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Human Villous Trophoblasts Express and Secrete Placenta-Specific MicroRNAs into Maternal Circulation via Exosomes[1]

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ABSTRACT

In this study, we performed small RNA library sequencing using human placental tissues to identify placenta-specific miRNAs. We also tested the hypothesis that human chorionic villi could secrete miRNAs extracellularly via exosomes, which in turn enter into maternal circulation. By small RNA library sequencing, most placenta-specific miRNAs (e.g., *MIR517A*) were linked to a miRNA cluster on chromosome 19. The miRNA cluster genes were differentially expressed in placental development. Subsequent validation by real-time PCR and in situ hybridization revealed that villous trophoblasts express placenta-specific miRNAs. The analysis of small RNA libraries from the blood plasma showed that the placenta-specific miRNAs are abundant in the plasma of pregnant women. By real-time PCR, we confirmed the rapid clearance of the placenta-specific miRNAs from the plasma after delivery, indicating that such miRNAs enter into maternal circulation. By using the trophoblast cell line BeWo in culture, we demonstrated that miRNAs are indeed extracellularly released via exosomes. Taken together, our findings suggest that miRNAs are exported from the human placental syncytiotrophoblast into maternal circulation, where they could target maternal tissues. Finally, to address the biological functions of placenta-specific miRNAs, we performed a proteome analysis of BeWo cells transfected with *MIR517A*. Bioinformatic analysis suggests that this miRNA is possibly involved in tumor necrosis factor-mediated signaling. Our data provide important insights into miRNA biology of the human placenta.

exosome, human placenta, maternal blood plasma, miRNA, placenta, pregnancy, syncytiotrophoblast, trophoblast, tumor necrosis factor

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INTRODUCTION

MicroRNAs are small noncoding RNAs approximately 22 nucleotides (nt) in length. To date, the available data indicate that miRNAs are involved in a variety of physiological and pathological processes in animals [1]. Generally, miRNAs are believed to interfere with RNAs posttranscriptionally (i.e., miRNAs bind to the 3′-untranslated regions of their target mRNAs), suppressing translation [2]. However, some miRNAs are known to potentially degrade their target mRNAs in the same fashion as small interference RNAs [3]. Moreover, a recent study [4] revealed that miRNAs are able to not only suppress but also stimulate translation from their target mRNAs in a cell cycle-dependent manner, indicating that the miRNA-dependent translational regulation is more complicated than previously understood. Although many miRNAs are ubiquitously expressed in mammals, some miRNAs exhibit specific expression patterns in a manner that is dependent on organ type and cell type [5]. Findings from recent studies [6–9] on vertebrates suggest that the differential patterns of miRNA expression are closely associated with particular phenotypes expressed by cells, tissues, or organs and that tissue-specific or tissue-predominant miRNAs are involved in tissue differentiation and the maintenance of tissue identity. To date, however, few such miRNAs have been linked with specific physiological and pathological events at the tissue and organ levels. It has been reported recently that RNA molecules, including miRNAs, are not only localized intracellularly but also secreted extracellularly via exosomes, unilamellar small vesicles of 50–100 nm in diameter [10]. Exosomes are originated from intraluminal vesicles present in multivesicular bodies (MVBs) and have been shown to be secreted by cells of hematopoietic origins [11]. In line with previous findings that exosomes can mediate intercellular communication in physiological processes such as antigen presentation, Valadi et al. [10] demonstrated that the RNAs secreted via exosomes are transferable to other cells; therefore, they are designated as "exosomal shuttle RNA."

The mammalian placenta serves as an auxiliary fetal organ at the interface between the mother and the fetus and supplies many nutrients, including ions and gases, to the developing fetus throughout gestation. Growth and differentiation of the placenta are fundamental to mammalian reproduction, including humans, and functional impairment of this organ occasionally leads to severely abnormal pregnancies. For example, preeclampsia (PE) is caused by poor placentation with impaired remodeling of the spiral arteries [12]. Little is known about the role of miRNAs in such physiological and

pathological processes during pregnancy. Analysis of the expression profiles of miRNAs in the human placenta and subsequent identification of tissue-specific miRNAs are the first step toward understanding the biological functions of these molecules. Cloning of miRNAs has contributed greatly to an accelerated advance in miRNA profiling [5, 13–15]. This method gives a relative cloning frequency of each miRNA, which is considered to represent its expression level [5, 16].

In this study, we performed small RNA library sequencing and miRNA histochemistry using human placental chorionic villi to reveal miRNA expression profiles in the human placenta. We next tested the hypothesis that human chorionic villi could secrete miRNAs extracellularly via exosomes, which in turn enter into maternal circulation during pregnancy. Furthermore, we performed a proteome analysis by miRNA overexpression using a trophoblast cell line to elucidate the biological functions of placenta-specific miRNAs.

MATERIALS AND METHODS

Sample Collection

Human placentas and blood plasma samples were obtained according to protocols approved by the Nippon Medical School Hospital Ethics Committee and the Jichi Medical University Ethics Committee. First-trimester and full-term placental tissues were obtained from elective terminations of pregnancy and uncomplicated cesarean deliveries, respectively. In all cases, tissue was processed as soon as possible following collection (within 20 min). The number of placentas used in this study varied among three types of analyses: 1) small RNA library sequencing analysis (three first trimester and five full term), 2) PCR analysis (six first trimester and six full term), and C) histochemical analysis (three first trimester and four full term). Terminal villus (TV)-rich pellets were also collected from the same placentas to examine the anatomical regional heterogeneity of miRNA expression in the placental chorionic villous tree. A modification of the method by Kacemi et al. [17] was used to collect TV-rich samples as previously described [18]. Samples of peripheral blood were obtained from the following three groups: 1) three first-trimester pregnant women (gestational age, 7–11 weeks), 2) three full-term pregnant women (gestational age, 36–38 weeks), and 3) three healthy nonpregnant women. For the six full-term women, postdelivery maternal blood samples and placental tissues were also collected 3 days after delivery.

miRNA Cloning and Sequencing

Placental samples used in the sequencing analysis were categorized into two groups: first-trimester placental tissues and full-term placental tissues. The full-term placental tissues were further divided into two groups to examine the anatomical regional heterogeneity of miRNA expression in the placental chorionic villous tree: TV-rich samples and whole-villous (WV) samples. Blood plasma samples used in this study were also categorized into three groups: blood plasma from first-trimester women, blood plasma from full-term women, and blood plasma from healthy nonpregnant women.

The total RNA of placental tissue samples was isolated using Isogen (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. To extract the total RNA from blood plasma samples, 10-ml blood samples were collected into ethylenediaminetetraacetic acid-containing vacuum blood drawing tubes (Venogect II; Terumo, Tokyo, Japan) and then centrifuged at 1700 × *g* for 15 min at 4°C. After collecting the supernatant into a 15-ml centrifuge tube, an equal volume of phenol:chloroform:isopropyl alcohol (PCI) (25:24:1 dilution) was added. After gentle rotation, the supernatant was retrieved by centrifugation at 12 000 × *g* for 2 min at 4°C. An equal volume of PCI was then added to the supernatant. The tube was rotated and centrifuged as already described. After ethanol precipitation was performed, the resultant precipitate was redissolved using Isogen.

Construction of the small RNA libraries was performed as described previously [13, 16]. Briefly, 50 μg and 5 μg of total RNA from placenta and plasma, respectively, were used. Linker ligation, cDNA synthesis, and PCR amplification of cDNAs were carried out. We then concatenated more than 20 cDNAs into a single fragment using a *Ban*I restriction enzyme (New England Biolabs, Ipswich, MA), a DNA ligation kit (version 2.1; Takara Bio, Shiga, Japan), and a Geneclean III kit (Qbiogene, Irvine, CA) before TA cloning. The concatenated products were then inserted into plasmids and sequenced.

Bioinformatic Analysis of Sequence Data

We performed a homology search for all cloned small RNAs and a secondary structural analysis for all novel miRNA candidates. Small RNA sequences were analyzed for homology with known RNAs and human genomic DNA sequences, including miRNAs, piwi-interacting RNAs (piRNAs), rRNAs, tRNAs, small nucleolar RNAs (snoRNAs), mRNAs, and genomic DNA. The databases used were as follows: miRNAs (mature and pre), Sanger database (miRBase) version 9.2 (http://microrna.sanger.ac.uk/sequences/index.shtml), rRNAs (European Ribosomal RNA database [http://bioinformatics.psb.ugent.be/webtools/rRNA/]), tRNAs (Genomic tRNA Database [http://lowelab.ucsc.edu/GtRNAdb/]), sn/snoRNAs (RNAdb [http://research.imb.uq.edu.au/rnadb/]), and NONCODE (http://www.noncode.org/). All searches were conducted on 10 July 2007. In this study, the miRNAs that were unregistered opposite-strand miRNAs of known "unpaired" miRNAs in the miRBase version 9.2 were designated as "miRNA (opposite)" (e.g., *MIR140 [opposite]*).

Clones with 100% homology to human genomic sequences but not identical to known RNAs were then subjected to secondary structural analysis. The two-dimensional pre-miRNA configurations of these clones were predicted as previously described [19]. We defined each of the clones as a novel miRNA candidate when its configuration with the least free energy met the following criteria: 1) it contains a stem-loop configuration, 2) the cloned mature miRNA sequence portion consists of more than 16 nt in its double-stranded region, 3) the loop is less than 20 nt long, 4) the internal loop is less than 10 nt long, and 5) the bulge is less than 5 nt long. In this analysis, novel sequences with overlapping positions in the genome were grouped together. These candidates were then subjected to immunoprecipitation analysis of a eukaryotic translation initiation factor 2C, 2 (EIF2C2 [also known as Argonaute-2]) protein described herein.

