European Journal of Human Genetics (2017) 25, 935–945

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ARTICLE

New insights into the imprinted *MEG8*-DMR in 14q32 and clinical and molecular description of novel patients with Temple syndrome

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The chromosomal region 14q32 contains several imprinted genes, which are expressed either from the paternal (*DLK1* and *RTL1*) or the maternal (*MEG3*, *RTL1as* and *MEG8*) allele only. Imprinted expression of these genes is regulated by two differentially methylated regions (DMRs), the germline *DLK1*/*MEG3* intergenic (IG)-DMR (*MEG3*/*DLK1*:IG-DMR) and the somatic *MEG3*-DMR (*MEG3*:TSS-DMR), which are methylated on the paternal and unmethylated on the maternal allele. Disruption of imprinting in the 14q32 region results in two clinically distinct imprinting disorders, Temple syndrome (TS14) and Kagami-Ogata syndrome (KOS14). Another DMR with a yet unknown function is located in intron 2 of *MEG8* (*MEG8*-DMR, *MEG8*:Int2-DMR). In contrast to the IG-DMR and the *MEG3*-DMR, this somatic DMR is methylated on the maternal chromosome and unmethylated on the paternal chromosome. We have performed extensive methylation analyses by deep bisulfite sequencing of the IG-DMR, *MEG3*-DMR and *MEG8*-DMR in different prenatal tissues including amniotic fluid cells and chorionic villi. In addition, we have studied the methylation pattern of the *MEG8*-DMR in different postnatal tissues. We show that the *MEG8*-DMR is hypermethylated in each of 13 non-deletion TS14 patients (seven newly identified and six previously published patients), irrespective of the underlying molecular cause, and is always hypomethylated in the four patients with KOS14, who have different deletions not encompassing the *MEG8*-DMR itself. The size and the extent of the deletions and the resulting methylation pattern suggest that transcription starting from the *MEG3* promoter may be necessary to establish the methylation imprint at the *MEG8*-DMR.
European Journal of Human Genetics (2017) **25,** 935–945; doi:10.1038/ejhg.2017.91; published online 21 June 2017

INTRODUCTION

Temple syndrome (TS14, OMIM #616222) and Kagami–Ogata syndrome (KOS14, OMIM #608149) are two clinically distinct disorders that are caused by genetic and epigenetic alterations in the imprinted domain on chromosome 14q32. TS14 is characterized by low birth weight and length, muscular hypotonia and feeding difficulties, motor delay, premature puberty and short stature.[1,2] KOS14 patients show a much more severe phenotype early in life with the most prominent sign being a bell-shaped thorax with coat hanger-like ribs. This is often associated with respiratory insufficiency and feeding difficulties leading to an increased lethality early in life. Other phenotypic signs include placentomegaly and polyhydramnios, developmental delay and dysmorphic features with full cheeks, a broad nasal bridge and a protruding philtrum.[3]

The molecular causes of TS14 and KOS14 include uniparental disomies (UPDs), imprinting defects (IDs) and deletions affecting the chromosomal region 14q32.[3,4] This region harbours a cluster of imprinted genes that are either expressed from the paternal allele only, like *DLK1* and *RTL1* or only expressed from the maternal allele like *MEG3*, *RTL1as*, *MEG8*, as well as a sno- and a microRNA gene cluster (Figure 1).[5] The imprinted expression of these genes is regulated by two differentially methylated regions (DMRs) that act as imprinting control regions: The intergenic (IG)-DMR (*MEG3*/*DLK1*:IG-DMR[6]), which is located between *DLK1* and *MEG3*, and the *MEG3*-DMR (*MEG3*:TSS-DMR) located in the promotor region of the *MEG3* gene.[7,8] Recently, Court and colleagues identified a third DMR within intron two of the *MEG8* gene (*MEG8*:Int2-DMR[9]). In contrast to the IG- and the *MEG3*-DMR which are methylated on the paternal and unmethylated on the maternal allele, the *MEG8*-DMR shows an opposite pattern and is methylated on the maternal allele. The IG-DMR is a primary DMR where the methylation is set already in the germline.[10] It could be shown that it governs the secondary *MEG3*-DMR in a hierarchical fashion.[11,12] Both the *MEG3*- and the *MEG8*-DMR are secondary DMRs where the methylation imprint is

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Received 21 December 2016; revised 4 April 2017; accepted 25 April 2017; published online 21 June 2017

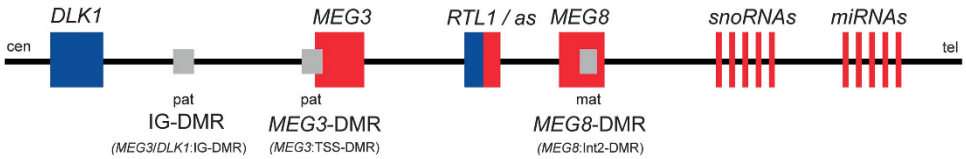

Figure 1 Overview of the imprinted region on chromosome 14q32. The figure shows in red the maternally only and in blue the paternally only expressed genes. The differentially methylated regions (DMRs) are represented by grey squares. The methylated allele is indicated below by pat (paternally methylated) and mat (maternally methylated), repectively. Tel, telomeric; cen, centromeric. Not drawn to scale. Modified from Beygo *et al.*[11]

set postzygotically.[6,12] To the best of our knowledge, the exact time of the methylation setting is known in mice, but not in humans.[13]

TS14 and KOS14 belong to the group of rare imprinting disorders. Up to now, only ~94 patients with TS14 and ~67 with KOS14 have been reported so far (TS14 reviewed in, Ioannides *et al.*[1] KOS14 reviewed in, Ogata *et al.*[3] additional patients described in Supplementary Information). Interestingly, of this group only 15 TS14 and 8 KOS14 patients have been reported to have a primary ID.

In the study at hand we describe eight new patients with TS14 due to different molecular alterations, including deletions, UPD and four patients with a primary ID increasing the number of TS14 ID patients considerably.

Furthermore we conducted extensive methylation analyses of all three DMRs on chromosome 14q32 in pre- and postnatal tissues by deep bisulfite sequencing. For the newly described *MEG8*-DMR we analysed the methylation in a relatively large cohort of 15 TS14- and 6 KOS14-patients (three of them familial cases with affected siblings) with different molecular defects including the patients reported here for the first time. The results yield new insights into tissue-specific methylation and the establishment of the methylation imprint of the *MEG8*-DMR.

MATERIALS AND METHODS

Samples

All samples were obtained after written informed consent.

