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Abundant expression and hemimethylation of C19MC in cell cultures from placenta-derived stromal cells

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ABSTRACT

MicroRNAs of the chromosome 19 microRNA cluster (C19MC) are known to be abundantly expressed in the placenta. Their genes are located on the long arm of chromosome 19 and seem to be part of a large imprinted region. Although the data available so far suggest important functions in the placenta, no data are available on their general expression patterns in cultures of placenta-derived mesenchymal stromal cells (PDMSC). Surprisingly, qRT-PCR on tissue cultures from first-trimester and term placenta mesenchymal stromal cells showed an abundant expression of the cluster members miR-517a-3p, miR-519a-3p, and miR-520c-3p. Accordingly, analyses of methylation patterns suggested that these cells had escaped methylation and epigenetic silencing, respectively, of the paternal allele. This was confirmed by the results of treatment of chorionic villous stromal cells by the demethylating agent 5-Aza-2′-deoxycytidine. Our results offer clear evidence that, in contrast to what is suggested in previous papers, members of C19MC are highly expressed in PDMSC indicating that their placenta-specific functions are not restricted to the trophoblast.

1. Introduction

For a successful pregnancy the proper development and function of the placenta are of utmost importance. This, in turn, seems to depend partly on the expression of microRNAs (miRNAs) which is implied by the fact that miRNAs in general are particularly abundant in the placenta [1]. Of the whole miRNA population expressed in the placenta the miRNAs of C19MC ("chromosome 19 microRNA cluster") constitute a signification portion [2] and some members of that cluster even belong to the most abundant placental miRNAs. C19MC encodes more than 50 mature miRNAs [3] sharing common seed sequences [4]. The cluster, mapping to chromosomal band 19q13, constitutes the largest human miRNA cluster known so far and is primate-specific [3].

Whereas miRNAs of C19MC have been found to be abundantly expressed in term placenta virtually no expression was observed in other normal adult human tissues [2,3,5] suggesting that its members serve crucial placenta-specific functions. By RT-PCR analysis Zhang et al. [6] detected the expression of 21 out of the 46 pre-miRNAs of C19MC in a human placenta from a 5-week embryo (Carnegie stage 13 or 14 embryo) and *in situ* data indicated that three miRNAs tested (miR-498, miR-516-5p, and miR-520e) were preferentially expressed in the cytoplasm of the syncytiotrophoblast in normal term placenta while being absent from the chorionic villi mesenchymal stroma core. Luo et al. [5] also addressed this issue by performing *in situ* hybridization (ISH) for miR-517b in first-trimester and term placenta chorionic villi. They obtained similar results detecting miR-517b exclusively in the trophoblast layer with dominant signals in the syncytiotrophoblast.

The expression of C19MC is highly correlated with the methylation state of a distal CpG-rich region located about 17.6 kb upstream of the miRNA cluster [7] and regulated by genomic imprinting with only the paternally inherited allele being expressed in the placenta [8]. Interestingly, C19MC maps near another imprinted gene, i.e. *ZNF331* gene [9], which underlies maternal expression [10,11] and may thus belong to a previously unrecognized large imprinted chromosomal domain. Members of

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C19MC have also been found to be expressed in human embryonic stem cells (hESCs) [4,12–14]. However, to the best of our knowledge, no studies on C19MC in cultures of placenta-derived stromal cells have been reported so far. We have thus quantified the expression of three members of C19MC, i.e. miR-517a-3p, miR-519a-3p, and miR-520c-3p in placenta-derived mesenchymal stromal cells. Cultures of these cells that are known to contain mesenchymal stem cells are of great promise in regenerative medicine [15–17].

2. Materials and methods

2.1. Tissue, cell culture and cytogenetic analyses

2.1.1. First-trimester chorionic villi stromal cells

For cell cultures of chorionic villi, first-trimester samples (10–14 weeks of gestation) were collected by transabdominal chorionic villus sampling (CVS) for prenatal cytogenetic diagnosis. Cell cultures were set up following routine methods for establishing cultures of chorionic villi stromal cells as, e.g., outlined by Portmann-Lanz et al. [18] and Yong et al. [19] with a few modifications. A cytogenetic analysis was performed by standard procedures. The surplus cell cultures were donated to this study after informed written consent given by the patients.