EIF2C2 Immunoprecipitation and PCR Analysis of Novel miRNAs

After bioinformatic analysis of the sequence data, we further validated novel miRNAs by EIF2C2 immunoprecipitation [16, 20], followed by PCR-based miRNA detection [21]. EIF2C2 immunoprecipitation from the human placenta was performed using a miRNA isolation kit (Wako, Osaka, Japan) according to the manufacturer's protocol. PCR-based detection of the novel miRNA candidates from the EIF2C2-associated RNAs was performed as previously reported [21]. The expected size of amplicons derived from mature miRNAs is approximately 120 base pairs (bp). The PCR conditions (primer sequence, annealing temperature, and PCR cycle) are given in Table 5. Complementary DNA libraries were also generated from small RNAs isolated from five reproductive organs, including first-trimester placenta, full-term placenta, cervix, ovary, and testis. The expression levels of novel miRNAs in the six organs were examined by semiquantitative PCR and scored as 4 for high, 3 for medium, 2 for low, 1 for little to none, and 0 for none.

Real-Time PCR

Real-time PCR was performed on an ABI7300 (Applied Biosystems, Foster City, CA). As an endogenous control, *5S rRNA* or *RNU6–2* (also known as *U6*) was amplified in parallel. The primers used for *MIR1* (catalog number 30008), *MIR21* (30102), *MIR23A* (30119), *MIR122A* (30019), *MIR124A* (30020), *MIR125B* (30022), *MIR200C* (30096), *MIR221* (30115), *MIR424* (30195), *5S rRNA* (30302), and *RNU6–2* (30303) were purchased from Ambion (Austin, TX). The primers used for *MIR15B* (part number 4373122), *MIR30B* (4373290), *MIR99A* (4373008), *MIR451* (4373209), *MIR497* (4373222), *MIR512-3p* (4381034), *MIR517A* (4373243), *MIR517B* (4373244), *MIR518B* (4373246), *MIR519A* (4373249), *MIR574* (4381019), and *RNU6–2* (4373381) were from Applied Biosystems. Real-time PCR analysis of *MIR1* and *MIR124A* represented controls, as *MIR1* and *MIR124A* were shown to be highly specific for brain and muscle, respectively [22].

In Situ Hybridization

In situ hybridization (ISH) was performed using miRCURY locked nucleic acid (LNA) detection probes (Exiqon, Vedbeak, Denmark) for *MIR122A*, *MIR125B*, and *MIR 517B* and a modified version of the method by Kloosterman et al. [23]. Briefly, human placenta samples were fixed with 4% paraformaldehyde at 4°C overnight. Small pieces of fixed chorionic villous tree were treated with proteinase K (10 μg/ml; Wako) at 37°C for 45 min in 2-ml microcentrifuge tubes, refixed with 4% paraformaldehyde at 22°C for 20 min, and then acetylated with a mixture of 0.25% acetic anhydride and 0.1 M triethanolamine at 22°C for 10 min. The samples were then prehybridized with H7782 hybridization solution (Sigma, St. Louis, MO) at 55°C for 2 h and subsequently hybridized overnight in the same hybridization buffer containing

10 nM 3′-digoxigenin (DIG)-labeled LNA probes at 55°C. After hybridization, the sections were incubated with sheep anti-DIG-alkaline phosphatase–Fab fragments (1:2000 dilution; Roche, Basel, Switzerland) at 4°C overnight and then visualized with nitroblue tetrazolium chloride/5-bromo-4-chloro-3′-indolylphosphatase p-toluidine salt (NBT/BCIP; Roche) at 22°C for 2–12 h. After visualization of the ISH signal, cryostat sections (5 μm in thickness) were made on a Microm (Walldorf, Germany) HM 550 cryostat, counterstained with 4′,6-diamidino2-phenylindole dihydrochloride (DAPI; Molecular Probes, Eugene, OR), and then mounted in aqueous mounting medium (Pristine Mount Research Genetics, Huntsville, AL). Control sections received the same treatment except that the probes were replaced with a 3′-DIG-labeled LNA scramble-miR control probe (Exiqon) as a negative control.

Some hybridized sections were subsequently immunostained with mouse anti-CD63 monoclonal antibodies (catalog number sc-5275, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA; and catalog number H5C6, 1:400; Developmental Studies Hybridoma Bank, Iowa City, IA). The CD63 signal was visualized by incubation with the secondary antibody, Alexa Fluor 488-labeled goat anti-mouse IgG (10 μg/ml; Invitrogen, Carlsbad, CA). Control sections received the same treatment except that the primary antibodies were replaced with nonimmune isotype-matched IgG_1 (catalog number M9269; Sigma).

The sections were examined under an Axioplan microscope (Carl Zeiss, Göttingen, Germany) equipped with a DP20 charge-coupled device (CCD) camera (Olympus, Tokyo, Japan) or under a BX60 microscope (Olympus) equipped with a Spot RT SE6 CCD camera (Diagnostic Instruments, Sterling Heights, MI). Bright-field, fluorescence, and differential interference contrast images were captured using the MetaMorph image analysis system (MDS Analytical Technologies, Toronto, ON, Canada). The figures were created using Photoshop CS software (Adobe Systems, San Jose, CA).

Cell Culture

BeWo, a trophoblast cell line derived from a human gestational choriocarcinoma, was provided by the Riken Bioresource Center (Tsukuba, Japan). The cell line was maintained in Ham F12 medium supplemented with 15% fetal bovine serum at 37°C in a humidified incubator with 5% CO_2.

Enhanced Green Fluorescent Protein-Tagged CD63 and Transfection

The open reading frame (ORF) of human *CD63* cDNA was amplified from the total RNA of the thymus (Ambion) by RT-PCR using PrimeStar HS polymerase (Takara Bio) and cloned into the plasmid-enhanced green fluorescent protein (pEGFP)-C1 vector (Clontech, Mountain View, CA) via *Bgl*II and *Hind*III sites, allowing the linkage of EGFP to the human *CD63* ORF in frame (pEGFP-hCD63). Validation of our plasmid construction was then performed by transfection of COS-7 cells with pEGFP-hCD63, followed by Western blotting with an anti-human CD63 antibody (data not shown). Based on this analysis, a single band representing the EGFP-CD63 protein was detected at the theoretical position described previously [24].

Transfection of BeWo cells was performed using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. Briefly, cells grown at semiconfluence were transfected with 20 pmol/ml of Alexa Fluor 594-labeled synthetic *MIRLET7B* (Nippon Bioservice, Tokyo, Japan) and 0.2 mg/ml of pEGFP-hCD63 or pEGFP-C1 and were cultured for 48 h. The cells were then observed to detect signals from both Alexa Fluor 594 and EGFP under an IX71-TIRFM-SP microscope (Olympus) equipped with a Photon MAX EMCCD camera (Princeton Instruments, Trenton, NJ) and processed using the MetaMorph system.

Isolation of Exosomes

BeWo cells grown at semiconfluence on 90-mm φ dishes were transfected with pEGFP-hCD63 (8 μg/dish) with or without the precursor molecule of *MIR517A* (pre-*MIR517A*) (Ambion) at a concentration of 45 nM according to the manufacturer's protocol and cultured for 48 h. Media from the cultures of transfected and untransfected BeWo cells were depleted from floating cells by centrifugation at 2300 × *g* for 5 min, passed through 0.8-μm disk filters (Pall, Ann Arbor, MI) to remove cell debris, and concentrated using Amicon Ultra centrifugal filter devices (molecular weight cutoff, 10 000; Millipore, Billerica, MA). The concentrated media were preincubated overnight with Dynabeads M280 anti-mouse IgG (Invitrogen) at 4°C to reduce the background noise in the following procedure: To capture exosomes immunologically, the concentrated media were incubated with Dynabeads M280 anti-mouse IgG bound to the anti-CD63 mouse monoclonal antibody (Santa Cruz Biotechnology) or the nonimmune mouse IgG_1 (Sigma) at 4°C overnight. The beads were then washed three times with PBS at room temperature and subjected to extraction of exosomal RNAs using RNAiso (Takara Bio). Because the yield of the exosomal RNAs was expected to be low, 5 μg of yeast tRNA (*Saccharomyces cerevisiae* tRNA; Roche Diagnostics, Mannheim, Germany) was spiked into the reagent as a carrier. In parallel, to evaluate if the anti-CD63 antibody works to trap CD63 protein in solution, the lysates of the transfected BeWo cells were incubated with anti-CD63 antibody-coated protein G-sepharose 4B beads (GE Healthcare, Uppsala, Sweden), washed three times with PBS, and observed under fluorescent microscopy to detect EGFP-derived fluorescence (Supplemental Fig. S1 available at www.biolreprod.org).

Proteome Analysis

BeWo cells grown at a semiconfluence were transfected with Pre-miR has-*MIR517A* or Pre-miR Negative Control #1 (Applied Biosystems) at a final concentration of 45 nM as already described. The Pre-miR-transfected BeWo cells were harvested 72 h after the initiation of transfection and subjected to the following proteome analysis: The cells were lysed with a thiourea lysis buffer (7.5 M urea, 2 M thiourea, 4% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate, 1 mM PMSF, 1 μM aprotinin, 1 μM pepstatin A, and 10 mM Tris-HCl [pH 8.8]). After removal of cell debris by centrifugation at 20 000 × *g* at 4°C for 20 min, proteins in the cell lysates were labeled with CyDye DIGE Fluors minimal dyes (GE Healthcare Bio-Sciences, Tokyo, Japan). The labeled proteins were subjected to two-dimensional difference gel electrophoresis using the Ettan DALTsix Large Electrophoresis System (GE Healthcare Bio-Sciences) (Supplemental Fig. S2) as described in the manufacturer's protocol. Protein samples obtained from three independent experiments were subjected to proteome analysis to exclude deviation by experimental errors. Spots with significant differences in intensity between the samples (upregulation or downregulation by more than 10%, $P \leq 0.05$) were excised from the gels using the Ettan Spot Picker (GE Healthcare Bio-Sciences). These criteria for spot selection were determined based on previous studies [25, 26] describing that the repression of cellular protein synthesis by miRNA overexpression is typically mild. Proteins in the spots were subjected to digestion with 10 ng/μl trypsin (Sigma) at 37°C, followed by elution in an acetonitrile buffer. The eluted proteins were then analyzed using a LC/MS mass spectrometer (LCQ DECA XP Plus; Thermo-Finnigan, San Jose, CA). Data from mass spectrometry were analyzed using MASCOT software (Matrix Science, London, England), and proteins hit with significantly high scores were subjected to Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Redwood City, CA) to delineate possible networks and pathways involving the proteins.

