Deleting maternal Gtl2 leads to growth enhancement and decreased expression of stem cell markers in teratoma

Nozomi TAKAHASHI1), Eito YAMAGUCHI1), Yukiko KAWABATA1) and Tomohiro KONO1)

1)Department of Bioscience, Tokyo University of Agriculture, Tokyo 156-0054, Japan

Abstract. The distal region of mouse chromosome 12 harbors the Dlk1–Dio3 domain, is essential for normal development and encodes maternally expressed noncoding RNAs (ncRNAs), including Gtl2 as well as paternally expressed proteins. Gtl2 works as a tumor suppressor in several types of human cancer cell lines; however, whether this reflects its function in vivo is unknown. Deleting Gtl2 from the maternal allele (Gtl2(–/+) results in loss of expression of Gtl2 and decreased expression of downstream ncRNAs, including many miRNAs. To determine the role of ncRNAs in tumorigenesis, we induced teratomas by engrafting E6.5 embryos (wildtype or Gtl2(–/+) under the kidney capsule of scid mice. Some teratomas derived from the Gtl2(–/+) embryos exhibited hypertrophic growth, suggesting that ncRNAs, including Gtl2, may act as tumor suppressors in vivo. Microarray analysis of miRNAs expressed by Gtl2(–/+) teratomas revealed decreased expression of 28 miRNAs encoded by the Dlk1–Dio3 domain, low expression of embryonic stem cell–specific miRNAs and dysregulation of miRNAs involved in tumorigenesis. This study suggests that downregulation of ncRNAs in the Dlk1-Dio3 domain leads to enhanced teratoma growth and repression of stem cell markers.

Key words: Dlk1-Dio3 domain, Genomic imprinting, miRNA, Noncoding RNA, Teratoma

Gtl2 (human MEG3) is expressed in the visceral yolk sac and embryonic ectoderm by early postimplantation mouse embryos [1]. During embryonic development, Gtl2 transcripts are abundant in the paraxial mesoderm, certain regions of the central nervous system and in epithelial ducts [2]. Splice variants of Gtl2 lack a consensus Kozak sequence for initiation of translation, suggesting that Gtl2 encodes an RNA [1]. Normal pituitary tissue expresses high levels Meg3 in contrast, Meg3 expression is undetectable in human pituitary tumors and several cancer cell lines [3]. Further, transfection of a cancer cell line with Meg3 inhibits its proliferation [4], indicating that Meg3 transcripts function as tumor suppressors.

Gtl2 is an imprinted gene that is expressed from the maternal allele [5]. Imprinted genes are regulated by parent-specific epigenetic modifications, including DNA methylation of the differentially methylated region (DMR). Gtl2 is located within the Dlk1–Dio3 paternally imprinted domain on chromosome 12 in mice and on chromosome 14 in humans [6]. This region includes three paternally expressed protein-coding genes (Dlk1, Rtl1 and Dio3) and multiple maternally expressed ncRNAs (Gtl2, Rtl1as, Rian and Mirg) (Fig. 1A), which are regulated by the primary intergenic DMR (IG-DMR) and the postfertilization-derived secondary Gtl2-DMR [7]. A long transcript may span the Gtl2–Mirg domain [8]. Moreover, deleting Gtl2 on the maternal allele decreases the expression of Rtl1as, Rian and Mirg that encode six small nucleolar RNAs and 59 microRNAs (miRNAs) (miRBase: http://www.mirbase.org/) [9].

The expression of the ncRNAs from the Gtl2–Mirg domain may correlate with the pluripotency of induced pluripotent stem cells, because the ncRNAs expressed by fully pluripotent cell lines are silenced in partially pluripotent cell lines [10, 11]. Moreover, miRNAs encoded by the Dlk1-Dio3 domain are involved in tumorigenesis. For instance, overexpression of miRNAs by human hepatocellular carcinomas correlates with poor survival [12]. In contrast, expression of the miRNAs encoded by the Dlk1-Dio3 domain is reduced in human epithelial ovarian cancer, which leads to increased tumor proliferation and shorter survival times [13]. These data suggest that both overexpression and repressed expression of the Dlk1-Dio3 miRNAs are correlated with worsening tumor grade.