2.1.2. Term placenta, chorioamniotic membrane and stromal cells

Samples of a term placenta as well as from the chorioamniotic membrane were obtained after delivery with informed written consent given by the mother. The collected tissues were fixed in 4% formalin and embedded in paraffin. A further sample of placenta was stored in Hank's solution and subjected to cell culture as described above.

2.1.3. Thyroid tumors

A subgroup of thyroid adenomas is shown to overexpress the C19MC due to chromosomal rearrangements [20]. Hence, an adenoma of this subtype served as positive control (S958). In contrast, a cytogenetically normal adenoma was used as a negative control (S925).

The tissue samples were obtained from patients undergoing thyroid resection and immediately stored in liquid nitrogen.

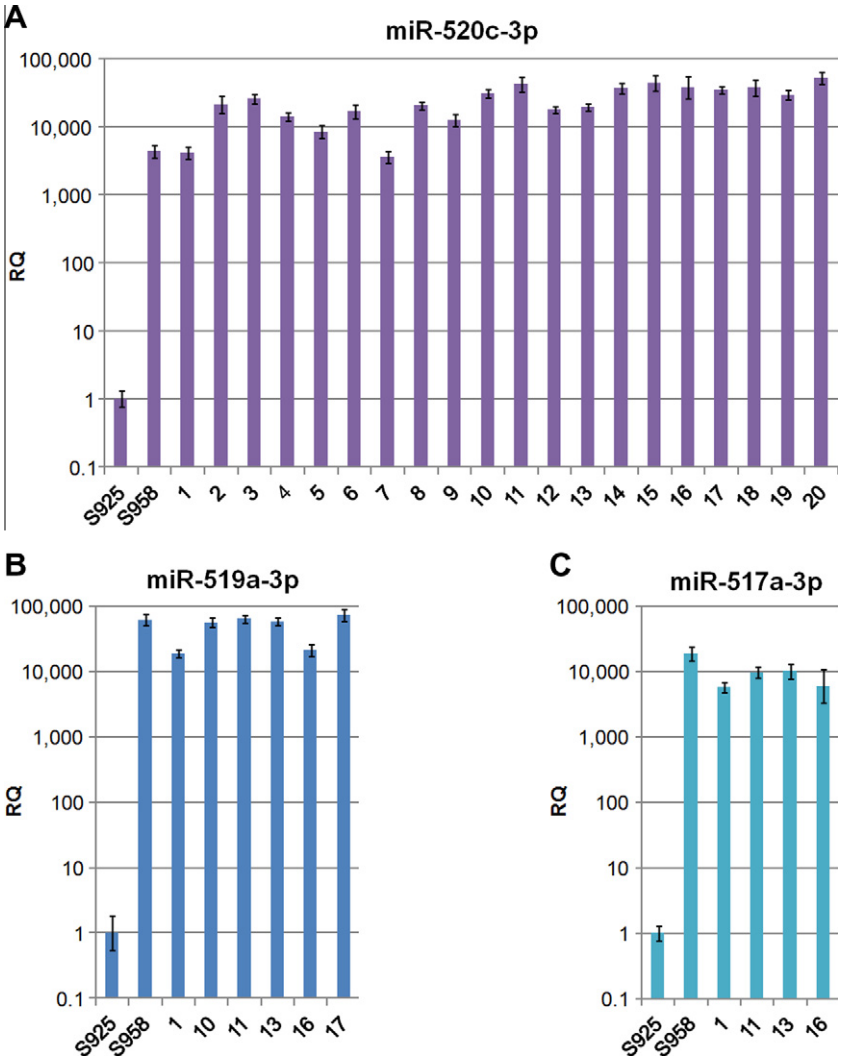

Fig. 1. First-trimester placental villous-derived stromal cells strongly express miR-520c-3p, miR-519a-3p, and miR-517a-3p. Relative expression of miR-520c-3p (A), miR-519a-3p (B), and miR-517a-3p (C) was quantified in first-trimester placental villous-derived stromal cells (1–20). The results obtained are compared with those from a thyroid tumor with 19q13 rearrangement known to overexpress C19MC (positive control S958) and a thyroid tumor without such a rearrangement showing low expression of C19MC (calibrator S925, set 1). RQ: relative quantity (logarithmic scale).