Western Blot Analysis

BeWo cells in culture were rinsed in PBS, scraped in PBS with a cell scraper, and centrifuged at 1000 × *g* for 3 min. Cell pellets were then resuspended in M-PER Mammalian Protein Extraction Reagent (Thermo-Fischer Scientific, Rochester, NY) containing Halt Protease Inhibitor Cocktail (Thermo-Fischer Scientific) as indicated in the manufacturer's protocol and subjected to sonication to complete cell lysis. The lysates were centrifuged at 20 000 × *g* at 4°C for 3 min to remove debris, and supernatants were subjected to Western blotting as follows: The lysates were separated in SDS-PAGE gels and transferred to Sequi-Blot polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA). Blots were then incubated at 4°C overnight with primary antibodies diluted as recommended in the manufacturer's instructions. This was followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Signals were detected using Immobilon reagent (Millipore) and visualized using an LAS-400 Lumino image analyzer (Fujifilm, Tokyo, Japan). The intensity of visualized signals was quantitatively analyzed using Multi-gauge software (Fujifilm). Antibodies for thioredoxin (TXN), voltage-dependent anion channel 1 (VDAC1), phosphorylated mitogen-activated protein kinase 8/9/10 (MAPK8/9/10 [previously designated as JNK1/2/3]), and total MAPK8/9/10 were purchased from Cell Signaling Technologies (Danvers, MA). An antibody for β-actin (ACTB) was purchased from Sigma.

RESULTS

miRNA Expression Profiling of the Human Placenta by Small RNA Library Sequencing

We obtained 25 100 small RNA clones from placental samples. The human placenta-derived small RNAs were classified as follows: 20 238 miRNAs (313 genes), 176 piRNAs, 191 rRNAs, 60 tRNAs, 17 small nuclear RNAs (snRNAs), 45 snoRNAs, and 166 mRNAs (Fig. 1). Supplemental Table S1 gives the profiling data for all of the known

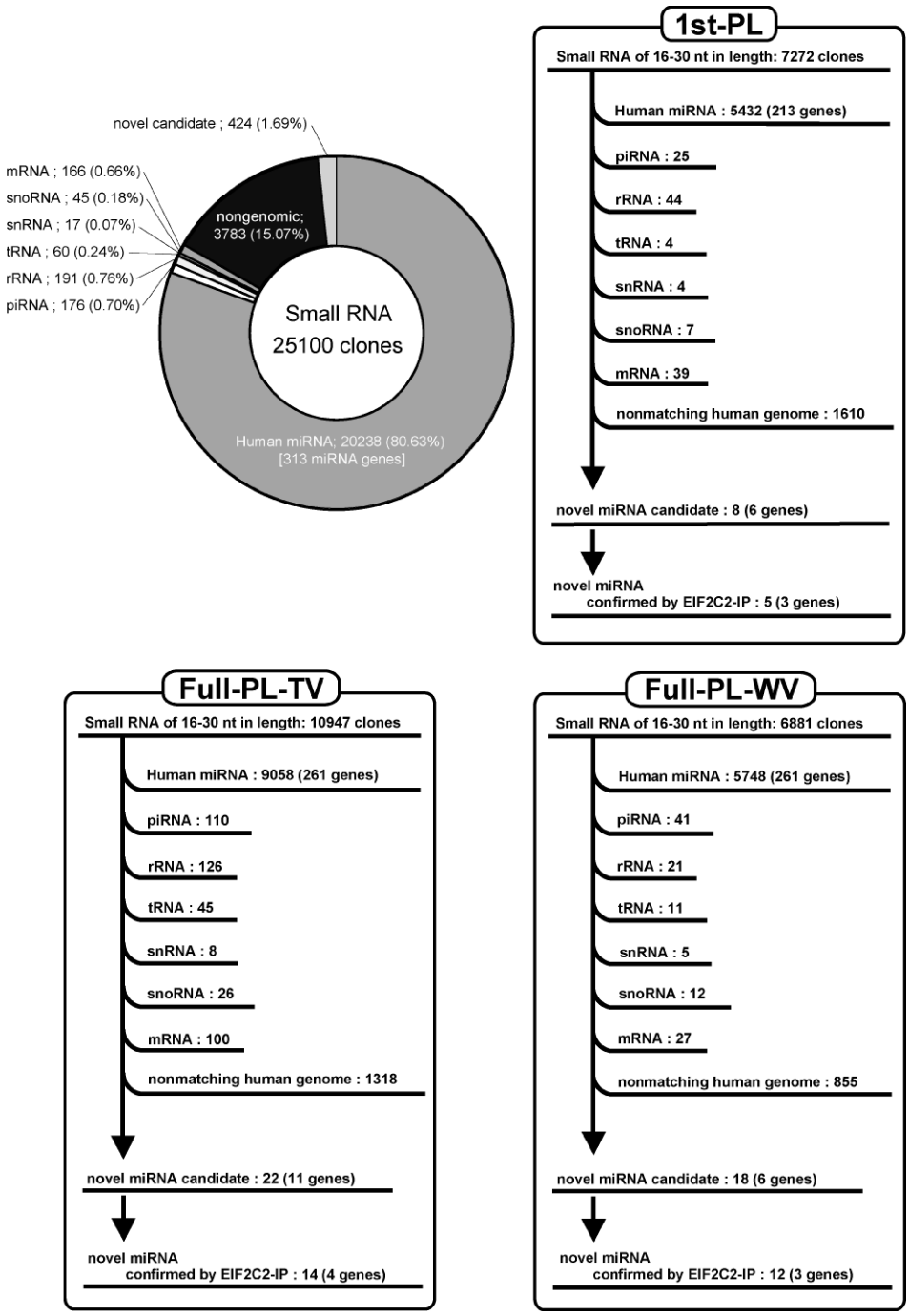

FIG. 1. Bioinformatic analysis of small RNA clones derived from the human placenta. The sequences, composed of 16–30 nt, were extracted as valid small RNAs and were compared with various RNA databases. Novel miRNAs were identified by computational prediction, followed by small RNA detection using EIF2C2 immunoprecipitation (EIF2C2-IP). 1st-PL, first-trimester placenta; Full-PL, full-term placenta; WV, whole villi; TV, terminal villi.

miRNAs, including sequence information (identification, representative clone sequence, and location in the 5′- and 3′-strand duplex of each miRNA stem loop), clone count, and cloning frequency. We attained high efficiency of miRNA cloning; miRNAs were approximately 81% of all small RNAs cloned. The most abundant miRNAs were 22 nt long. Placental tissue samples used in this sequencing analysis were categorized into three groups, namely, first-trimester placental tissue, full-term placental tissue WV samples, and full-term placental tissue TV-rich samples (see *Materials and Methods*).

We first focused on highly cloned miRNAs (i.e., >1% of the entire miRNA clone population in each placental group) from each placental group as summarized in Table 1. Sixteen miRNAs (*MIR21*, *MIR23A*, *MIR24*, *MIR27A*, *MIR30B*, *MIR30D*, *MIR122A*, *MIR125B*, *MIR143*, *MIR199A**, *MIR200C*, *MIR221*, *MIR424*, *MIR517A*, *MIR517B*, and *MIR518B* [approximately 50% of the entire miRNA clone population in each placental group]) were common among the three groups. Genes encoding the miRNAs cloned from the human placenta are located on all chromosomes but the Y chromosome. The genes for highly cloned miRNA, however, appear to be biased toward those from chromosome 19; approximately 30%–40% of them were derived from this chromosome. We next directed our attention to the miRNAs that were differentially detected between two placental groups (i.e., first-trimester placental tissue vs. full-term placental tissue WV samples and full-term placental tissue WV samples vs. full-term placental tissue TV-rich samples). For each comparison, miRNAs cloned at a frequency higher by at least fourfold compared with others and greater than 0.1% of the entire

TABLE 1. Highly cloned miRNAs from human placenta by small RNA library sequencing.[a]

First-trimester placenta				Full-term placenta							
				Whole-villi				Terminal-villi			
MIR[b]	Percentage[c]	Chromosome	Cluster[d]	*MIR*[b]	Percentage[c]	Chromosome	Cluster[d]	*MIR*[b]	Percentage[c]	Chromosome	Cluster[d]
21	8.82	17		*21*	9.31	17		*517A*	8.46	19	C3
125B	7.49	11		*517A*	7.52	19	C3	*21*	6.62	17	
517A	5.71	19	C3	*125B*	6.94	11		*125B*	5.69	11	
122A	3.98	18		*200C*	4.82	12		*200C*	4.45	12	
*199A**	3.07	19		*30B*	3.90	8	C1	*23A*	3.61	19	C2
30B	2.98	8	C1	*424*	3.74	X		*221*	3.35	X	
99B	2.82	19		*122A*	2.59	18		*30B*	3.31	8	C1
23A	2.80	19	C2	*27A*	2.49	19	C2	*424*	2.70	X	
200C	2.69	12		*518B*	2.23	19	C3	*451*	2.33	17	
143	2.45	5		*221*	2.17	X		*26A*	2.01	3	
191	2.10	3		*30D*	2.16	8	C1	*517B*	1.91	19	C3
34A	1.99	1		*23A*	2.09	19	C2	*27A*	1.85	19	C2
424	1.93	X	C4	*143*	2.07	5		*24*	1.69	9	C5
15B	1.91	3		*24*	1.91	9		*519A*	1.62	19	
30D	1.86	8	C1	*517C*	1.72	19		*199A**	1.56	19	
221	1.66	X		*519A*	1.44	19		*122A*	1.44	18	
27A	1.56	19	C2	*518A*	1.34	19	C3	*99B*	1.38	19	
503	1.49	X	C4	*517B*	1.32	19	C3	*30D*	1.35	8	C1
512–3p	1.47	19		*512–3p*	1.29	19		*181A*	1.32	1	
29B	1.27	7		*26A*	1.27	3		*LET7A*	1.30	11	
24	1.25	9		*100*	1.25	11	C6	*27B*	1.09	9	C5
99A	1.25	21		*199A**	1.18	19		*517C*	1.06	19	
518B	1.25	19	C3	*LET7A*	1.08	11	C6	*518E*	1.06	19	C3
30C	1.18	11		*181A*	1.06	1		*518B*	1.05	19	C3
449	1.18	20		*LET7B*	1.03	22		*143*	1.04	5	
517B	1.16	19	C3								
25	1.10	7									
512–5p	1.10	19									