Seven of the 15 TS14 and all of the KOS14 patients have been described previously: TS patient 1 in Mitter *et al.*[14] (as patient 5), in Bena *et al.*[15] (as patient 3) and Buiting *et al.*[16] TS patient 4 in Mitter *et al.*[14] (as patient 2); TS patient 6 in Mitter *et al.*[14] (as patient 9); TS patient 10 in Mitter *et al.*[14] (as patient 6) and in Bena *et al.*[15] (as patient 4); TS patient 11 in Bena *et al.*[15] (as patient 1); TS patient 12 in Mitter *et al.*[14] (as patient 10); TS patient 13 in Bens *et al.*[17] (as TS3); KOS patient 1 in Irving *et al.*[18] KOS patients 2, 4 and 5 in Beygo *et al.*;[11] KOS patient 3 in Kagami *et al.*[12] and Beygo *et al.*;[11] KOS patients 6–9 in van der Werf *et al.*[19] Information regarding the newly described patients has been submitted to the LOVD (patient IDs: #00100510, 00100511, 00100513, 00100514, 00100516 - 00100519; http://databases.lovd.nl/shared/individuals).

Sets of postnatal tissues were derived from persons who consented to donate their body to research. The individuals investigated in this study were a 44 year old male (individual 1, I1) and a 45-year-old female (individual 2, I2) who both died of severe heart attacks. Individual 2 was informative for a SNP (C>G rs10135782) in the amplicon within the *MEG8*-DMR. Additional brain samples were obtained from a 74-year-old female (individual 3, myocardial infarction), a 79-year-old male (individual 4, heart failure), a 4-month-old female (sinus arrest) and a previously described fetal brain sample.[20]

Chorionic villi (CVS) samples 1–3 were taken around week 11–12 of gestation and amniotic fluid (AF) samples 1–3 around week 15–18 of gestation. AF samples 1–3 were cultured prior to DNA retrieval. One naive amniotic fluid sample (sample 4) was taken at week 20 of gestation. Placenta samples were obtained from the Institute of Molecular Biology/Department of Gynecology and Obstetrics, University Hospital Essen, Germany after informed consent.[21] First trimester placenta samples (Placenta 1–3) were taken at week 9, 10 and 11 of gestation, respectively, after abortions that were not medically indicated. Third trimester placenta samples (Placenta 4 and 5) were taken after the birth of healthy babies at week 36 and 37 of gestation. Samples were taken from the middle part of the term placentas, were washed three times with PBS to remove residual blood and then snap frozen at −80 °C.

DNA extraction

DNA was extracted using the Flexigene Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol for the different tissue types. Maternal contamination was excluded in all fetal samples (CVS, AF and placenta) using the PowerPlex 16 System (Promega, Mannheim, Germany) prior to the subsequently described analyses. DNA was extracted from naive CVS samples.

Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA)

Gene dosage and methylation analyses of the chromosomal region 14q32 including the *MEG3*-DMR were carried out using the SALSA MLPA Kit ME032-X1 or A1 (MRC Holland, Amsterdam, The Netherlands) according to the manufacturer's manual. Amplification products were analysed on an ABI3130XL capillary sequencer (Applied Biosystems, Darmstadt, Germany) followed by data analysis with the Gene Marker Software (Softgenetics, State College, PA, USA).

DNA methylation analysis of the *MEG8*-DMR, *MEG3*-DMR and IG-DMR by next generation bisulfite sequencing

Bisulfite treatment of DNA was performed with the EZ DNA Methylation-Gold Kit (Zymo Research Europe, Freiberg, Germany) according to the manufacturer's instructions. For each sample, bisulfite amplicon libraries were generated and tagged with sample-specific barcode sequences. The amplicons were purified, diluted and clonally amplified in an emulsion PCR before sequencing on the Roche/454 GS junior system (Branford, CT, USA). A detailed description has been published previously.[22] Primer sequences are listed in Supplementary Table 1. For subsequent data analysis the Amplikyzer software was used.[21] Only reads with a conversion rate over 95% were considered.

Microsatellite analysis

Microsatellite analysis for chromosome 14 was performed applying standard protocols. Fluorescence-tagged PCR products were analysed on an ABI3130XL capillary sequencer (Applied Biosystems) and the GeneMarker software (Softgenetics).

RESULTS

Patients

Clinical features of all 15 TS14 patients are summarized in Table 1 (seven which have already been published and eight described here for the first time). Detailed clinical descriptions of the new identified patients are given in the Supplementary Information and Supplementary Figure 1.

Table 1 Clinical features of the TS14 patients

	TS 1	*TS 2*	*TS 3*	*TS 4*	*TS 5*	*TS 6*	*TS 7*	*TS 8*	*TS 9*	*TS 10*	*TS 11*	*TS 12*	*TS 13*	*TS 14*	*TS 15*
m/f	F	F	M	F	M	M	M	M	F	M	F	M	F	F	F
Described in	Mitter *et al.*[14] (patient 5), Buiting *et al.*[16] (patient 3)	This study	This study	Mitter *et al.*[14] (patient 2)	This study	Mitter *et al.*[14] (patient 9)	This study	This study	This study	Mitter *et al.*[14] (patient 6), Buiting *et al.*[16] (patient 4)	Buiting *et al.*[16] (patient 1)	Mitter *et al.*[14] (patient 10)	Bens *et al.*[17] (TS3)	This study	This study
Molecular diagnosis	1.1 Mb deletion	1.1 Mb deletion	UPD(14) mat rob(13;14)	UPD(14)mat rob(13;14)	UPD(14)mat, small supernumerary marker chromosome	UPD(14)mat	ID	ID	ID	ID	ID	ID/UPD	ID	ID mosaic	UPD(14)mat
Iugr	+	–	+	+	ND	+	+	–	–	+	+	mild	+	–	
Low birth weight	+	–	+	+	ND	+	+	–	–	+	+	+	+	–	+
Low birth lenght	+	–	+	–	ND	+	–	–	–	+	+	–	–	–	+
Birth head circumference	Normal	Normal	Normal	Normal	ND	Mild microcephalic	Normal	Normal	Normal	Low-normal	ND	Normal	ND	Normal	Normal
Reduced fetal movement	+	+	ND	–	ND	+	ND	ND	–	–	ND	ND	ND	ND	
Hypotonia	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Feeding problems	+	+	+	+	+ (tube feeding 3days)	–	+	+	+ (partial tube feeding)	+	+	ND	+	+ (tube feeding for 4–5 weeks)	
Head circumference	normal	low-normal	normal	low normal	normal	ND	Microcephaly	Low–normal	Normal	Normal	Microcephaly	ND	Low normal	Normal	Normal
Microcephaly	–	–	–	(+) resolved	–	ND	+	(–)	–	–	+	ND	(–)	–	–
Facial dysmorphism															
Frontal bossing / prominent forehead	+	–	+	–	–	ND	+	+	+	(+)	–	ND	ND	–	–
Short philtrum	–	–	–	mild	–	+	–	+	ND	mild	–	ND	ND	mild	mild
Broad nose	–	+	–	+	(+)	–	–	+	ND	mild	–	ND	ND	+	mild
Depressed nasal bridge	–	+	(–)	–	ND	ND	+	+	ND	–	–	ND	ND	ND	ND
Almond–shaped eyes (Epikanthus)	–	–	–	–	'+/–	+	-	+	ND	+	–	+	ND	+	ND
Micrognathia	–	+/–	+	–	mild	–	+	mild	+	–	–	+	+	–	–
High palate	–	+	–	–	+	+	+	+	+	–	–	+	ND	+	ND
Anteverted nares	–	–	–	–	–	–	+	+	ND	–	–	ND	ND	–	–
Clinodactyly	–	–	–	–	ND	ND	+	–	ND	ND	ND	ND	ND	+	–
Short stature	+	+	(+)	+	+	–	+	+	+	+ (mild)	+	ND	+	–	+
Obesity	–	–	too young	+	+	+	too young	–	+	+	+	ND	+	+	+
Small hands / feet	+	–	–	+	+	–	+	+	+	+	+	ND	+	–	+
Motor delay	+	+	+	+	+	ND	+ (slightly)	+	–	+	+	+	+	ND	normal
Intellectual disability	+	+	too young	–	–	learning disability	–	too young	–	–	no	ND	ND	–	+ (slight)
Premature puberty	+ (11 years)	+ (5 years)	too young	+	+	ND	too young	too young	+ (9–10 years)	+ (11 years)	+ (10 years)	+	+ (9 years)	+	+ (11 years)
Accelerated bone age	+	+	ND	+	ND	ND	–	ND	ND	+	ND	ND	+	+	ND
Other features				hyperextensible joints	cryptorchidism	hypogonadism myopia	bilateral cryptorchidism unilateral inguinal hernia	hypoglycemia as a newborn	hyperextensible joints			hyperextensible joints, epilepsy			
Previous tentative clinical diagnoses	PWS	PWS	PWS	Turner syndrome, Hypochondroplasia	PWS	PWS	SRS	PWS	PWS	PWS		PWS		PWS	Turner syndrome, Hypochondroplasia
Cytogenetics	46,XX	46,XX	45,XY,rob (13;14)(q10; q10)	45,XX,rob(13;14) (q10;q10)	47,XY,+del(14) (q11.1)/46,XY	46, XY		46,XY			46,XX	ND	46,XX	46,XX	46,XX