Table 1
Karyotypes and weeks of gestation of first-trimester chorionic villi samples used in this study.

Sample no.	Karyotype	Week of gestation
1	46,XX	11 + 6
2	45,X	12 + 4
3	46,XY	12 + 4
4	47,XY, + mar/46,XY	12 + 5
5	46,XX	14 + 1
6	47,XXX	12 + 0
7	47,XX, + 18	12 + 1
8	47,XY, + 21	12 + 2
9	46,XY	10 + 3
10	46,XY	13 + 2
11	46,XY	13 + 4
12	46,XX	10 + 0
13	46,XY	14 + 2
14	45,XY, + 21	13 + 2
15	46,X	12 + 4
16	46,XY	12 + 4
17	46,XX	10 + 0
18	46,XX	12 + 5
19	45,X	12 + 2
20	47,XX + 21	12 + 5

2.2. RNA isolation, reverse transcription and real-time PCR

Total RNA from cell culture and frozen samples was isolated using QIAGEN miRNEasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

Total RNA isolation from formalin-fixed paraffin-embedded (FFPE) samples was carried out using the innuPREP Micro RNA Kit (Analytik Jena AG, Jena, Germany) for RNA isolation according to the manufacturer's instructions with the following modifications: Lysis of the paraffin sections preceding RNA isolation was conducted using TLS-Lysis Solution and Proteinase K from the innuPREP DNA Micro Kit (Analytik Jena AG, Jena, Germany) without prior deparaffinization. Sections were incubated for 1 h at 60 °C and 15 min at 80 °C.

To quantify the expression of miR-520c-3p, miR-519a-3p, and miR-517a-3p reverse transcription and real-time PCR were performed as described previously [20] using TaqMan microRNA RT Kit, microRNA assays and Universal PCR Master Mix (Life Technologies Corporation, Carlsbad, USA). 200 ng of total RNA were used for reverse transcription. Relative quantity (RQ) was calculated using the ΔΔCt method [21]. RNU6B served as endogenous control for normalization.

2.3. DNA methylation analysis

To determine the methylation status of the CpG island upstream of C19MC DNA was isolated from one sample of cultured first-trimester chorionic villi cells (sample 10) using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Two micrograms of DNA was then subjected to bisulphite conversion using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, USA) following the manufacturer's protocol. PCR was performed using ZymoTaq DNA Polymerase (Zymo Research, Irvine, USA) with the following conditions: initial denaturation for 10 min at 95 °C, then 35 cycles of 95 °C for 30 s, 51 °C for 30 s and 72 °C for 30 s, then followed by a final elongation phase of 7 min at 72 °C. The primers (biomers, Ulm, Germany) used were AAGGTTGGTTTTTTTATTTGTAAA (forward primer) and ACAAATTCTAATCCCTCAAAAA (reverse primer). The PCR product was then cloned into pGEM-T Easy vector (Promega, Mannheim, Germany) and 20 clones were sequenced.

2.4. Treatment of cell cultures with the DNA methyltransferase inhibitor 5-Aza-2′-deoxycytidine

A first-trimester chorionic villi cell culture (case 3) was treated with 2.5 μM 5-Aza-2′-deoxycytidine (5-Aza-dC) in culture media for 72 h. An untreated cell culture of the same case served as control.