[a] Greater than 1% of the total miRNA clone population in each placenta group.
[b] The miRNA IDs in the miRBase version 9.2 are employed in this study.
[c] Percentage of miRNA genes in the total miRNA clone population in each placenta group.
[d] Clusters (C1–C6) indicate miRNA gene clusters.

miRNA clone population in either group are summarized in Table 2. In a comparison between the first-trimester placental tissue and full-term placental tissue WV sample groups, 16 and 10 miRNAs were cloned in favor of the former and the latter, respectively (Table 2). Regarding the chromosomal distribution of the predominant miRNA genes in a comparison between the first-trimester placental tissue vs. full-term placental tissue WV samples, five of 10 full-term placental tissue WV samples-predominant miRNA genes are derived from chromosome 19; in contrast, none of 16 first-trimester placental tissue-predominant miRNA genes are from this chromosome. Based on these results, the expression of chromosome 19-linked miRNAs likely increases during placental development. We also analyzed miRNAs that were cloned exclusively from one group in each pairwise comparison. Table 3 summarizes the exclusive miRNAs (>0.05% in one placental group and not found in the other group). Most of the exclusive miRNAs had a low cloning frequency.

Validation of the High-Level Expression of Placenta-Specific miRNAs by Real-Time PCR and ISH

The expression levels of 20 miRNAs (*MIR1*, *MIR15B*, *MIR21*, *MIR23A*, *MIR30B*, *MIR99A*, *MIR122A*, *MIR124A*, *MIR125B*, *MIR200C*, *MIR221*, *MIR424*, *MIR451*, *MIR497*, *MIR512-3p*, *MIR517A*, *MIR517B*, *MIR518B*, *MIR519A*, and *MIR574*) that had high cloning frequencies in the human placenta as already described were examined by real-time PCR. The level of expression was compared among 10 organs (brain, heart, lung, liver, kidney, small intestine, skeletal muscle, testis, ovary, and placenta). Our analysis revealed that five of them (*MIR512-3p*, *MIR517A*, *MIR517B*, *MIR518B*, and *MIR519A*) were specifically expressed in the placenta (Fig. 2). The other miRNAs examined were likely ubiquitously expressed in human organs, albeit with variation in expression levels (e.g., *MIR21* and *MIR125B* in Fig. 2). *MIR1* and *MIR124*, which are highly specific for the brain and for heart and skeletal muscles, respectively (data not shown), as previously reported by Mishima et al. [22], were also analyzed as controls.

For some of the miRNAs already described, we further investigated their in vivo localization in human placental villi in the first trimester and full term by ISH. *MIR517B* was detected exclusively in the trophoblast layer; little signal (if any) was observed in villous stroma cells or fetal endothelial cells (Fig. 3, A–C). Intense signals were present mostly in the syncytiotrophoblast (STB), whereas weak but positive signals were discernible in the cytotrophoblast (CTB) (Fig. 3C). We also examined the in vivo localization of *MIR122A* (data not shown) and *MIR125B* (Fig. 3, D and E, for *MIR125B*) in the human placenta. These miRNAs were expressed not only in the villous trophoblasts but also in some villous stroma cells. Specific staining was absent from sections that were incubated with a scramble-miR control probe (Fig. 3A, inset).

Alteration in the Levels of Placenta-Specific miRNAs in Maternal Plasma Before and after Delivery

By small RNA library sequencing, we obtained 8940 small RNA clones from human plasma samples, which were

TABLE 2. Predominant miRNAs revealed by comparison between first-trimester and full-term placentas.[a]

First–trimester placenta vs. full-term placenta (whole-villi)								Full-term placenta whole-villi vs. terminal-villi							
First-trimester/full-term ratio				Full-term/first-trimester ratio				Whole-villi/terminal-villi ratio				Terminal-villi/whole-villi ratio			
MIR[b]	Ratio	Chromosome	Cluster[c]	*MIR*[b]	Ratio	Chromosome	Cluster[c]	*MIR*[b]	Ratio	Chromosome	Cluster[c]	*MIR*[b]	Ratio	Chromosome	Cluster[c]
99A	14.4	21		*141*	15.4	12		*141*	7.0	12		*483*	16.5	11	
324–3p	11.6	17		*30E-5p*	11.3	1		*181A**	6.3	1		*124A*	8.9	8	
497	11.6	17		*516–5p*	9.5	19		*519E**	5.9	19		*376A*	7.0	14	
331	9.9	12		*450*	6.6	X		*450*	5.5	X		*LET7D*	5.7	9	
342	8.5	14		*520D**	6.4	19	C1					*365*	5.1	16	
483	8.5	11		*100*	6.2	11						*542–5p*	5.1	X	
449	7.5	5		*524*	5.5	19	C1					*93*	4.2	*7*	
210	7.4	11		*196B*	5.2	*7*									
25	6.3	7		*524**	4.7	19	C1								
92B	6.3	1		*525**	4.3	19									
503	6.1	X													
214	6.0	1													
191	4.8	3													
34A	4.6	1													
93	4.2	*7*													
15B	4.1	3													

[a] More than 4-fold difference between samples in cloning frequency, and greater than 0.1% in either placenta group.
[b] The miRNA IDs in the miRBase version 9.2 are employed in this study.
[c] Clusters (C1–C4) indicate miRNA gene clusters.

TABLE 3. Exclusive miRNAs revealed by comparison between first-trimester and full-term placentas.[a]

First–trimester placenta vs. full-term placenta (whole-villi)								Full-term placenta whole-villi vs. terminal-villi							
Exclusive miRNAs in first-trimester				Exclusive miRNAs in full-term				Exclusive miRNAs in whole-villi				Exclusive miRNAs in terminal-villi			
MIR[b]	Percentage[c]	Chromosome	Cluster[d]	*MIR*[b]	Percentage[c]	Chromosome	Cluster[d]	*MIR*[b]	Percentage[c]	Chromosome	Cluster[d]	*MIR*[b]	Percentage[c]	Chromosome	Cluster[d]
LET7D (opposite)	0.35	9		*181A**	0.14	1		*32*	0.05	9		*532*	0.07	X	
34B (opposite)	0.31	11	C1	*140*	0.09	16						*532 (opposite)*	0.07	X	
34B	0.20	11	C1	*106B*	0.07	*7*						*1*	0.06	20	
532 (opposite)	0.11	X		*342 (opposite)*	0.07	14						*20B*	0.06	2	
99B (opposite)	0.09	19		*551B*	0.07	3						*323*	0.06	14	
125B (opposite)	0.07	21		*151*	0.05	8						*520A*	0.06	19	
31	0.06	9		*361 (opposite)*	0.05	X									
34C	0.06	11	C1	*517C (opposite)*	0.05	19									
520A	0.06	19													

[a] Greater than 0.05% in one placenta sample and none in the other sample.
[b] The miRNA IDs in the miRBase version 9.2 are employed in this study.
[c] Percentage of miRNA genes in the total miRNA clone population in each placenta group.
[d] C1 indicates a miRNA gene cluster.

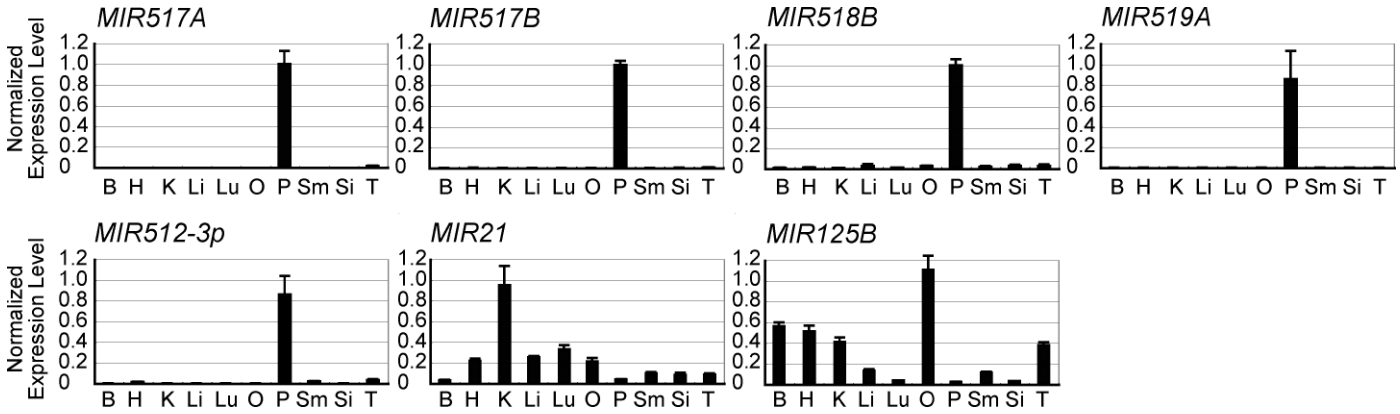

FIG. 2. Tissue distribution of human placenta-specific miRNAs. The expression levels of 20 miRNAs with high cloning frequencies from the human placenta were examined by real-time PCR (see *Results*). The level of expression was compared among 10 organs (brain [B], heart [H], kidney [K], liver [Li], lung [Lu], ovary [O], placenta [P], skeletal muscle [Sm], small intestine [Si], and testis [T]). Of these, *MIR512-3p*, *MIR517A*, *MIR517B*, *MIR518B*, and *MIR519A* exhibit placenta-restricted expression patterns. The rest are more broadly distributed (the distribution of *MIR21* and *MIR125B* are shown as representatives). The data are normalized to the expression levels of *RNU6–2* that were analyzed in parallel. For each miRNA, a normalized level (miRNA/*RNU6–2*) in a tissue where the miRNA is expressed most abundantly (e.g., placenta for *MIR517A*) is assigned a value of 1. Values are the mean ± SD from three experiments.