In the present study, we generated teratomas by engrafting scid mice with Gtl2(–/+) embryos to gain a better understanding of the contribution of ncRNAs encoded by the Dlk1–Dio3 domain to tumorigenesis. This teratoma formation assay is used to prove that H19, which is a paternally imprinted and maternally expressed ncRNA, acts in vivo as a tumor suppressor [14]. The results of histopathological and gene expression analyses showed that Gtl2 deletion induced decreased expression of 28 miRNAs in the Dlk1–Dio3 domain and led to increased cell proliferation and decreased expression of stem cell markers in the teratoma.

Materials and Methods

Experimental animals

Gtl2 knockout mice [9] were maintained by mating with B6D2F1 (BDF1) mice for up to 10 generations. Immunodeficient scid mice were purchased from CLEA Japan. This study was approved by

Received: July 11, 2014
Accepted: September 14, 2014
Published online in J-STAGE: October 30, 2014
©2015 by the Society for Reproduction and Development
Correspondence: T Kono (e-mail: tomohiro@nodai.ac.jp)
This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License <http://creativecommons.org/licenses/by-nc-nd/3.0/>.
Teratoma formation

Gtl2(−/+) and wild-type (WT) embryos at embryonic day (E) 6.5 were obtained by mating male WT (BDF1) and female Gtl2(+/–)
mice. To produce teratomas, E6.5 embryos were placed under the kidney capsules of scid mice. After four weeks, the teratomas were surgically removed, weighed and fixed or homogenized in TRIzol (Life Technologies, Carlsbad, CA, USA). Fixed sections were cut and stained with hematoxylin and eosin. For genotyping, DNAs were isolated from untreated teratomas.

**Cell culture**

Mouse embryonic fibroblasts (MEFs) were harvested from Gtl2<sup>–/–</sup> and WT E13.5 embryos, which were pooled separately for males and females (N = 2, each genotype), and cultured according to the 3T3 protocol using 3 x 10<sup>5</sup> cells seeded on a 10-cm diameter petri dish. The number of cells was counted every three days [15]. The cells were passaged at least 50 times in Dulbecco’s Modified Eagle Medium (Life Technologies), 10% fetal bovine serum, penicillin and streptomycin at 37°C in an atmosphere containing 5% CO<sub>2</sub>. Medium (Life Technologies), 10% fetal bovine serum, penicillin and streptomycin at 37°C in an atmosphere containing 5% CO<sub>2</sub>.

**miRNA array analysis**

Total RNAs extracted from the teratomas using TRIzol reagent were used for the miRNA array. The miRNAs were labeled using miRNA labeling reagent (Agilent) and hybridized to mouse miRNA array v1.5 (Agilent). Images were scanned using an Agilent microarray scanner, and data were extracted with the supplied Feature Extraction software. The GeneSpring GX10 software was used to analyze the microarray results. The per-chip 90th percentile method was used for normalization. We selected as a cutoff point a raw signal > 50 for at least one sample.

**Quantitative real-time RT-PCR**

Total RNA was extracted from teratomas with TRIzol reagent and treated with DNase, and cDNA was synthesized using SuperScript III and Oligo(dT) Primer (Life Technologies). Quantitative real-time PCR was performed using an ABI Real-Time PCR instrument. Seven (0.1221–0.9451 g) Gtl2<sup>–/–</sup> and nine (0.0567–0.1823 g) WT teratoma samples were used for tests, respectively. The following primers were used for the analysis: P53F, TGAAACGCCGACCTATCCTTA; p53R, GGCACAAACACGAACCTCAAA; Oct4F, GTTGGA-GAAGGTGGAACCAA; Oct4R, TCT TCTGCTTCAGCAGCTTG; p53R, GGCACAAACACGAACCTCAAA; Oct4F, GTTGGA-GAAGGTGGAACCAA; Oct4R, TCT TCTGCTTCAGCAGCTTG; NanogF, CAGAAAAACCAGTGTGGAAGACTG; NanogR, GCAATGGATGCTGGGATACTC.

**Statistical analysis**

All analyses were performed with PRISM (GraphPad, La Jolla, CA). The tests used for each analysis are noted in the figure legends.