3. Results

3.1. Abundant expression of miR-520c-3p, miR-519a-3p, and miR-517a-3p in stromal cells from first-trimester chorionic villi

Since so far no studies on C19MC in cultures of placenta-derived stromal cells have been conducted we have quantified the expression of three members of C19MC, i.e., miR-520c-3p, miR-519a-3p, and miR-517a-3p in first-trimester chorionic villi-derived mesenchymal stromal cells.

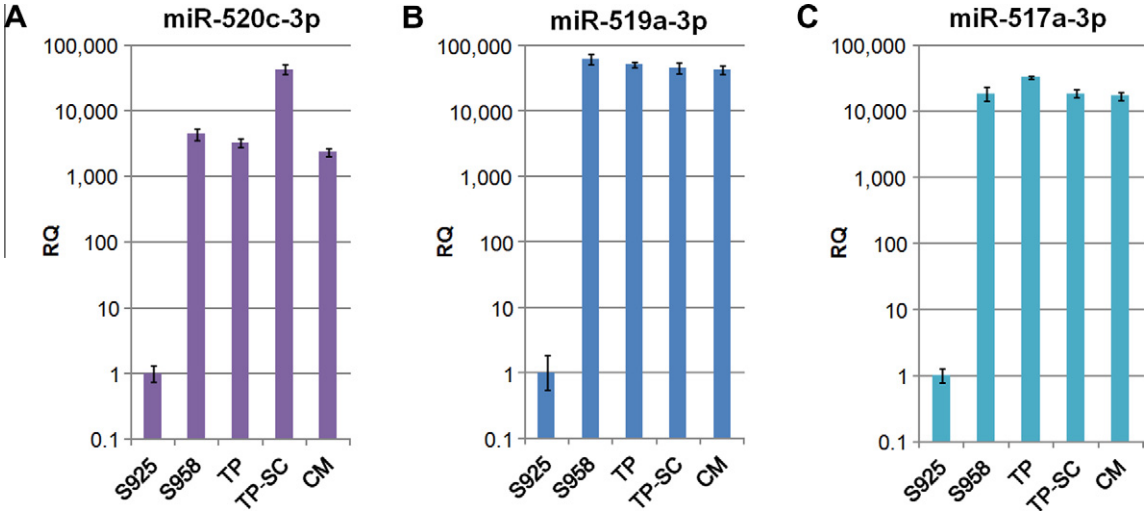

Fig. 2. Term placenta-derived stromal cells strongly express miR-520c-3p, miR-519a-3p, and miR-517a-3p. Relative expression of miR-520c-3p (A), miR-519a-3p (B), and miR-517a-3p (C) was quantified in term placenta-derived stromal cells (TP-SC) as well as in native term placenta (TP) and chorioamniotic membrane (CM). The results obtained are compared with those from a thyroid tumor with 19q13 rearrangement known to overexpress C19MC (positive control S958) and a thyroid tumor without such a rearrangement showing low expression of C19MC (calibrator S925, set 1). RQ: relative quantity (logarithmic scale).