classified as follows: 5035 miRNAs (142 genes), 525 piRNAs, 109 rRNAs, 86 tRNAs, 6 snRNAs, 355 snoRNAs, and 479 mRNAs. Supplemental Table S2 gives the profiling data for all of the known miRNAs, including sequence information (identification, representative clone sequence, and location in the 5′- and 3′-strand duplex of each miRNA stem loop), clone count, and cloning frequency. The population of miRNAs accounts for approximately 56% of all small RNAs cloned. The most abundant miRNAs were 22 nt long. We examined the highly cloned miRNAs (i.e., >0.5% of the entire miRNA clone population) in each of three groups, namely, blood plasma from first-trimester women, blood plasma from full-term women, and blood plasma from healthy nonpregnant women (see *Materials and Methods*). The results of the highly cloned miRNAs in each blood plasma group classified in this study are summarized in Supplemental Table S3. Ten miRNAs (*MIR23A*, *MIR25*, *MIR29A*, *MIR143*, *MIR484*, *MIR503*, *MIR517A*, *MIR518B [opposite]*, *MIR526B*, and *MIRLET7A*) were likely pregnancy-dominant miRNAs that were cloned at a much lower frequency in blood plasma from healthy nonpregnant women. The cloning frequency of the three chromosome 19-derived miRNAs (*MIR517A*, *MIR518B [opposite]*, and *MIR526B*) in blood plasma from first-trimester women was lower than that in blood plasma from full-term women (Supplemental Tables S2 and S3). It may correlate with the growth of the placenta (e.g., the increase in surface area of

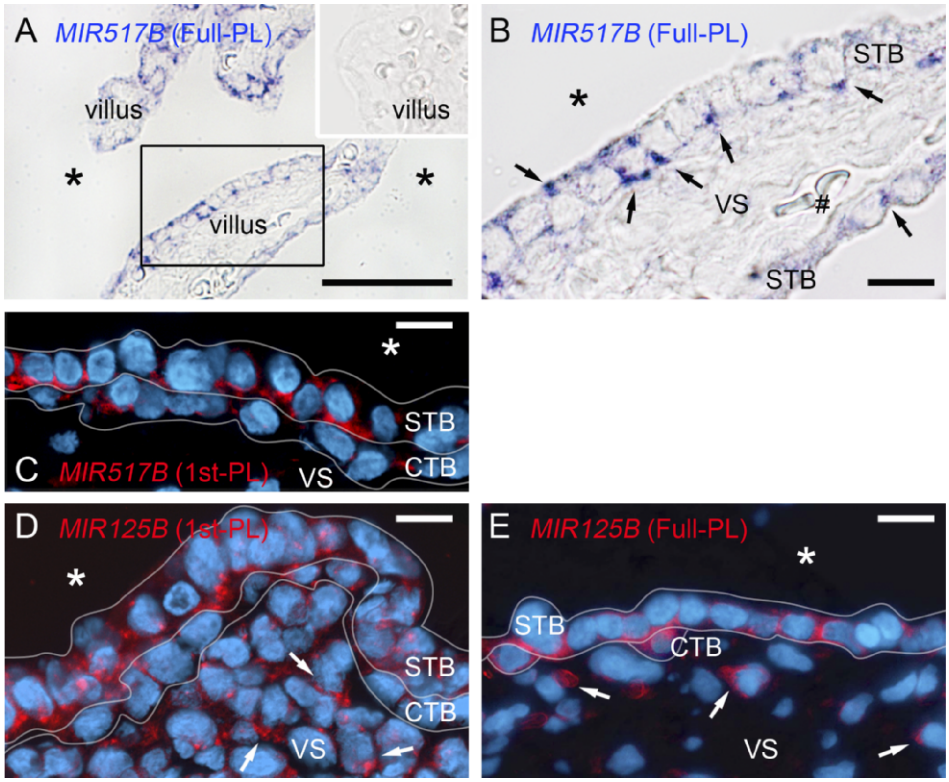

FIG. 3. In vivo localization of *MIR517B* in the human placenta by ISH. **A**) ISH for *MIR517B* in the terminal villi of a full-term placenta (Full-PL). The inset shows the section of a negative control for which a placental section was incubated with a scramble-miR control probe. Intervillous space is indicated (*). **B**) A higher-magnification view of the inset in **A**. Dark purple hybridization signals, indicating *MIR517B*, are present predominantly in the STB layer of the placenta (black arrows). Fetal endothelium (#) in the villous stroma (VS) is evident. **C**) Merged image of the pseudocolored signals, indicating *MIR517B* (red) and DAPI-stained nuclei (blue), in the TV of the first-trimester placenta (1st-PL). The CTB is also positive for *MIR517B* expression. **D** and **E**) In vivo localization of *MIR125B* in the human placenta by ISH. Pseudocolored signals, indicating *MIR125B* (red) and DAPI-stained nuclei (blue), are merged. Unlike *MIR517B*, *MIR125B* signals are observed not only in STBs and CTBs but also in villous stroma cells (white arrows). Bars = 50 μm (**A**) and 10 μm (**B**–**E**).

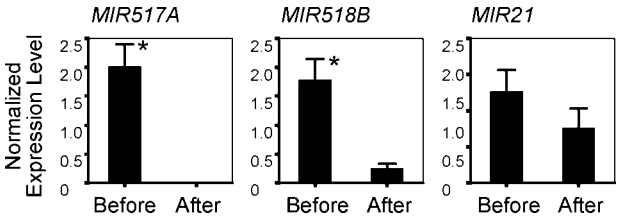

FIG. 4. Real-time PCR analysis of placenta-specific miRNAs (*MIR517A* and *MIR518B*) in maternal blood plasma before and after delivery. Maternal blood plasma was collected 1 day before delivery; postdelivery blood plasma in the same women was also collected 3 days after delivery. For each miRNA, a normalized level (miRNA/*RNU6–2*) in maternal plasma before delivery is assigned a value of 1. Values are the mean ± SE from three measurements. Significant differences in the levels of *MIR517A* and *MIR518B* were detected in the preterm and postterm groups ($*P < 0.05$). *MIR21* is probably derived from many organs.

the placenta, the arborization of the villous trees, and the changes in the uteroplacental circulation).

We focused on two placenta-specific miRNAs, *MIR517A* and *MIR518B*, that were frequently cloned in the maternal plasma of pregnant women (0.79% and 1.11%, respectively, of miRNA genes in the total miRNA clone population in the blood plasma from full-term women [Fig. 2 and Supplemental Table S3]) and examined whether the levels of these miRNAs in maternal plasma reflect the state of pregnancy. As expected, our real-time PCR analysis revealed that the levels of these miRNAs in maternal plasma decreased dramatically after delivery (Fig. 4). Although the expression level of *MIR21* in blood plasma (which was considered to originate from many organs [Fig. 2]) appeared to decrease after delivery, no significant differences in its expression level were detected in the preterm and postterm groups (Fig. 4). Therefore, the placenta-specific miRNAs should be excellent pregnancy-associated markers.

Identification of Novel miRNAs

We next evaluated whether any of our cloned cDNAs represent novel miRNA genes. After comparison with existing gene databases to exclude clones matching known RNAs and those missing in the human genome (Fig. 1), the remaining small RNA clones (424 clones) were screened in silico for the ability of their putative precursor sequences to form thermodynamically stable stem-loop structures. Based on this analysis, 48 clones (16 genes) were identified as novel miRNA candidates. We then examined whether these 16 candidates are associated with EIF2C2, a key component in the RNA-induced silencing complex, to demonstrate that they are indeed novel miRNAs. Six of 16 candidates were specifically detected in the immunoprecipitates with anti-human EIF2C2 antibody from full-term placental lysates (e.g., *miR-PL1* in Fig. 5). Specific bands for the other candidates were undetectable (data not shown). We assigned tentative miRNA names (*mirR-PL1–6*) to the six novel miRNA genes in this study. Table 4 summarizes the data on the novel miRNAs, including sequence information (representative clone sequence, clone count, locus in the human genome, and minimum free energy [ΔG] of the miRNA/miRNA binding-site duplex). We further examined the relative expression levels of the novel miRNAs in representative reproductive organs (Table 5). Four of six novel miRNAs (*miR-PL1*, *miR-PL2*, *miR-PL4*, and *miR-PL6*) were expressed either preferentially or exclusively in the placenta (e.g., *miR-PL1*, *miR-PL2*, and *miR-PL6* in Fig. 5). The other two miRNAs (i.e., *miR-PL3* and *miR-PL5*) were detected in the examined

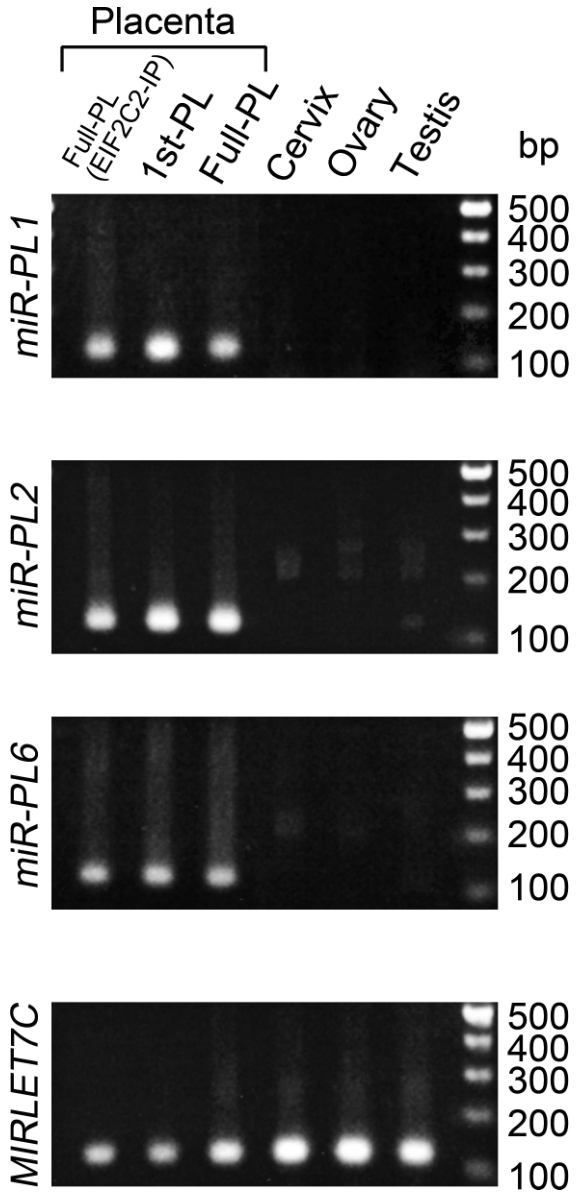

FIG. 5. Analysis of novel miRNAs using EIF2C2 immunoprecipitation (EIF2C2-IP), followed by semiquantitative PCR analysis. A representative gel picture shows the expression levels of *miR-PL1*, *miR-PL2*, and *miR-PL6* in the reproductive organs. *MIRLET7C* was used as a positive control. Sizes of DNA ladders run in each gel are indicated on the right. Novel miRNAs detected in the full-term placenta (Full-PL) by EIF2C2-IP are shown in the left lane of each gel. Data are representative of two independent experiments giving similar results. 1st-PL, first-trimester placenta.