Abbreviations: F, female; M, male; ND, not determined; PWS, Prader–Willi syndrome

Methylation analyses in different postnatal tissues at the *MEG8*-DMR

The *MEG8*-DMR is one of the few intronic DMRs and so far, not much data are available regarding its methylation status in different pre- and postnatal tissues. Therefore we established a deep bisulfite sequencing approach on the Roche/454 GS junior system covering 24 consecutive CpG dinucleotides within the DMR.

First analyses in normal controls showed a methylation level of ~50% in DNA from blood and liver confirming previous data.[9] Separation of the parental alleles showed an allele-specific methylation pattern (Supplementary Figure 2). Similar results could be obtained for skin, lung, spleen, skeletal muscle and heart although the methylation level did not reach 50% in all investigated samples. In some tissues the distribution of methylation among the CpGs seems to be a bit more uneven than in others, maybe due to different cell types present in some of the tissues (Figure 2a and Supplementary Figure 2). For kidney we observed methylation levels of 26–31%. Allele separation showed one nearly completely unmethylated allele and one allele with about 44%, suggesting that maternal methylation might only be present in a subset of cells or a certain type of cells.

Interestingly, in brain tissue we observed a methylation level that seems to increase slightly with age (Figure 2b). While little methylation was detectable in fetal brain tissue (2%) and in brain tissue from a 4-month-old child (9%), a level of about 14–30% (average: 22%) was present in four individuals aged 44, 45, 74 and 79 years. No increase in methylation was observed when comparing the mid-forty and the mid-seventy individuals. Methylation is acquired predominantly on one allele in all three informative individuals. This is probably the maternal allele, although we had no parental DNA samples to prove this.

Methylation analyses in different prenatal tissues at the IG-, *MEG3*- and the *MEG8*-DMR

The methylation of the primary IG-DMR is set already in the germline, in contrast to the secondary *MEG3*- and *MEG8*-DMR where the methylation imprint is set somatically. When this takes place in humans is, to the best of our knowledge, unknown. We thus analysed the methylation at all three DMRs in different prenatal tissues of embryonic and extraembryonic origin. As expected we observed a methylation level of about 50% at the IG-DMR (CpGs6-8, Figure 3a) in three chorionic villi samples (CVS) and three amniotic fluid samples (AF). The placenta samples showed slightly lower methylation levels between 39 and 46%. As shown previously[11] and also here, the methylation levels at the surrounding CpGs (CpGs1-5 and 9–15) are in the range of about 60–70%. In CVS and placenta samples methylation levels are even higher (80–90%). In contrast, the amniotic fluid samples have a methylation pattern similar to the one observed in skin and blood (Figure 3a).

For the secondary *MEG3*-DMR, the methylation in the CVS and placenta samples ranges between 42 and 63% with an average of 52.4%, but the distribution of the methylation among the single CpGs is uneven (Figure 3b and Supplementary Figure 3a). On the other hand the methylation patterns in the three AF samples show a methylation level of 45 to 47% with a clear pattern of nearly completely methylated and unmethylated reads. Furthermore, the distribution of methylation in AF is quite uniform among all analysed CpGs resembling the pattern observed in blood and fibroblasts, thus indicating that the methylation is already set in the embryonic fibroblasts in AF at this stage in development.

At the *MEG8*-DMR all samples, CVS, placenta and AF show low methylation levels between 16 and 30% (Figure 3c). Furthermore, the methylation in these cases is distributed unevenly among the CpGs and also among the alleles (Supplementary Figure 3b). To evaluate whether culturing might have an effect on the methylation levels observed in AF, we investigated a naive AF sample taken at week 20 of gestation (amniotic fluid 4). Methylation level was ~39% and the pattern resembled those of (cord) blood or skin (Supplementary Figure 3b).

Methylation analyses at the *MEG8*-DMR in patients with different 14q32 molecular defects

Following previous reports on three TS14 patients with a primary imprinting defect who showed hypermethylation of the paternal allele at the *MEG8*-DMR[17] we wanted to investigate the impact of different molecular causes of TS14 and also of KOS14 on the methylation level of the *MEG8*-DMR. In total we investigated 15 patients with TS14 and 6 patients with KOS14, three of them familial cases with affected siblings (Figure 4). Of the TS patients two have the recurrent 1.1 Mb deletion spanning the entire imprinted region and fourteen non-imprinted genes, five have upd(14)mat, two of them due to a Robertsonian translocation (13;14), and one due to a small supernumerary marker chromosome. One patient has either a maternal ID or a upd(14)mat and seven have a primary ID, one of those in a mosaic state (Table 1). The patients have high methylation levels ranging from 74.7 to 93%, irrespective of the underlying molecular defect.