**Results**

**Teratoma induction and growth rate of MEFs**

E6.5 Gtl2<sup>–/–</sup> and WT embryos were placed under the kidney capsules of scid mice and examined for tumorigenicity in vivo after four weeks (Fig. 1B). Eight teratomas with weight of more than 0.2 g were obtained by grafting Gtl2<sup>–/–</sup> embryos (Fig. 1C). This was significantly greater compared with the WT teratomas. Most of the teratomas weighed less than 0.2 g, and only 4 WT teratomas weighed over 0.2 g (Fig. 1C). Histological analysis showed that the differentiation of the three germ layers and gut-like structures was more advanced in the Gtl2<sup>–/–</sup> teratomas (Fig. 1D and E) (Supplementary Fig. S1: online only).

We compared the proliferation in vitro of MEFs derived from the Gtl2<sup>–/–</sup> and control embryos. The Gtl2<sup>–/–</sup> and control MEFs grew at similar rates during the first several passages. In later passages, the growth rates of Gtl2<sup>–/–</sup> MEFs surpassed those of the controls (Fig. 1F). These data suggested that deleting Gtl2 from the maternal allele enhanced cell proliferation in vivo and in vitro.

**Expression of miRNAs by teratomas**

The miRNAs expressed from the Dlk1-Dio3 domain may be involved in tumorigenesis [12, 13]. Using miRNA array analysis, we attempted to establish a link between the expression of specific miRNAs and the growth of the larger Gtl2<sup>–/–</sup> teratomas (0.26–0.94 g). The miRNA array contained 80 miRNAs (including minor variants) encoded by the Dlk1-Dio3 domain, and we detected expression of 31 miRNAs by the teratomas derived from WT embryos. Their expression levels in the Gtl2<sup>–/–</sup> teratomas were decreased by a factor of at least 2, except for miR-299* (1.9), miR-3072* (1.3) and miR-494 (no difference) (Fig. 2A, Table 1) (Supplementary Table S1: online only). The target genes are known for approximately one half of these miRNAs, which act as tumor suppressors (Suppl Table S1). Therefore, we hypothesized that downregulation of their expression might affect the growth of the Gtl2<sup>–/–</sup> teratomas. When we compared miRNA expression between the normal (0.12–0.13 g) and larger teratomas (0.26–0.94 g) in the Gtl2<sup>–/–</sup>, unexpectedly, there was no difference between expression levels of miRNAs encoded by the Dlk1-Dio3 domain (Fig. 2A). These findings suggested that the downregulation of the Dlk1-Dio3 miRNAs was not enough to promote teratoma growth. Therefore, we focused on miRNAs located outside of the Dlk1-Dio3 domain.

We detected ≥ 2-fold differences in expression levels between the larger Gtl2<sup>–/–</sup> and WT teratomas for only 12 miRNAs that map to regions other than the Dlk1-Dio3 domain (Fig. 2B) (Supplementary Tables S2 and S3: online only). Among the downregulated miRNAs, miR-292-3p, 293, 295, 302a, 302c and 367 are embryonic stem cell markers [16], and miR-18a functions as a tumor suppressor [17]. Further, the expression levels of oncogenic miR-378, tumor-related miR-19b [18, 19] and tissue-specific miRNAs (miR-122, endoderm; miR-206, muscle; and miR-203, epidermis) were increased in the Gtl2<sup>–/–</sup> teratomas [20–22]. Thus, the miRNA expression profile suggested the attenuation of stemness and increased differentiation of the Gtl2<sup>–/–</sup> teratomas. Interestingly, miRNA expression levels tended to recover to the level of normal size Gtl2<sup>–/–</sup> teratomas except for miR-292-3p, 293 and 295 in the miR-290/295 cluster (Fig. 2B). In particular, expression of miR-378 and miR-196b returned to the control level, indicating that the dysregulation of these tumor-related miRNAs contributed to enlargement of the Gtl2<sup>–/–</sup> teratomas.

