Oct4, Sox2 and Klf4), which are abundantly expressed in tetraploid-derived blastocysts.

To understand the mechanisms that reverse silencing or induce activation in ICM lineages as a result of tetraploid induction during transdifferentiation, we investigated genome-wide gene expression using a DNA chip. In this study, granzyme N, heat shock protein 8, aristaless-like homeobox 3, cytochrome P450 family 2 and cathepsin D were significantly upregulated in tetraploid-derived blastocysts, whereas voltage-dependent anion channel 1 and glutathione S-transferase alpha 4 were significantly downregulated. It is wellknown that aristaless-like homeobox 3-deficient embryos show increased apoptosis, and morphometric analysis has indicated that such embryos are, on average, of smaller size than islets from control mice. Also, voltage-dependent anion channel 1 has been implicated in apoptosis, as it forms an open pore that allows the release of cytochrome *c* (cyto *c*) and other proteins from the mitochondrial intermembrane space into the cytosol. In addition, “death” receptors [tumor necrosis factor receptor 1

(TNFR1)/Fas] engage caspase 8, whereas various stress conditions induce calpain, caspases, cathepsins or granzyme B to cleave and activate BID, which is a proapoptotic BH3-only BCL2 family member [22]. Depletion of endogenous zyxin, which colocalizes with HIPK2 at the cytoskeleton and in the cell nucleus, stimulates proteasome-dependent HIPK2 degradation. Also, it is wellknown that cathepsin D is a very good marker for areas of embryonic cell death [23]. As implied in the above discussion, the compromised ICM seen in tetraploid blastocysts may be caused by an increase in apoptotic protein expression as well as a dose-dependent loss of expression of ICM-related genes such as Oct4, Sox2 and Klf4.

Previously, Kawaguchi *et al.* (2009) reported the profiling of global gene expression using microarray analysis to identify molecular and cellular pathways that were affected by tetraploid and diploid hatched blastocysts of mice. However, in this study, we compared gene expression profiles of the tetraploid blastocyst with those of parthenote- and diploid-derived blastocysts [19]. As a result of differences in the methods of statistical analysis, Kawaguchi's group may have found that genes involved in cell division and the cell cycle in tetraploid blastocysts were mainly downregulated, whereas the present study showed that the main differential expressions among tetraploid-, parthenote- and diploid-derived blastocysts involved apoptosis-related genes. Therefore, this discrepancy may be caused by 2 reasons, 1) comparison of diploid and tetraploid blastocysts vs. parthenote, diploid and tetraploid blastocysts and 2) the blastocyst stages used, such as hatched vs. middle blastocyst stages.

Fig. 3. Validation of DNA chip data by real-time RT-PCR analysis. mRNA from pools of 10 embryos at the blastocyst stage was reverse transcribed and subjected to real-time quantitative PCR using transcript-specific primers (Table 1). All reactions were conducted in triplicate and normalized for β -actin mRNA expression. Data are presented as means $\pm$ SEM (bars) from 3 determinations. Different superscripts (a, b) indicate values with significant ($P < 0.05$) differences in relative transcript abundance between diploid and tetraploid embryos at the blastocyst stage. All RT-PCR data examined in this study mirror DNA chip data, with increases or decreases in the tetraploid-derived blastocyst as compared with the diploid-derived blastocyst. Data represent values from 3 independent experiments.

Mouse *Jmjd1c* mRNA is expressed in the fertilized egg, blastocyst, undifferentiated embryonic stem cells, embryonic germ cells, c-Kit⁺/Sca-1⁺/Lin⁻ hematopoietic stem cells and other tissues, indicating that POU5F1-mediated expression of JMJD1C histone demethylase is implicated in the reactivation of silenced genes in undifferentiated ES cells [24]. In this study, however, JMJD1C mRNA expression in tetraploid embryos was significantly increased. This is especially intriguing in the context of tetraploid-derived blastocysts because tetraploid blastocysts have a compromised inner cell mass. We also observed the upregulation of chromodomain helicase DNA-binding protein (CHD). CHD is known to affect transcription through its ability to remodel chromatin and modulate histone deacetylation. Notably, earlier studies have shown the importance of differentiation and cell type-specific transcriptional programming during the terminal differentiation of hematopoietic cells [25]. Therefore, these observations suggest that the JmjC domain and CHD play pivotal roles in trophoblast differentiation in the absence of the ICM.

The microarray-based genomic survey is a high throughput approach that allows parallel study of the expression patterns of thousands of genes [26]. In this study, we discovered previously unknown gene expression patterns related to tetraploid-derived

blastocysts. In agreement with the phenotypic alterations found in tetraploid-derived blastocysts, many differentially expressed genes were shared between the gene lists for diploid- and parthenote-derived blastocysts, suggesting that common pathways are affected by the technique and culture system used. However, the numerous unique gene functions and pathways affected solely in tetraploid-derived blastocysts imply that many of these unique genes are good candidates for studying blastocysts with compromised ICMs.

Acknowledgments

This work was partially supported by Woo Jang-Choon (PJ007849) projects from the RDA, Republic of Korea. We thank Dr P Hughes (BioEdit) for assistance with the manuscript preparation.

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