TS patient 14 has an imprinting defect in a mosaic state, which explains the relatively low increase in methylation (74.7%).

In two TS14 patients we observed a reduced hypermethylation (TS patients 2 and 15) compared to patients with the same molecular defect. In both patients MS-MLPA and in TS patient 2 also a SNP array revealed no evidence for somatic mosaicism. Thus, the discrepancies in the methylation levels of these patients remain unclear.

In contrast to the high levels of methylation observed for the patients with TS14, all KOS14 patients show very low levels of methylation (1–4%) regardless of the molecular defect. For KOS patient 1 (2.3%) who has a upd(14)pat this is due to the absence of the methylated maternal allele and the presence of two unmethylated paternal alleles. KOS patient 2 (4.2%) has a deletion of 165 kb on the methylated maternal allele that includes the *MEG8* gene with the *MEG8*-DMR which explains the hypomethylation here. KOS patient 3 and the siblings 4 and 5 have a deletion of the *MEG3*-DMR alone, which is located about 77 kb upstream of the investigated *MEG8*-DMR. Nevertheless, the deletion of the *MEG3*-DMR leads to a complete loss of methylation at the *MEG8*-DMR in all three cases (1.6, 4.2 and 3.0%, respectively). Furthermore, KOS patient 6 and his brother (KOS patient 7), who have a 130 kb deletion that includes the IG- and the *MEG3*-DMR, also show a complete loss of methylation at the *MEG8*-DMR (3.1 and 3.0%). Most interestingly, though, is the result for the two siblings (KOS patients 8 and 9) who have a 66 kb deletion with an insertion of 16 kb between the breakpoints. The deletion spans the region from the *MEG3* to the *MEG8* gene without affecting any of the DMRs. Here again, a complete loss of methylation could be observed (2.5 and 2.6%).

All results are summarized in Table 2.

DISCUSSION

Clinical findings

We here add eight additional patients with different molecular causes to the rare number of TS14 patients known to date. All patients display phenotypic hallmarks of TS14, namely reduced birth measurements, hypotonia and feeding difficulties early in life as well as short

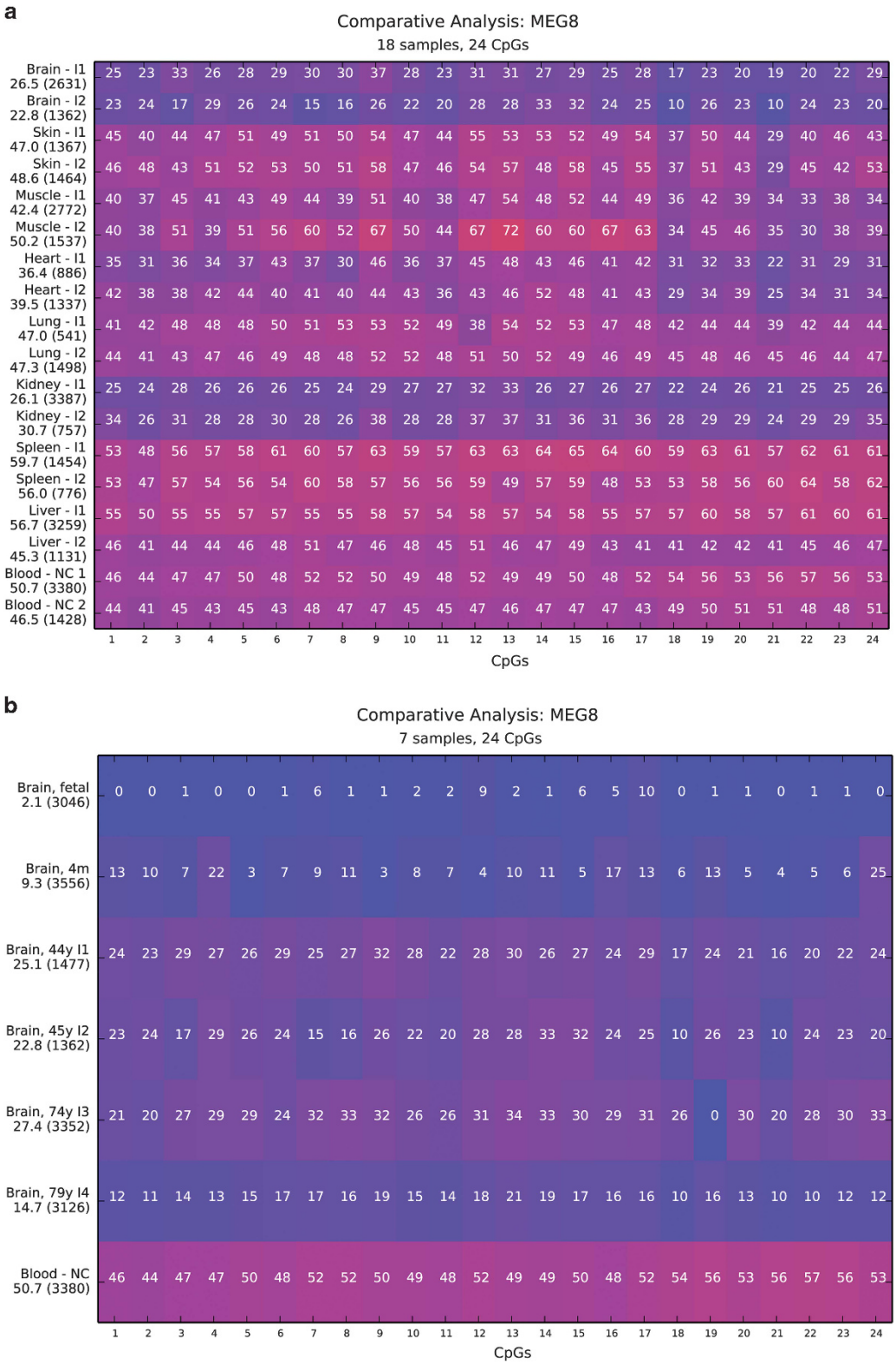

Figure 2 Methylation patterns of the *MEG8*-DMR in different postnatal tissues. The comparative view of the methylation at the *MEG8*-DMR is given in (**a**) for eight different postnatal tissues taken from two normal individuals each and in (**b**) of six brain samples taken from donors of different ages and blood from an adult normal control (NC) for comparison. I1, individual 1; 44-year-old male; I2, individual 2; 45-year-old female; I3, individual 3; 74-year-old female; I4, individual 4, 79-year-old male; NC1, normal control 1; NC 2, normal control 2. Every line represents a specific sample, every square an analysed CpG with its average methylation. The average methylation over all 24 analysed CpGs is given on the left in percentage together with the number of analysed reads in brackets. Red is methylated, blue is unmethylated.