reproductive organs (Table 5). In addition, four of six novel miRNAs were embedded in a primate-specific miRNA cluster on chromosome 19, as identified by Bentwich et al. [27]. After additional analysis for homology for the six novel RNAs using miRBase version 12.0, three of six miRNAs are new (i.e., *MIR1283*, *MIR1185*, and *MIR1323* [Tables 4 and 5]).

Secretion of miRNAs from Human Trophoblast Cells via Exosomes

In correspondence with our results, miRNAs were recently reported to be detectable in human blood serum [28–31]. In particular, the study by Taylor and Gercel-Taylor [31] is

TABLE 4. Novel miRNAs cloned from human placenta.

MIR[a]	Representative sequence (5′-3′)	Chromosome	Start	End	Strand	ΔG	Clones[b] Total	1st-PL	Full–PL-WV	Full-PL-TV
PL1	TCTACAAAGGAAACGCCTTTCTGT	19	58953311	58953334	+	−45.0	21	3	7	8
			58883560	58883581	+	−45.4				
PL2	GAAATCGCTTCCCTTTGGAGTGT	19	58953349	58953371	+	−45.0	9	1	3	4
PL3	AAAGTGCACCCAGTTTTGGGGA	19	58867079	58867100	+	−49.1	2	0	2	0
PL4	AGAGGATACCCTTTGTATGTTCA	14	100580302	100580324	+	−43.2	1	0	0	1
PL5	AGGAACCACACTCAGGGT	1	45044943	45044960	+	−49.6	1	1	0	0
PL6	TCAAAACTGAGGGGCATTTTCT	19	58867044	58867065	+	−49.1	1	0	0	1

[a] Tentative miRNA names (*miR-PL1* through *miR-PL6*) assigned to the six genes in this study.
[b] 1st-PL, first-trimester placenta; Full-PL, full-term placenta; WV, whole villi; TV, terminal villi.

TABLE 5. Expression of the novel miRNAs in reproductive organs and primer information on PCR analysis.

MIR[a]	Expression profiles by PCR[b,c] 1st-PL	Full-PL	Cervix	Ovary	Testis	Primers	Annealing temp. (°C)	PCR cycles	Remark[d]
PL1	4	4	0	0	0	5′-CTACAAAGGAAAGCGCTTTCT-3′	50	30	*MIR1283*
PL2	4	4	0	0	0	5′-AAATCGCTTCCCTTTGGAGTGT-3′	50	30	
PL3	4	4	4	4	4	5′-AAAGTGCACCCAGTTTTGGGGA-3′	50	30	
PL4	2	2	0	0	0	5′-AGAGGATACCCTTTGTATGTTCA-3′	50	30	*MIR1185*
PL5	2	2	1	0	1	5′-AGGAACCACACTCAGGGT-3′	55	40	
PL6	2	2	0	0	0	5′-TCAAAACTGAGGGGCATTTTCT-3′	50	30	*MIR1323*

[a] Tentative miRNA names (*miR-PL1* through *miR-PL6*) assigned to the six genes in this study.
[b] The expression levels of the miRNAs were classified as follows: 4 for high, 3 for medium, 2 for low, 1 for little to none, and 0 for none.
[c] 1st-PL, first-trimester placenta; Full-PL, full-term placenta.
[d] *miR-PL1*, *miR-PL4*, and *miR-PL6* are newly registered as *MIR1283*, *MIR1185*, and *MIR1323*, respectively, in the miRBase current version 12.0.

notable in that it describes the presence of miRNAs in circulating exosomes. We then hypothesized that human chorionic villi, especially the surface-covering STB, could secrete miRNAs extracellularly via exosomes, which in turn enter into maternal circulation. Based on this hypothesis, we performed ISH for *MIR517B* in combination with immunostaining for CD63, a typical marker of exosomes and MVBs [32], in the same section of the human placenta. CD63-positive signals were distributed throughout the STB in the full-term placenta; morphometric analysis using MetaMorph demonstrated that the percentage of the CD63-positive pixel area in the total pixel area of the selected outline of the villous surface (STB) was approximately 60% (data not shown). As shown in Figure 6B, spotted immunostaining for CD63 was observed in the STB layer of the human chorionic villous villi, indicating that STB cells have CD63-positive vesicular structures, which presumably represented MVBs. The localization of CD63-positive signals partly overlapped that of *MIR517B*, supporting our hypothesis that placenta-derived miRNAs are released from the STB via exosomes (Fig. 6). No immunofluorescence signal above background was detected in isotype-matched control sections (data not shown).

Next, as a model system to test the hypothesis in vitro, BeWo trophoblast cells overexpressing CD63 were examined. BeWo cells endogenously expressed CD63, which was localized on intracellular vesicle-like structures (Fig. 7A). The endogenous CD63 protein level of BeWo cells was not so high, albeit detectable by real-time PCR. Therefore, CD63 was overexpressed for this experiment to isolate CD63-positive exosomes efficiently by immunoprecipitation (Fig. 7D). First, the cells were transfected with pEGFP-CD63 together with Alexa Fluor 594-labeled *MIRLET7B*. Forty-eight hours later, localization of EGFP-CD63 was observed in vesicular structures in the cells, which are considered MVBs-containing exosomes to be released (Fig. 7D). Transfected *MIRLET7B* (Fig. 7E) was localized inside the CD63-positive MVBs (Fig. 7F). Although observation of each exosome in the MVBs at a light microscopic level is difficult, this result indicates that

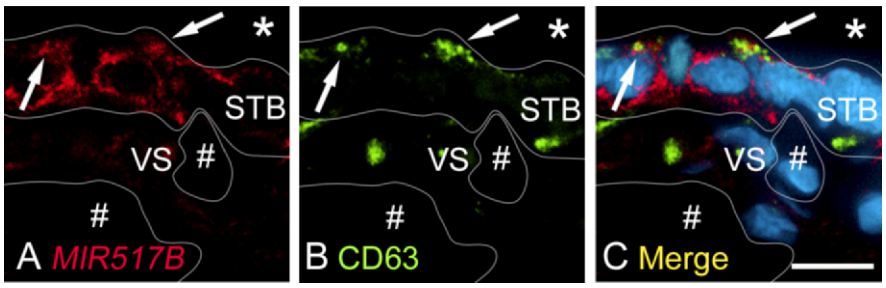

FIG. 6. In vivo localization of *MIR517B* and CD63 in the human placenta. The same section of the full-term human placenta was subjected to ISH for *MIR517B* (**A** [red]) and subsequent immunostaining for CD63 (**B** [green]), a marker of the MVB/exosome. **C**) The merged image of *MIR517B* and CD63 signals with DAPI-stained nuclei (blue). Some *MIR517B* signals are colocalized with CD63-positive compartments (arrows) in the STB. The boundaries of STB and fetal capillaries (#) are indicated by white lines. Intervillous space (*) and villous stroma (VS) are indicated. Bar = 10 μm.

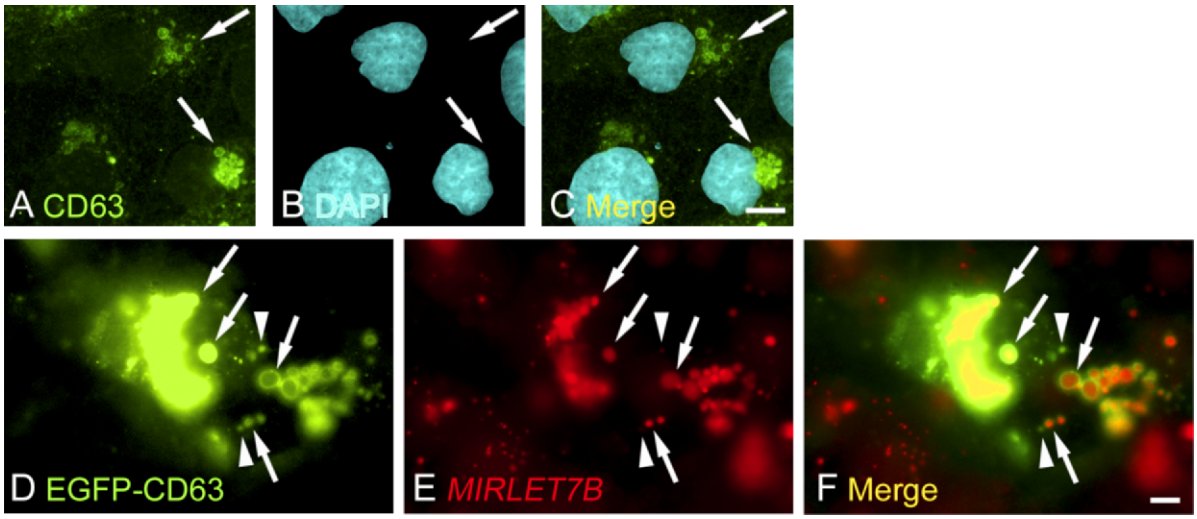

FIG. 7. In vitro localization of *MIRLET7B* and CD63 in trophoblast cells. (**A**–**C**) Endogenous CD63 in BeWo cells. **A**) Immunostaining of CD63. The cells contain CD63-positive vesicular structures (arrows). **B**) DAPI-stained nuclei of the same cells. **C**) The merged image of CD63 and DAPI. **D**–**F**) Alexa Fluor 594-labeled *MIRLET7B* was introduced in BeWo cells together with the expression plasmid of EGFP-CD63 as a marker of the MVB/exosome. **D**) EFGP-CD63-transfected BeWo cells (green). **E**) Exogenously introduced *MIRLET7B* in the same EGFP-CD63-expressing cells (red). **F**) The merged image of CD63 and *MIRLET7B* signals. Some *MIRLET7B* signals are colocalized with CD63-positive compartments (arrows). *MIRLET7B*-negative CD63 compartments are also indicated (arrowheads). Bar = 10 μm.

miRNAs are partially introduced intracellularly and become localized in exosomes in MVBs.