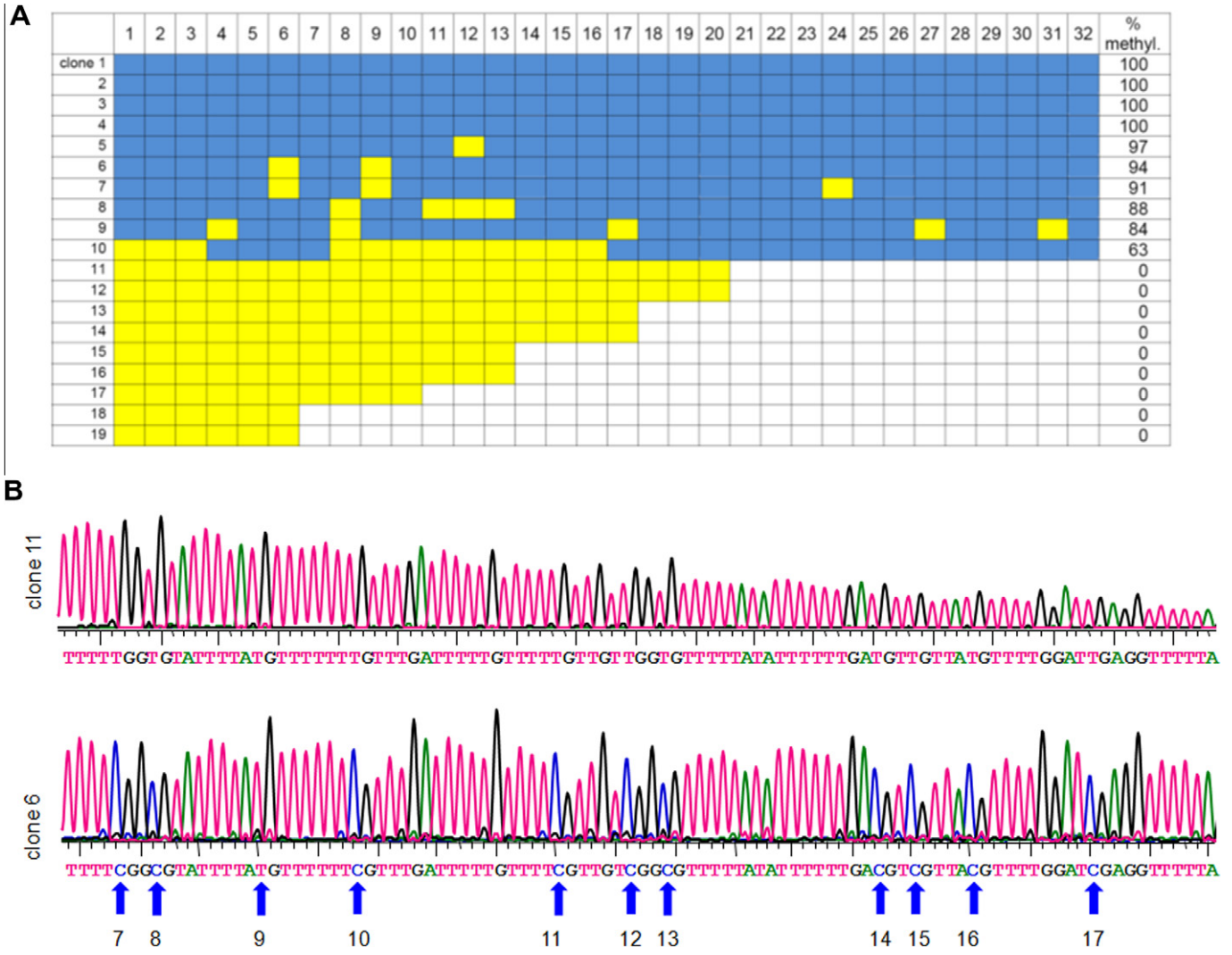

Fig. 3. Hemi-methylation of the CpG island regulating the expression of C19MC in first-trimester placental villous-derived stromal cells. Genomic DNA isolated from first-trimester placental villous-derived stromal cells (case 10) was bisulphite treated and part of the C19MC CpG island amplified by PCR. The PCR product was cloned and 20 clones were sequenced. (A) Diagram showing the methylation pattern of 19 clones analyzed. Methylated sites are represented by blue boxes and unmethylated sites by yellow boxes. White boxes: sites not analyzed. In roughly one half of the clones at least 84% of the sites were found to be methylated. (B) As an example parts of the chromatograms of two different clones are shown. The lower chromatogram shows the sequence of a methylated clone, the upper one the sequence of an unmethylated clone. Blue arrows indicate the potential methylation sites. Numbers below the arrows indicate the CpG sites as in (A).

Long-term chorionic villi cell cultures are considered to consist of only mesenchymal cells as they originate from the villous mesenchymal core [22]. They have been shown to be free of trophoblast cells [19] and the cells obtained from long-term chorionic villi cell cultures display typical characteristics of mesenchymal stem cell (MSCs) [18,23]. Expression of miR-520c-3p was quantified in 20 long-term cell cultures from first-trimester chorionic villi between 10 and 14 weeks of gestation (Fig. 1 A, Table 1). In addition, miR-519a-3p and miR-517a-3p were quantified in six and four of these samples, respectively (Fig. 1B and C).