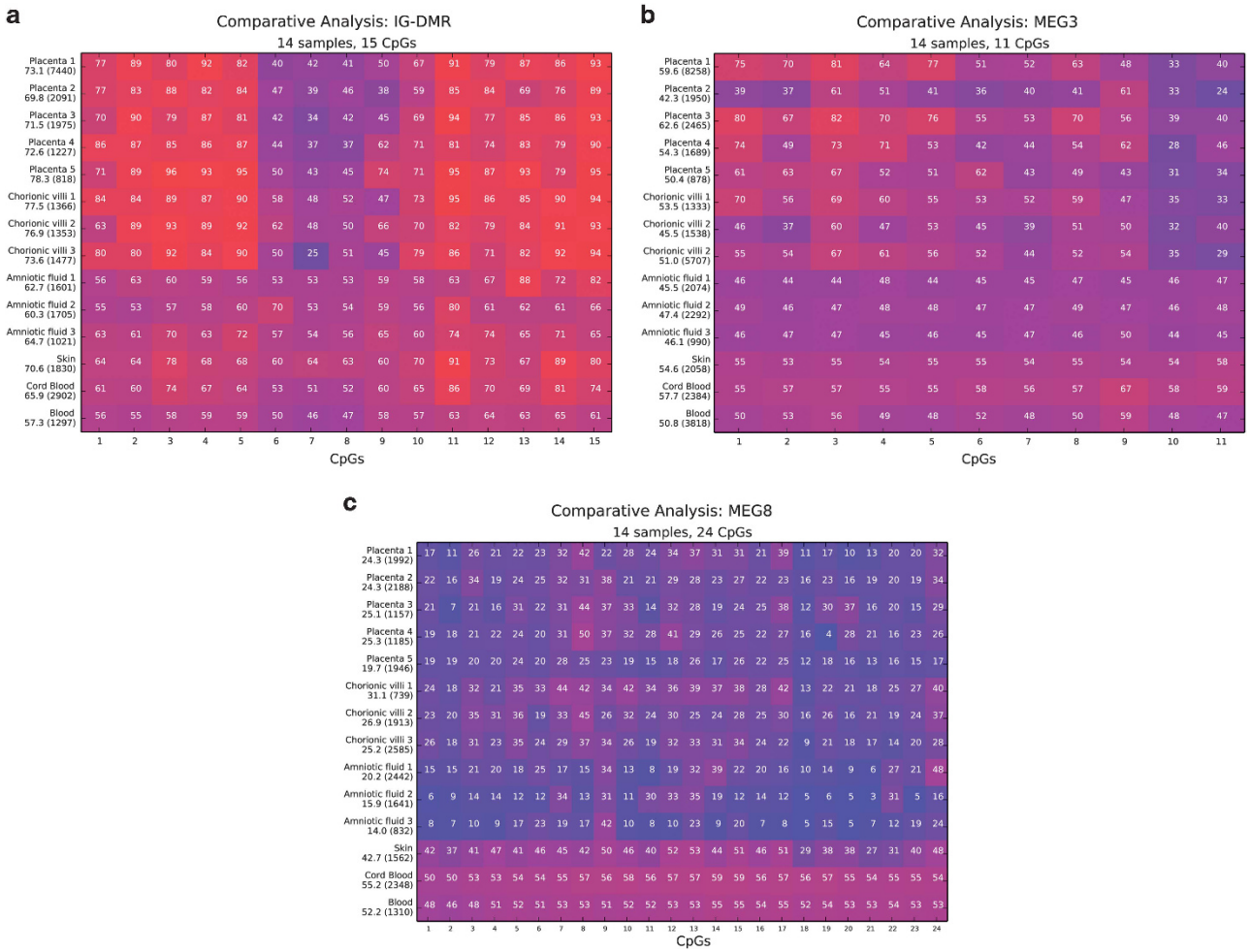

Figure 3 Analyses of methylation patterns in prenatal tissues at (**a**) the IG-DMR, (**b**) the *MEG3*-DMR and (**c**) the *MEG8*-DMR. The figure shows comparative methylation heatmaps for the three DMRs on chromosome 14q32 for three first trimester, two third trimester placenta samples (1–5), three chorionic villi samples (1–3) and three amniotic fluid samples (1–3). For comparison a cord blood sample, a blood sample from an adult normal control and a skin sample from a four month old individual are given in the bottom lines. Every line represents a specific sample, every square an analysed CpG with its average methylation. The average methylation over all analysed CpGs (IG-DMR: 15, *MEG3*-DMR: 11, *MEG8*-DMR: 24) is given on the left in percentage together with the number of analysed reads in brackets. Red is methylated, blue is unmethylated.

stature with small hands and feet. In nearly all patients older than 4 years, obesity and a premature puberty were present, too. Signs of facial dysmorphism like frontal bossing, a short philtrum, a broad nose with a depressed nasal bridge or micrognathia were only present in the minority of patients. Furthermore, three patients were described to have hyperextensible joints, which is less than in previous reports[1] although this feature might not always be reported.

One of the newly described patients reported in this study has a 1.1 Mb deletion with breakpoints inside TGG repeats (TS patient 2). Highly similar deletions have been described in nine cases so far, adding further support to the existence of a recurrent microdeletion syndrome on chromosome 14q32.[2,14–16,23–25] TS patient 2 shows severe intellectual disability usually not observed in TS14 patients with upd(14)mat or ID but present in all 1.1 Mb deletion cases.[2] As hypothesized before this might be due to the deletion of the *YY1* gene, which has been linked to intellectual disability.[2,24]

One of the two patients with TS14 and upd(14)mat newly described in this study has a balanced Robertsonian translocation (13;14) that occurred *de novo* (TS patient 3) and the other one has a supernumerary marker chromosome (TS patient 5). Thus, conventional cytogenetic analysis should be performed in patients with TS14 and upd(14)mat and their parents to uncover underlying molecular mechanisms and to enable a better estimation of the recurrence risk.