**Expression by teratomas of p53, pluripotency and differentiation-related markers**

To identify another cause of the teratoma enlargement, we analyzed the expression of p53 by the Gtl2<sup>–/–</sup> teratomas, because the human homolog (MEG3) of Gtl2 activates p53 and inhibits cell proliferation [4]. Gtl2<sup>–/–</sup> teratomas expressed p53 at significantly lower levels compared with WT-induced teratomas, which showed a trend,
Fig. 2. Expression by teratomas of miRNAs, p53 and pluripotency markers. (A) Expression of miRNAs encoded within the Dlk1-Dio3 domain. Twenty-eight miRNAs were decreased by a factor of at least 2 in the Gtl2(+/−) teratomas. (B) Twelve miRNAs mapping outside of the Dlk1-Dio3 domain were differentially expressed (> 2-fold) in the larger Gtl2(+/−) teratomas compared with the controls. Six ES cell-specific miRNAs and the tumor suppressor miR-18a were downregulated, and the oncogene miR-378, proliferation-related miR-196b and 3 tissue-specific miRNAs were upregulated in the larger Gtl2(+/−) teratomas. The vertical line represents the raw signal. The values represent means ± SD. One-way ANOVA followed by Fisher’s LSD test was used to compare two Gtl2(+/−) groups with the control. *** P < 0.01; ** P < 0.05; *P < 0.1. (C) Expression of p53 was downregulated (** P < 0.05, unpaired t-test with Welch’s correction), and a trend toward a negative correlation was found between the teratoma weight and p53 expression in the Gtl2(+/−) teratomas. P = 0.069, Spearman correlation test. (D) The level of expression of Oct4 and Nanog in WT teratomas varied considerably and were significantly decreased in the Gtl2(+/−) teratomas. Gapdh was used as the internal control. ** P < 0.05, unpaired t-test with Welch’s correction.
Table 1. The number of miRNAs that expressed in the teratomas and differentially expressed by > 2-fold in the growth promoted Gtl2(-/-) teratomas

<table>
<thead>
<tr>
<th>Location of miRNAs</th>
<th>Total number</th>
<th>Detected</th>
<th>up (&gt; 2-fold)</th>
<th>down (&lt; 2-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All chromosome</td>
<td>717</td>
<td>212</td>
<td>5</td>
<td>35</td>
</tr>
<tr>
<td>Dlk1-Dio3 domain</td>
<td>80</td>
<td>31</td>
<td>0</td>
<td>28</td>
</tr>
</tbody>
</table>

Discussion

The expression of Dlk1–Dio3 ncRNAs is altered in several types of tumors and cancer cell lines in humans [3, 4]; however, it is unclear whether their expression pattern affects tumor formation or is changed by tumorigenesis. Deletion of the maternal Gtl2 locus causes significantly decreased expression of downstream miRNAs in embryos [9]. Therefore, teratomas derived from Gtl2(-/-) embryos serve as a model for evaluation of the role of Dlk1–Dio3 ncRNAs in tumorigenesis. We show here that Gtl2 and miRNAs located downstream control teratoma growth and stem cell markers in vivo.