In all samples of chorionic villi stromal cells the expression levels of the three miRNAs miR-520c-3p, miR-519a-3p, and miR-517a-3p were comparable to those observed in the positive control S958. In case of miR-520c-3p the expression in most samples even clearly exceeded that of S958.

3.2. Abundant expression of miR-520c-3p, miR-519a-3p, and miR-517a-3p in stromal cells from term placenta

To check whether the high expression of C19MC in chorionic villi derived stromal cells is restricted to early pregnancy or is maintained throughout pregnancy we next quantified the expression of miR-520c-3p, miR-519a-3p, and miR-517a-3p in term placenta-derived mesenchymal stromal cells (Fig. 2A–C). The expression levels of miR-519a-3p and miR-517a-3p were comparable to those observed in the positive control S958 and also to the expression levels of native term placenta tissue obtained from the same placenta. The expression of miR-520c-3p in cultured stromal cells again exceeded that of S958 and native term placenta. We also observed a comparably strong expression of all miRNAs in chorioamniotic membrane.

3.3. In stromal cells from first-trimester chorionic villi the imprinting patterns are identical to those previously observed in term placentae

Since the expression of C19MC is known to depend on the methylation of an upstream CpG island [7,8] we were interested to see if this island shows a heterozygous methylation pattern in the stromal cells as well. We thus isolated DNA from one of the samples of stromal cells from first-trimester chorionic villi also used for miRNA quantification (case 10). After bisulphite modification, PCR amplification, and cloning 20 clones were sequenced. Of

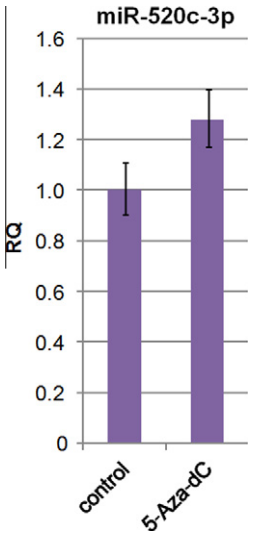

Fig. 4. Treatment of first-trimester placental villous-derived stromal cells with 5-Aza-dC leads to a slight increase of the expression of miR-520c-3p. First-trimester placental villous-derived stromal cells were treated with 2.5 μM 5-Aza-2′deoxycytidine (5-Aza-dC) for 72 h. The relative expression of miR-520c-3p was quantified and compared to that of the untreated control. RQ: relative quantity.

these, nine clones revealed methylation of 84 to 100% of CpG sites analyzed. One clone showed methylation of 63% of the CpG sites analyzed. Of the remaining clones nine did not show evidence for methylation of the analyzed sites (Fig. 3) and one clone was inadequate for analysis. This pattern reflects a 1:1 ratio of methylated to unmethylated clones.

To further confirm this finding we used 5-Aza-2′-deoxycytidine (5-Aza-dC) to demethylate CpG sites of a sample of stromal cells (case 3) and measured the expression of miR-520c-3p after 72 h of treatment with 5-Aza-dC. Given the fact that these cells, as was demonstrated above, possess one unmethylated allele which presumably gives rise to the high expression of miR-520c-3p (cf. Fig. 1A), the demethylation of the second allele can at most cause a twofold increase in expression. As expected the treatment of the cells with 5-Aza-dC for 72 h resulted in a slight increase (1.3fold) of the expression of miR-520c-3p as compared to the untreated control (Fig. 4).

4. Discussion

This is the first report aimed at a detailed analysis of the expression of miRNAs of C19MC in cultures of placenta-derived stromal cells and at the regulation of their expression.