We next concentrated exosomes from the conditioned medium of the BeWo cells in culture and examined if miRNAs were detected in the exosomal fraction. We used placenta-specific *MIR517A* and organ-ubiquitous *MIR21*, as these miRNAs were highly cloned in the human placenta (Table 1). As expected, we detected significantly higher levels of these miRNAs in the exosomal fraction than in negative controls (Fig. 8). Furthermore, when exogenous *MIR517A* was introduced in the cells, the *MIR517A* level in the exosomal fraction, but not that in the negative controls, greatly increased, while the endogenous *MIR21* level was unaffected (Fig. 8). Taken together, these results suggest that miRNAs are indeed secreted from human trophoblast cells via exosomes and that the amount of miRNA in exosomes is correlated with the intracellular level of miRNAs.

Proteome Analysis of MIR517A-Regulated Proteins in BeWo Cells

Our proteome analysis by two-dimensional difference gel electrophoresis resulted in 40 spots with differential intensities between BeWo cells transfected with pre-*MIR517A* and the negative controls. Subsequent mass spectrometry analysis identified 58 proteins as promising candidates of *MIR517A*-regulated proteins, 47 and 11 of which were upregulated and downregulated, respectively (Supplemental Table S4). In contrast, none of these proteins are included in the in silico targets of *MIR517A* as predicted by two online programs, TargetScan (http://www.targetscan.org/) and miRanda (http://microrna.sanger.ac.uk/targets/v5/), raising the suspicion that they are secondarily regulated rather than directly targeted by this miRNA. To validate this result, we examined if *MIR517A* regulated the protein levels of TXN and VDAC1, representative proteins identified in the spots (Supplemental Fig. S2) in BeWo cells by Western blot analysis. As shown in Figure 9, the normalized levels of TXN and VDAC1 were indeed upregulated and downregulated, respectively, by *MIR517A* overexpression in accord with the results of proteome analysis. A network-based analysis using Ingenuity Pathway Analysis suggested that most of the *MIR517A*-regulated proteins were mapped to two networks (Supplemental Fig. S3). The first network contains numerous proteins associated with nuclear factor of kappa light polypeptide gene enhancer in B cells 1 (NFKB1) and MAPK. In contrast, the second network consists of proteins associated with tumor necrosis factor (TNF)-related signaling. Because TNF elicits the activation of both MAPK and NFKB1, accompanied by apoptosis induction in many types of cells, the two networks can be merged (Supplemental Fig. S3). By Western blot analysis, we demonstrated that the phosphorylation of MAPK8/9/10 proteins was attenuated by *MIR517A* overexpression in BeWo cells (Fig. 10), suggesting that *MIR517A* could be associated with the pathway. A recent study [33] demonstrated that overexpression of TXN attenuated cigarette smoke extract-mediated cell death and MAPK8/9/10 activation in human bronchial epithelial cells. Thus, a possible function of *MIR517A* could be to regulate signal transduction mediated by TNF and/or other death ligands, although more detailed studies are necessary.

DISCUSSION

Generation of expression profiles of miRNAs in the human placenta is a prerequisite for a thorough understanding of their roles in placental development. In the present study, we demonstrated miRNA expression profiles of the human placenta. Small RNA library sequencing showed that most placenta-specific miRNAs were linked to a miRNA cluster on chromosome 19. The miRNA cluster genes were upregulated in placental development. Histochemical analysis demonstrated that human chorionic villi, especially the surface-covering STB, expressed *MIR517B*, one of the placenta-specific miRNAs. We next demonstrated the presence of placenta-specific miRNAs in maternal plasma during pregnancy and their rapid clearance from the human plasma after delivery. We inferred from these results that the STB should secrete miRNAs extracellularly via exosomes, which in turn enter into maternal circulation, and then tested the hypothesis using a human trophoblast cell line (BeWo cells).

To our knowledge, this study is the first to show detailed miRNA profiles of human placentas using small RNA library sequencing. The focus of most previous cloning studies for miRNA was the discovery of novel miRNAs rather than the organ-specific profiling of miRNA expression. However, recent cloning-based studies [5, 16] indicate that the relative cloning frequencies of miRNAs represent a measure of miRNA expression. Thus, we used the cloning method as a valuable lead to uncover miRNAs that have important physiological and pathological roles in tissues of interest. Landgraf et al. [5] presented an atlas of mammalian miRNA expression (including that of human placenta) by cloning (1568 miRNA clones for the human placenta). Our cloning results are in good agreement with those by Landgraf et al. [5]. In a comparison between their data and ours, eight of 13 miRNAs highly cloned in this study (i.e., *MIR21*, *MIR23A*, *MIR27A*, *MIR125B*, *MIR143*, *MIR200C*, *MIR424*, and *MIR517A*) are common highly cloned miRNAs in their data (compare Table 1 herein with Table S5 in the study by Landgraf et al. [5]). Moreover, both studies showed the chromosome bias in the distribution of the highly cloned miRNAs in the human placenta; herein, approximately 30%–40% of the highly cloned miRNAs were derived from chromosome 19-containing *MIR506* and *MIR515* family genes, as well as *MIR23* family genes (Table 1). The former family of genes represents a primate-specific miRNA cluster [25]; they are embedded among Alu repeats, a short interspersed nuclear element specific to primates, that are involved in the amplification of the miRNA family genes [34, 35]. Our cloning results are consistent with recent findings that the chromosome 19 miRNA cluster genes are placenta specific [27, 35]. Furthermore, we discovered four new members of the chromosome 19 miRNA cluster; three of four miRNAs were placenta specific (Tables 4 and 5). We also profiled circulating miRNAs in blood plasma to investigate the feasibility that placenta-specific miRNAs are released into maternal circulation (Supplemental Tables S2 and S3). We found pregnancy-dependent variation in the cloning frequency of individual miRNAs detected in blood plasma herein. Taken together, the present findings suggest that changes in the expression of the chromosome 19-linked miRNAs mirror the physiological status of the human placenta. In terms of miRNA expression profiling, the small RNA library sequencing technique used in this study has an advantage over other methods of identifying novel miRNAs (e.g., miRNA microarray is designed to analyze only known miRNAs). Another advantage of this technique is distinguishing the expression of highly homologous miRNAs (e.g., those of the *MIRLET7* family) that sometimes give high background in microarray analysis due to cross-hybridization).

Because our results of miRNA expression profiles of placenta tissue and maternal blood plasma indicate that the STB secretes miRNAs into maternal circulation during pregnancy, the miRNA release pathway was examined using the BeWo cell line as a model of villous trophoblasts. BeWo cells exhibit physiological and morphological characteristics of normal trophoblasts [36]. Other groups have also reported that miRNAs are detected in blood plasma or serum [28–31]. An important question then is how stable are the circulating miRNAs, as RNAs are generally thought to be extremely unstable molecules, which makes reliable and reproducible detection difficult. The study by Taylor and Gercel-Taylor [31] describes the association of miRNAs with circulating tumor-derived exosomes, which possibly prevents miRNAs from degradation. This finding is supported by Valadi et al. [10], who demonstrated that miRNAs and mRNAs are released extracellularly from mast cells via exosomes. Although Chim et al. [28] also reported that placenta-derived miRNAs are detected in maternal plasma, they did not address the question as to whether placental cells indeed release exosomes containing miRNAs. In this study, we successfully demonstrated significant amounts of *MIR517A* and *MIR21* in an exosome-enriched fraction isolated from the culture media of BeWo cells (Fig. 8). This result strongly supports the idea that placenta-derived miRNAs are transferred into maternal circulation in an exosome-mediated manner. Although tumor cells can secrete exosomes, the exosome-releasing cells reported previously are mainly immune cells, including reticulocytes, dendritic cells, B cells, T cells, mast cells, intestinal epithelial cells, and platelets [11, 37]. Our data suggest that trophoblast cells that are not maternal immune cells are capable of releasing exosomes. Although the pathophysiological roles of