Furthermore, we describe four new patients with TS14 due to a primary imprinting defect (TS patients 7–9 and 14), increasing the small number of known patients with this particular molecular defect considerably to now 19 (reviewed in [refs 17,26–29]). On the basis the methylation pattern obtained by independent methods, the imprinting defect in TS patient 14 is present in a mosaic state, that is she has normally methylated cells and cells with an ID. This indicates that the ID occurred postzygotically due to a problem in maintaining the correct methylation imprint, whereas in the non-mosaic patients the methylation defect is more likely due to a failure in imprint erasure or establishment. To the best of our knowledge, patients with mosaic methylation defects have only been described in very rare cases.[14,30]

As shown in Table 1, ten of the 15 TS14 patients (patients 1–3, 5, 6, 8–10, 12 and 14) - although having different molecular causes of TS14 —were initially suspected of having Prader–Willi syndrome (PWS). PWS and TS14 show some overlapping phenotypical features, the

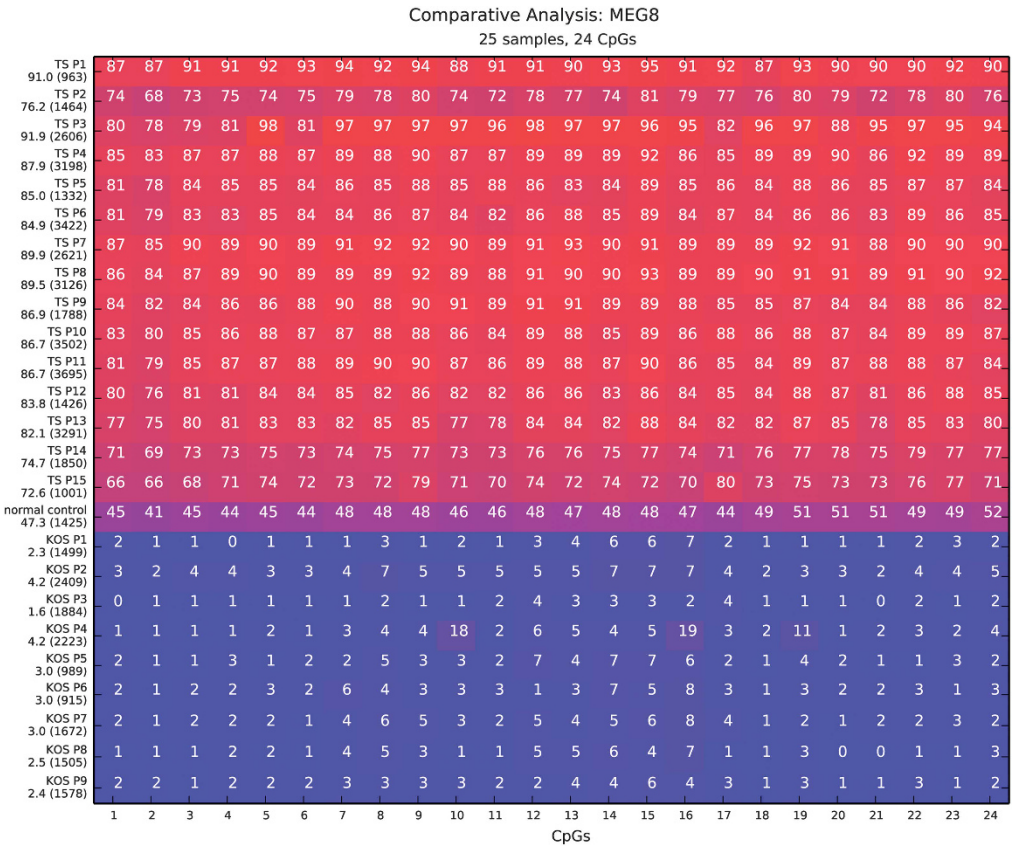

Figure 4 Methylation pattern at the *MEG8*-DMR in patients with Temple and Kagami-Ogata syndrome due to different molecular disturbances. Results for all 15 analysed TS14 patients and for all six analysed KOS14 patients and three also affected sibs are shown. For comparison the result for blood from a normal control is given, too. All TS14 patients show a severe hypermethylation, while all KOS14 patients show a severe hypomethylation. TS patients 1 and 2–1.1 Mb deletion, TS patients 3 and 4—UPD(14)mat rob(13;14), TS patient 5—UPD(14)mat, supernumerary marker chromosome, TS patient 6 UPD(14) mat, TS patients 7-11, 13—ID, TS patient 12—UPD or ID, TS patient 14—mosaic ID, TS patient 15 UPD(14)mat (Table 1), KOS patient1—UPD(14)pat, KOS patient 2–165 kb deletion including the *MEG3*- and the *MEG8*-DMR, KOS patients 3–4.3 kb deletion including the *MEG3*-DMR, KOS patients 4 and 5–5.8 kb deletion including the *MEG3*-DMR, KOS patients 6 and 7–130 kb deletion including the IG- and the *MEG3*-DMR, KOS patients 8 and 9–66 kb deletion with an insertion of 16 kb between the breakpoints (Supplementary Figure 4). Every line represents a specific sample, every square an analysed CpG with its average methylation. The average methylation over all analysed CpGs is given on the left in percentage together with the number of analysed reads in brackets. Red is methylated, blue is unmethylated.

most prominent being muscular hypotonia in the newborn infants and the later onset of obesity.[14] These high numbers emphasize again that TS14 should be considered as a differential diagnosis for PWS, as has been noted before.[2,14] Interestingly, it has been suggested that a subset of PWS phenotypic features may arise from the dysregulation of maternally expressed genes in 14q32.[31] Stelzer and colleagues found the long noncoding RNA *IPW*, located in the imprinted region critical for PWS on chromosome 15q11q13, to be a regulator of the imprinted region 14q32. They could show that overexpression of *IPW* resulted in downregulation of the maternally expressed genes in 14q32 thus providing a possible mechanistic link for the overlapping phenotypes of TS14 and PWS.

Of note is that one patient (TS patient 7) had a tentative clinical diagnosis of Silver–Russell syndrome (SRS). SRS is also characterized by intrauterine growth retardation (IUGR) and a short stature, but SRS patients only rarely show premature puberty or develop obesity although follow up data is scarce. The prominent pointed chin in SRS is usually not observed in patients with TS14. Up to date at least eight patients with an initial clinical diagnosis of SRS have been identified to have TS14. In two cases only a hypomethylation of the *MEG3*-DMR was reported, but the underlying defect remains unclear.[25,27–30,32] Thus, in cases with a clinical diagnosis of SRS where molecular analyses of chromosomes 11p15 and 7 yielded a normal result, TS14 should be considered as a differential diagnosis.[33]

Molecular findings

So far, it was not known when the methylation imprint at the somatic *MEG3*- and especially the *MEG8*-DMR is established in humans and if there are differences between embryonic and extraembryonic tissues. As expected, the methylation at the germline IG-DMR is already fully established in AF samples as well as in CVS and first and third trimester placenta when focusing on the CpGs6-8.[10,34] The neighbouring CpGs (CpG1-5 and CpG9-15) tend to be hypermethylated in placenta and CVS, which is in accordance with previous reports showing a higher than average methylation of about 70%.[34] Similar results have also been obtained in extraembryonic tissue of E6.5 and 7.5 mice where methylation was present on both parental alleles.[13]

The methylation levels of the investigated CpGs are highly variable with an unequal distribution, especially in CVS and placenta samples but also in AF, skin and blood as has been noted earlier.[11,35] This again emphasises the need for a careful evaluation of the analysed CpGs within the IG-DMR and the technique used, as only a small subset of