Compared to other organs the human placenta displays unique characteristics of invasive growth and modulation of the mother's immune system to tolerate the semi-allogenic embryo. In addition, it is a rich source of mesenchymal stem cells. Recently, research into the expression of miRNAs in the placenta has attracted a lot of interest. C19MC located on the long arm of chromosome 19 harbors numerous of these miRNA genes whose regulation underlies epigenetic silencing in almost all adult tissues. The miRNAs of this cluster not only share common seed sequences [4] but are also supposed to originate from a common ancestor, i.e., a member of the *miR-371-3* cluster [6] that maps in close proximity to C19MC. Previous papers showed a placenta-specific expression of all tested C19MC genes and, more specifically, in the trophoblast. Herein, we were able to demonstrate abundant expression of C19MC members in cultures of stromal cells of first-trimester as well as term placentae. Apparently, the high expression results from a methylation pattern as described for native placental tissue with a methylated maternal allele and an unmethylated paternal allele [8]. As the most likely explanation, these cells seem to escape epigenetic silencing. As an alternative explanation, demethylation of the regulatory CpG island [7] may occur as a result of the cell culture conditions. A similar mechanism has recently, e.g., been described to be responsible for the upregulation of miR-519d, another member of C19MC, in hepatocellular cancer [24]. However, these tumors revealed gradual undermethylation of the respective genomic region. In contrast, the placenta stromal cells displayed a methylation pattern clearly distinguishing between two alleles (methylated/unmethylated) speaking in favor of the former hypothesis. Accordingly, demethylation by 5-Aza-dC leads to moderate effects only because one completely unmethylated allele already results in an abundant expression of the miRNAs from C19MC. Thus, the maximal effect that could be expected would be a twofold increase of the expression. In contrast, 5-Aza-dC treatment of cells characterized by two-allele methylation results in a strong increase of expression [25].

Some C19MC's miRNAs were classified as so-called oncomiRs because of their association with invasiveness and metastazation. As to the mechanisms explaining this association suppression of CD44 has been discussed [26,27]. Certainly, both functions fit well with a role of this cluster in the human placenta in particular during early pregnancy. Trophoblast invasion of the decidua and the myometrium has to occur early during pregnancy (for review see [28]). Simultaneously, the size of the placenta grows dramatically accompanied by strong proliferation of the villi. Similar functions supporting proliferation and invasiveness have also been described for miR-520c-3p, one of the members of C19MC analyzed herein in detail. Furthermore, Liu and Wilson [29] were able to show that along with miR-373 miR-520c efficiently suppresses the translation of SIRT1 and mTOR thus activating the Ras/Raf/MEK/Erk signaling pathway. On the other hand a strong overexpression resulting from clonal chromosomal rearrangements is a recurrent finding in adenomas of the thyroid [20] and there is no evidence that corresponding tumors are at higher risk to undergo malignant transformation. Directly conflicting data to those associating miR-520c-3p with metastazation [26] have been reported quite recently by Keklikoglou et al. [30] who showed a metastasis-suppressive role of miR-520/373 attributed to direct suppression of TGFBR2.

However, apparently many of the functions of C19MC still remain to be elucidated.

Of note, in a recent paper by Donker et al. [31] it was shown that C19MC miRNAs are the predominant miRNA species expressed in trophoblast-derived exosomes. Therefore, it seems tempting to speculate that at least some of its miRNAs do not exert their effects in the placenta cells but in target cells of exosomes secreted by the trophoblast and stromal cells of the villi. The trophoblast is known to "secrete" a high amount of exosomes that seem to serve important functions during pregnancy, e.g. placenta-derived exosomes (PDE) have been shown to interact with immune cells thus numbing the immune response [32]. Accordingly, miRNAs of C19MC when packed into exosomes may serve important functions in the feto-maternal communication not restricted to the trophoblast.

In summary, the preferential expression of members of C19MC in cultures of placenta-derived stromal cells suggests important functions in this organ. Its miRNAs may serve quite different functions depending as to whether they act in the cells they are expressed in or in target cells of exosomes they release.

Acknowledgments

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