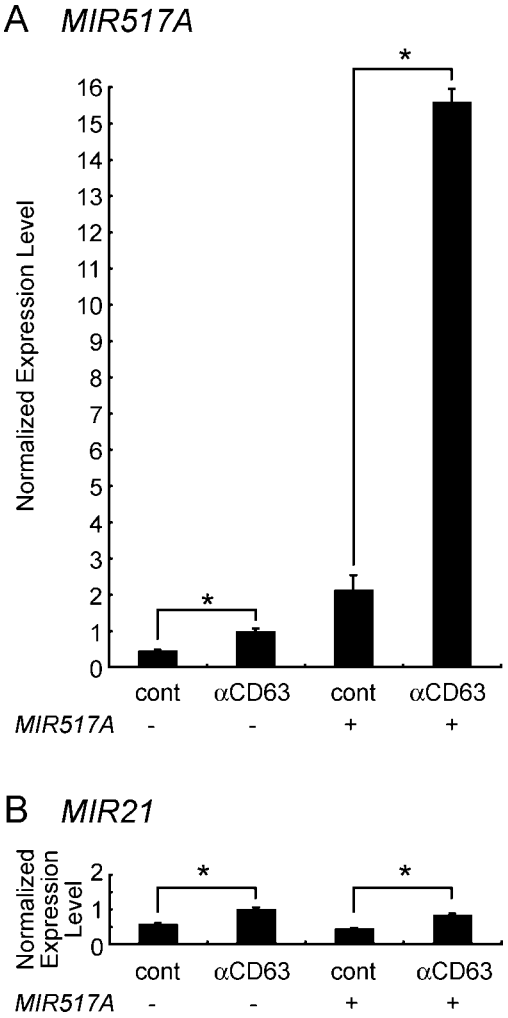

FIG. 8. Extracellular release of miRNAs from trophoblast cells via exosomes. RNAs in exosomes enriched from the conditioned medium of EGFP-CD63-expressing BeWo cells were immunoprecipitated using sepharose 4B beads conjugated with isotype-matched nonimmune IgG (cont) or anti-CD63 (αCD63) antibody. The immunoprecipitates were then subjected to real-time RT-PCR analysis for extracellularly released *MIR517A* (**A**) and *MIR21* (**B**). Plus sign (+) indicates that *MIR517A* was exogenously introduced in EGFP-CD63-expressing BeWo cells. *Saccharomyces cerevisiae* tRNA (sc-tRNA) spiked in each RNA sample was used as a control. A normalized miRNA level (miRNA/sc-tRNA) in the immunoprecipitates with anti-CD63 antibody from EGFP-CD63-expressing BeWo cells without exogenous *MIR517A* is assigned a value of 1. Values are the mean ± SD from three measurements. Significantly high levels of both endogenous and exogenous miRNAs were detected in the exosome-enriched fraction compared with those in the negative controls ($*P < 0.001$).

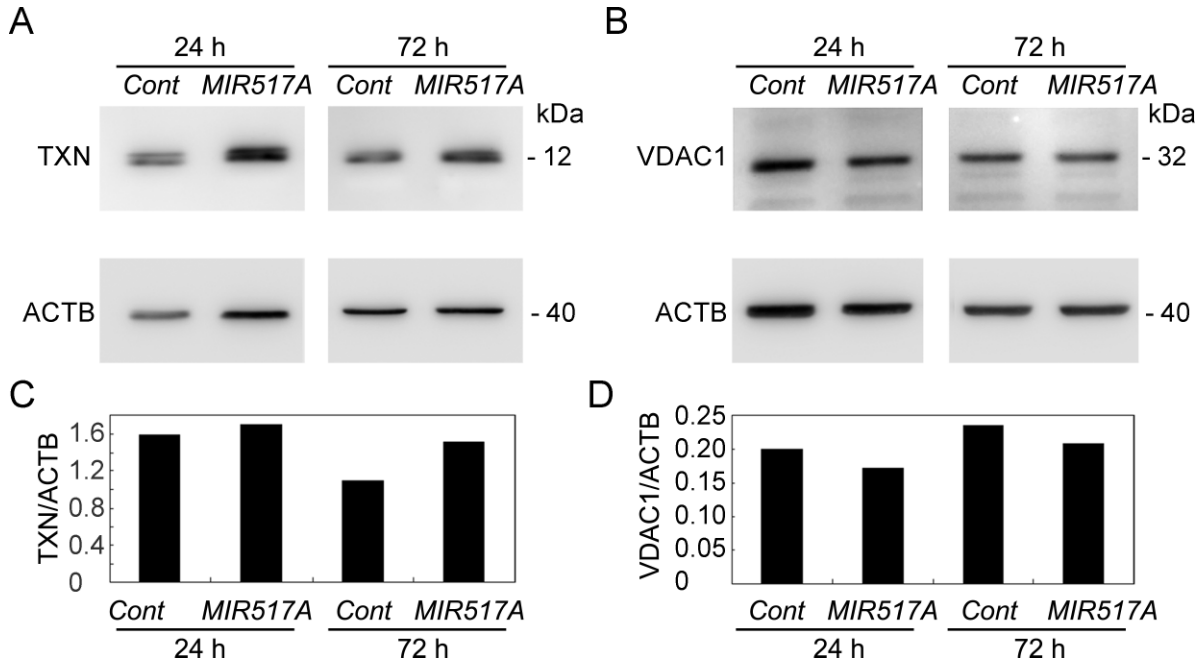

FIG. 9. Western blot analysis for representative proteins identified by the proteome analysis of BeWo cells overexpressing *MIR517A*. Pre-miR *MIR517A* (*MIR517A*)- or Pre-miR Negative Control #1 (*Cont*)-transfected cells were harvested 24–72 h after the initiation of transfection and subjected to proteome analysis. We identified 58 proteins that were upregulated or downregulated in intensity by more than 10% (Supplemental Table S4). TXN (**A**) and VDAC1 (**B**) proteins in the *Cont*- and *MIR517A*-overexpressing BeWo cells were probed as described in *Materials and Methods*. β-actin was also probed on the same blots as an internal control. The relative levels of TXN (**C**) and VDAC1 (**D**) proteins normalized to those of ACTB protein are shown. Protein samples obtained from two independent experiments were subjected to Western blot analysis, which gave similar results.

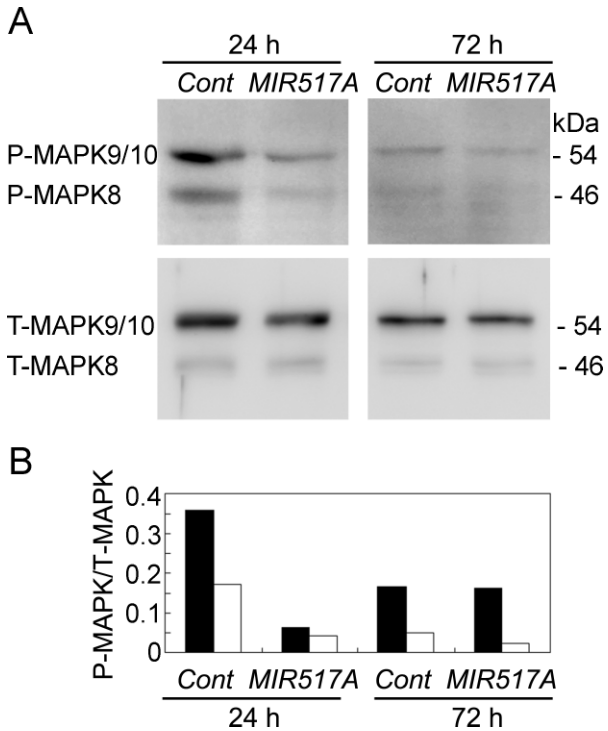

FIG. 10. Attenuation of the phosphorylation of MAPK8/9/10 in BeWo cells by *MIR517A*. Pre-miR *MIR517A* (*MIR517A*)- or Pre-miR Negative Control #1 (*Cont*)-transfected cells were harvested 24–72 h after the initiation of transfection and subjected to Western blot analysis. **A**) Western blot of phosphorylated MAPK8/9/10 (P-MAPK8/9/10) and total MAPK8/9/10 (T-MAPK8/9/10) in the *Cont*- and *MIR517A*-overexpressing BeWo cells. **B**) The protein levels of P-MAPK8 (filled columns) and P-MAPK9/10 (open columns) normalized to those of T-MAPK8 and T-MAPK9/10, respectively, are quantitatively analyzed. Protein samples obtained from two independent experiments were subjected to Western blot analysis, which gave similar results.

exosomes are largely unknown, exosomes are considered to have an important role in cell-cell communication [10, 11, 37]. Valadi et al. [10] demonstrated that mast cell exosomal mRNAs transferred to another cell can be translated in the new location. Therefore, placenta exosomal RNAs may act locally to communicate RNA signaling to adjacent cells and act on distant target cells through the circulation in a similar way that hormones do. Whether placenta exosomal miRNAs have functional roles in fetomaternal immune interaction and maternal homeostasis during pregnancy remains to be determined. In addition, it is likely that circulating placenta-derived miRNAs reflect the physiological status of the human placenta and could be used diagnostically. Pathogenesis is an important issue in the development of diagnostic methods for abnormal pregnancy, including PE, because it threatens both maternal and fetal lives. To explore promising novel biomarkers, we are investigating whether the expression of the chromosome 19 miRNA cluster genes is indeed significantly different between PE and normal blood plasma samples.

We are also interested in the biological functions of the placenta-specific miRNAs in the development and/or maintenance of the placenta. In this study, we performed a proteome analysis by placenta-specific miRNA overexpression using BeWo trophoblast cells. Although we could not detect the in silico targets of *MIR517A* by this analysis, conceivably because of the poor visualization of low-copy number proteins separated by two-dimensional difference gel electrophoresis, our proteome analysis combined with bioinformatics revealed that proteins regulated by the overexpression of *MIR517A* are possibly involved in the process of TNF signaling-mediated events (Supplemental Fig. S3). Tumor necrosis factor signaling through two receptors (i.e., TNFR1 and TNFR2) is known to elicit a variety of biological effects on placental trophoblasts such as apoptosis and inhibition of syncytialization and hormone production [38]; however, it remains to be elucidated in detail how *MIR517A* is involved in the signaling. Zhang et

al. [35] suggested that, because the chromosome 19-linked placenta-specific miRNA family produces variable miRNA genes, the family might lead to functional diversification. Further investigations of the diverse functional roles of the individual miRNA of the family would provide novel insights into molecular mechanisms governing placental development.

In conclusion, we identified placenta-specific miRNAs that are probably continuously and extracellularly released via exosomes from chorionic villous trophoblasts into maternal circulation, where they could target maternal tissues (e.g., maternal endothelium) during pregnancy. Proteome analysis suggests the involvement of *MIR517A*, one of the placenta-specific miRNAs, in the process of TNF signal transduction. Our data provide important insights into miRNA cellular and molecular biology of the human placenta. The investigations of miRNA expression in severe pregnancy disorders (such as PE, intrauterine growth retardation, and hydatidiform moles) could serve as searches for diagnostic biomarkers, and studies are important to define the functional roles of circulating placenta-derived miRNAs.

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