Table 2 Summary of aims, results and conclusions of the molecular findings

Experiments/questions	*Reason*	*Results*
Investigation of the methylation level of the *MEG8*– DMR in different postnatal tissues	Methylation of the *MEG8*-DMR only known for blood, liver and brain	Verification of results for blood and liver
		Methylation patterns compatible with imprinting in skin, lung, spleen, skeletal muscle and heart
		Kidney showed preferential methylation of one allele (maternal allele?), but methylation level of 26–31%
		Verification of the unmethylated status for brain tissue in young donors. Methylation seems to increase slighty with age
Investigation of the methylation level in extraembryonal and embryonal tissue	So far it is unknown when the methylation imprint is set at the secondary *MEG3*- and *MEG8*-DMR in humans	Germline IG-DMR: methylation imprint established in extraembryonic chorionic villi and placenta tissue and embryonic amniotic fluid, as expected
		Secondary *MEG3*-DMR: methylation imprint not established in extraembryonic chorionic villi and placenta tissue, but established in embryonic amniotic fluid
		Secondary *MEG8*-DMR: methylation imprint not established in extraembryonic chorionic villi and placenta tissue. For amniotic fluid: low methylation level in three cultutred amniotic fluid samples, pattern resmbling the pattern in blood in naive amniotic fluid sample
Investigation of the methylation at the *MEG8*-DMR in patients with TS14 and KOS14 with different molecular defects	It is not known whether the molecular defect has an effect on *MEG8* methylation	The molecular defect has no effect on the methylation level of the *MEG8*-DMR
	Did the results show something else?	Hypothesis: Transcription is necessary to establish the methylation imprint at the *MEG8*-DMR

Abbreviation: DMR, differentially methylated region

the CpGs of the DMR seem to be suitable at all, as has been discussed previously.[11]

For the *MEG3*-DMR we observed that the methylation imprint must be fully established in fibroblasts from amniotic fluid before sampling took place in week 15 of gestation. Recent studies indicate that methylation at the *MEG3*-DMR is established between the blastocyst stage and week six or ten of gestation based on total methylation levels.[36,37] Furthermore, a fully or nearly fully established methylation has been described for DNA from umbilical cord blood taken as early as day 57 of gestation based on total methylation levels observed by pyrosequencing.[34] Studies in mice showed similar results as methylation at the *Meg3*-DMR was not detectable by bisulfite sequencing in early stages of development, including the morula.[13,38] Nowak *et al* described that while at 3.5 dpc (days post coitum) no methylation could be detected, 6.5 dpc embryos showed a much enhanced methylation level and by 9.5 dpc the *Meg3*-DMR was nearly fully methylated on the paternal allele.[39] Another study could observe the same trend for E5.5, 6.5 and 7.5 embryos.[13]

Interestingly, the extraembryonic CVS and placenta samples investigated here showed a methylation level of around 50% at the *MEG3*-DMR as well, but without a clear pattern of methylated and unmethylated reads as present in blood and AF (Supplementary Figure 3a). In addition, the methylation level between the different CpGs analysed show a much higher variance so that presumably methylation is present on both parental alleles. Unfortunately, no informative SNP was present in the investigated samples, so that the allele-specificity cannot be clearly determined. In mice, methylation at the *Meg3*-DMR was present on both parental alleles in extraembryonic tissue of E6.5 and 7.5 embryos.[13] These data indicate that in extraembryonic tissues the *MEG3*-DMR acquires methylation on both alleles and is thus not allele-specific.

The low methylation levels of the *MEG8*-DMR observed in naive CVS and placenta could be explained by previous observations where extraembryonic tissues, especially placenta, often adopts a different methylation profile compared to somatic tissues.[9] As the CVS and the first and third trimester placenta samples show a methylation level of around 20–25%, this seems to reflect the final methylation status in this tissue. In embryonic tissues methylation imprints at somatic DMRs are usually set early in development before the blastocyst stage. Thus one would expect a ~50% methylation level as found in adult fibroblasts also in fibroblasts from AF. However, in all three cultured AF samples tested low levels of DNA methylation were observed, indicating either an extremely late setting of the methylation imprint or a consequence of culturing of the AF cells. It is known that DMRs for some imprinted loci tend to become hypomethylated during cell culture whereas other are relatively stable, for example, the *SNRPN*-DMR.[40,41]

Methylation analyses in nine different postnatal tissues showed a diverse pattern. In blood the *MEG8*-DMR is differentially methylated, and liver has a methylation level of ~50% as shown before.[9] For the other investigated tissues skin, lung, spleen, skeletal muscle and heart methylation levels between 40 and 50% were observed. Allele separation showed patterns compatible with imprinting in all those tissues, that is, one allele nearly completely unmethylated and the other allele nearly completely methylated. For kidney on the other hand the results were ambiguous with methylation levels of 26–31%, respectively, while allele separation in the informative sample showed a nearly completely unmethylated allele and a preferentially methylated allele with 44% methylation. The methylated allele was the same as in the other tissues from the same donor. Whether the methylation in kidney is preferentially on the maternal allele or if it is imprinted as well and the low methylation results due to technical or biological or

reasons, for example, a more diverse mixture of cell types, remains elusive and needs further testing with a higher number of samples being analysed.

The methylation levels in brain are quite interesting. In fetal brain and brain from a 4-month-old donor, no or very low levels of methylation were detectable as seen before in Court *et al.*[9] In four individuals aged 44, 45, 74 and 79 years a methylation of 26–30% was observed. Lokk *et al.* also detected methylation levels around 30% in medulla oblongata samples from four donors aged 40–60 years which is in line with our results for adult brain tissue.[42] Thus, the methylation level in brain seems to increase with age. To the best of our knowledge, this would be the first report of an imprinted locus acquiring methylation with age in a tissue-specific manner. Alternatively, our findings might reflect for example, the relative increase in cell number of a cell type with a methylated allele.

One question that remains unsolved is the function of the *MEG8*-DMR as there is no evidence that it influences monoallelic expression of *MEG8* itself, since it is methylated on the expressed maternal allele. One possible function could be that it serves as a promoter for an antisense transcript starting at the *MEG8*-DMR, a situation similar to the one of the imprinted locus on chromosome 11p15 with the maternally expressed *KCNQ1* and its paternally expressed antisense transcript *KCNQ1OT1*.[43] Here the expression of *KCNQ1OT1* and thereby of *KCNQ1* is regulated by the *KCNQ1OT1*-DMR (imprinting control region 2, *KCNQ1OT1*:TSS-DMR) which is also an intragenic DMR, located within intron 11 of the *KCNQ1* gene. However, evidence for a *MEG8*-antisense transcript has not been described so far.