Our analysis of the miRNA array reveals that 31 of 80 miRNAs encoded within the Dlk1–Dio3 domain were expressed in WT teratomas, and the expression levels of 30 (except miR-494) were downregulated in Gtl2(-/-) teratomas. Because the promoter of miR-494 is uniquely activated by the ERK1/2 pathway [23], its expression was likely unaffected by the deletion of Gtl2. Interestingly, mice injected with miR-494-transfected lung cancer cells produce larger tumors than controls [23] on the other hand, 13 of 28 miRNAs downregulated by the Gtl2 deletion act as tumor suppressors, and their target genes are known (Table S1). For example, miR-410, which was highly expressed in WT teratomas, targets the MET proto-oncogene, and transfection with miR-410 inhibits proliferation in human glioblastoma cells [24]. Nevertheless, the expression levels of these tumor-related miRNAs were similar between the larger and normal size teratomas induced by Gtl2(-/-) embryos (Fig. 2A). This suggests that Gtl2 deletion and the accompanying reduction of Dlk1–Dio3 miRNAs are not enough to promote teratoma growth. Additionally, considering that the teratomas derived from the H19-deficient embryos clearly show enhanced growth [14], the tumor repressor activity of Gtl2 may be moderate. According to the results of others [4], we reasoned that p53 expression might account for the Gtl2(-/-) teratoma size. This assumption was supported by findings that p53 expression was significantly decreased in the Gtl2(-/-) teratomas compared with the controls (Fig. 2C). Moreover, when we analyzed miRNA loci mapping outside of the Dlk1–Dio3 domain, we found that the levels of expression of 12 miRNAs by the larger Gtl2(-/-) teratomas differed by a factor of ≥ 2 compared with controls. Among them, miR-378, miR-18a and miR-196b are involved in cell proliferation [17–19]. Previously published studies show that mice injected human glioblastoma cells transfected with miR-378 develop larger tumors than controls [18], overexpression of miR-196b induces proliferative activity in human leukemia cells [19], and growth is stimulated by transfection of anti-miR-18a in bladder cancer cell lines [17]. Therefore, increased expression levels of miR-378 and miR-196b as well as decreased expression of miR-18a correlate with the formation of larger teratomas in the Gtl2(-/-) embryo-engrafted mice. Additionally, miR-378 and miR-196b expression recovered to the control level in the normal size Gtl2(-/-) teratomas. MiR-378 targets the tumor suppressor genes Sufu and Fus1, whereas miR-196 targets the tumor suppressor genes Fus [18, 19]. These data suggested that dysregulation of these miRNAs may be moderate. According to the miRNA target prediction program microRNA.org [25] showed that Ppparg1b and Hoxa9 are target genes of miR-543 (Ppparg1b), miR-154 (Ppparg1b) and miR-485 (Hoxa9), which are encoded by the Dlk1-Dio3 domain (Supplementary Fig. S2: online only). Both miR-543 and miR-154 were expressed in the teratoma (Fig. 2A), suggesting that downregulation of these miRNAs induced overexpression of miR-378 encoded within Ppparg1b in the Gtl2(-/-) teratomas. On the other hand, miR-485 expression was low in the teratomas (average raw signal of 10.5 in WT and 2.8 in Gtl2(-/-)), and therefore, there might be a different mechanism that enhances miR-196b expression.

Interestingly, the expression levels of 6 ES cell-specific miRNAs in the miR-302/367 and miR-290/295 clusters [16, 26] were downregulated by factors of 5–16. The promoters of these two clusters are bound by Oct4 and Nanog [27], the expression of which showed a significant decrease in the Gtl2(-/-) teratomas (Fig. 2D). It is reported that Oct4 and Nanog bind around the upstream region of Gtl2 and that these pluripotent markers regulate the Dlk1-Dio3 imprinted domain [28]. Our study showed that Gtl2 deletion induced repression of these transcription factors, indicating that the Dlk1-Dio3 ncRNAs themselves may be involved in regulation of Oct4 and Nanog. The expression levels of Oct4 and Nanog may not affect teratoma size, as their levels of expression in four of the nine WT teratomas were lower than the average expression of the Gtl2(-/-) teratomas. We expected accelerated differentiation of the Gtl2(-/-) teratomas because of the lower expression of stemcell markers and...
advanced gut-like structures in the Gut(2/−) teratomas. Though we were unable to detect a difference in the expression of genes encoding differentiation markers, tissue-specific miRNAs (miR-122, miR-206 and miR-203) showed increased expression in the Gut(2/−) teratomas. The expression of these miRNAs is induced during differentiation [20–22], suggesting the possibility that differentiation is promoted in Gut(2/−) teratomas. 

Taken together, our present findings demonstrate that a proper expression dosage of Dlk1–Dio3 ncRNAs is necessary to maintain the growth and expression of stem cell genes in teratomas. Thus, decreased expression levels of these miRNAs are associated with increased tumor proliferation [13]. Moreover, increased expression of them directly correlates with poor survival of patients with hepatocellular carcinoma [12].

Acknowledgments

We thank K Hata and K Nakabayashi (National Research Institute for Child Health and Development, Japan) for technical advice. This study was supported by Research Fellowships for Young Scientists of the Japan Society for the Promotion of Science to N.T. and Grants-in-Aid for Scientific Research on Priority Areas, and for Scientific Research A from the Ministry of Education, Culture, Sports, Science and Technology of Japan to TK.

References