By analysing KOS14 patients with different deletions affecting both, the IG- and the *MEG3*-DMR (KOS patient 6 and 7), only the *MEG3*-DMR (KOS patients 3, 4 and 5) or no DMR at all (KOS patient 8 and 9; Supplementary Figure 4) we could demonstrate in all cases, that the methylation of the *MEG8*-DMR was completely absent indicating a failure in establishing the methylation imprint on the maternal allele.

Of interest the 66 kb deletion between *MEG3* and *MEG8* also leads to a complete loss of methylation at the *MEG8*-DMR, although the *MEG3*-DMR is not affected (KOS patients 8 and 9). Taking into account that there is also an insertion of 16 kb between the deletion breakpoints, this deletion probably impairs transcription through the locus. This is of particular interest, as expression studies in mice could show that a transcript starting at the *Meg3* promotor seems to traverse all maternally expressed imprinted genes, including *Rtl1as*, *Meg8* and the sno- and microRNA clusters.[44–46] Recently a study in human embryonic stem cells showed that low levels of *MEG3* expression were accompanied by equally low levels of expression of *MEG8* and several miRNAs located further downstream thus corroborating the hypothesis of the existence of one long transcript spanning all maternally expressed genes including the snoRNA and miRNA gene cluster.[47]

Other studies in mice demonstrated that transcription through the imprinted *Gnas* locus is necessary to establish the correct methylation imprint at the different DMRs in the female germline.[48] Later, the same mechanism of transcription transition has been observed at the *SNRPN* locus.[49] Furthermore, recent data also suggested that the transcription of *KCNQ1* through the *KCNQ1OT1*-DMR is necessary to establish the methylation imprint.[50] On this background, it seems feasible to hypothesize that the same mechanism applies for the

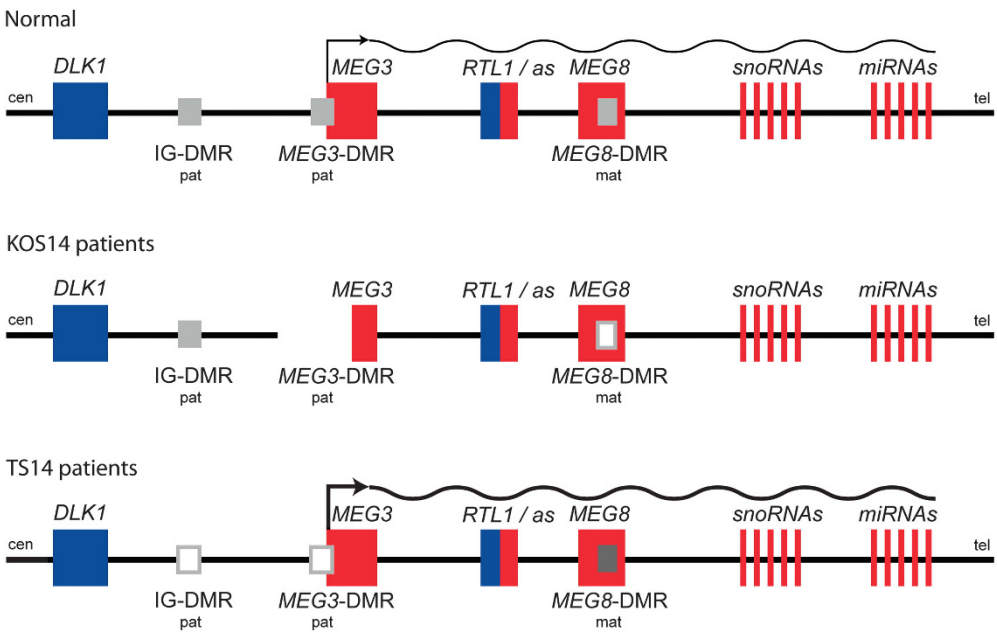

Figure 5 Model for the establishment of the methylation imprint at the *MEG8*-DMR by transcription transition. Three different situations of the region 14q32 in respect to expression and methylation status at the three DMRs are given. At first the normal situation is depicted with the presumed transcript starting at *MEG3* on the maternal allele and traversing through all other maternally expressed genes (depicted in red) including *MEG8*. The IG- and the *MEG3*-DMR are methylated on the paternal allele (pat) only and are given in grey due to their hemimethylated status. The *MEG8*-DMR is methylated on the opposite, the maternal allele (mat) only. Paternally expressed genes are shown in blue. Below the situation in KOS14 patients is shown with a deletion of the *MEG3*-DMR on the maternal allele as an example. Transcription cannot be initiated at the *MEG3*-DMR therefore no transcription transition through the *MEG8*-DMR takes place and the *MEG8*-DMR is hypomethylated (framed square). At the bottom the situation in TS14 patients, for example, with an epimutation, is shown. The expression of the long transcript starting from the *MEG3*-DMR takes place from both parental alleles as the IG- and the *MEG3*-DMR are unmethylated. Transcription transition of the *MEG8*-DMR on both alleles leads to methylation on both alleles and thus the observed hypermethylation (dark grey square).

MEG8-DMR and that transcription starting at the *MEG3* promotor is required to be transcribed through the *MEG8*-DMR to set the methylation imprint (Figure 5). If this transcription is disturbed, for example, by a deletion of the *MEG3* promotor or the region between *MEG3* and *MEG8*, the transcript does not reach the *MEG8*-DMR and the methylation is not established as seen here in the KOS patients 4–9.

Of note in this regard is that the establishment of the methylation imprint at the *Gnas/GNAS*, *SNRPN* and *KCNQ1OT1* locus takes place in the growing oocyte while the *MEG8*-DMR is a secondary DMR where methylation seems to be established around or after week 17 of the fetal development as shown by the methylation analyses in amniotic fluid samples here.[36,37,48,49] It also fits in that *MEG3* is not yet transcribed in the oocyte.[38]

Taken together, we describe eight new patients with TS14 adding them to the rare number of TS14 patients known to date and conducted extensive methylation analyses of the three DMRs in the chromosomal region 14q32, especially for the recently described *MEG8*-DMR. Analyses in 15 TS14 and 6 KOS14 patients lead to the hypothesis that the *MEG8*-DMR is yet another imprinted locus where transcription transition seems to be required for the establishment of the methylation imprint.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We are grateful to the patients and their families for their participation in the study, Sabine Kaya, Claudia Mertel and Christina Lich for expert technical assistance, Andreas Dufke (Tübingen) for obtaining photos and informed consents, Konrad Platzer (Lübeck, now Leipzig) and Andrew Green (Dublin) for providing patient material, Markus Schmidt (Duisburg) for providing the placenta samples and Dietmar Lohmann (Essen) for providing tissue sample. This work was funded by the Bundesministerium für Bildung und Forschung (BMBF; Imprinting diseases, grant No. 01GM1513A and D).

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