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Hypomethylation of functional retrotransposon-derived genes in the human placenta

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Abstract DNA hypomethylation is assumed to be a feature of the mammalian placenta; however, its role in regulating placental gene expression is not well defined. In this study, MeDIP and Sequenom MassARRAY were used to identify hypomethylated gene promoters in the human placenta. Among the genes identified, the hypomethylation of an alternative promoter for *KCNH5* was found to be restricted to the placenta and chorion. Complete methylation of this promoter correlates with a silenced *KCNH5* transcript in embryonic tissues, including the amnion. Unusually, this hypomethylated promoter and the alternative first exon are derived from a SINE (*AluY*) retrotransposon. Examination of additional retrotransposon-derived gene promoters in the placenta confirmed that retrotransposon hypomethylation permits the placenta-specific expression of these genes. Furthermore, the lineage-specific methylation displayed by *KCNH5*, *INSL4*, and *ERVWE1* revealed that dichotomous methylation establishes differential retrotransposon silencing between the extra-embryonic and embryonic lineages. The hypomethylation of the retrotransposons that regulate these genes, each of which arose during recent primate evolution, is consistent with these genes having functional roles that are unique to the invasive haemochorial placentas of humans and recent primates.

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Introduction

Placenta-specific epigenetic modifications such as DNA hypomethylation have been loosely associated with the unique functions of the mammalian placenta, such as the simultaneous nourishment and protection of the fetus throughout pregnancy (Coan et al. 2005; Constancia et al. 2004; Ferguson-Smith et al. 2006; Hemberger 2007; Rahnama et al. 2006; Reiss et al. 2007; Serman et al. 2007). However, the intersect of placental hypomethylation with placental gene expression has not been well documented. Previously, placental gene hypomethylation was reported for *ERVWE1* (the syncytin-1 gene), which has a key role in syncytium formation, but the data on *ERVWE1* were limited to a semiquantitative analysis of only two term placentas and four somatic tissues (Matouskova et al. 2006). Relative hypomethylation has also been observed in the promoters of chromosome X genes in female placentas, which, on average, showed 27% less methylation than in female blood (Cotton et al. 2009).

Although the human placenta grows continuously during pregnancy, the most important developmental stages occur during the first trimester. After fertilisation, a series of cell divisions prepare the embryo for implantation, the stage at which placental development begins (Benirschke et al. 2006). Documentation of the earliest stages of human embryonic development is based on studies in animals given the lack of human specimens available from these early stages of gestation (Cross 2005; Norwitz et al. 2001).

During primate embryonic development, the earliest lineage specifications occur when the extra-embryonic lineages differentiate from the embryonic lineage. The first cell fate decision is made when the inner-cell mass is distinguished from the trophectoderm, and the second event is when the inner-cell mass divides into the primitive endoderm and the epiblast (Enders and Schlafke 1981; Enders et al. 1986). The trophectoderm and primitive endoderm lineages give rise to the extra-embryonic structures such as the placenta, chorion, and yolk sac, whereas the epiblast lineage gives rise to the embryonic tissues, including the embryo, amnion, and umbilical cord (Benirschke et al. 2006; Cross et al. 1994; Enders and Schlafke 1981; Rossant 2007). The lineage origin of the placental villous mesenchyme, however, remains unsolved (Downs 2009; Enders and King 1988; Luckett 1978).

Epigenetic modifications play a central role in directing the early lineage commitment of cells (Reik et al. 2003; Santos et al. 2002). After fertilization, both paternal and maternal genomes are reprogrammed to remove the methylation marks from the gametes (Mayer et al. 2000; Reik et al. 2001). Once implantation occurs, de novo methylation patterns are established to nonimprinted genes in the embryonic (epiblast) lineage, but remethylation occurs to a lesser degree in the extra-embryonic lineages (trophectoderm and primitive endoderm) (Dean et al. 2003; Reik et al. 2003; Santos et al. 2002). Although the function of differential epigenetic modification between the lineages remains unclear, the growth and survival of the embryo relies on the formation of the placenta. In this study, a genome-wide assessment of DNA methylation in first-trimester placenta, followed by subsequent confirmatory experiments, was performed to identify placental hypomethylation that may be biologically relevant to the development or function of this relatively unexplored yet fascinating transient human tissue.

Materials and methods

Sample collection and processing

Thirty-one first-trimester human pregnancy tissues were collected from consenting patients undergoing medical terminations of pregnancy according to the provisions of the Lower South Regional Ethics Committee (New Zealand). Gestational age was estimated by ultrasound and tissues were processed 1–2 h after being passed. Most samples were the complete products of conception containing an intact gestational sac (chorion) lined with trophoblast villous (placental) cells. Before dissection, the gestational sac was washed with PBS buffer to remove residual (maternal) blood from the tissue. Trophoblast villous cells were teased away from the outer chorionic membrane, washed with PBS buffer, and snap-frozen in liquid nitrogen. Six samples were of 35–41 days gestation, 12 samples were of 42–48 days gestation, and 13 samples were of 49–55 days gestation. One human term placenta was used in this study and was processed approximately 3 h after delivery. The term placenta was dissected into components: maternal-facing section, middle section, fetal-facing section, chorion membrane, amnion membrane, and umbilical cord. The fetal human tissues (brain, liver, heart, kidney, and adrenal) were previously collected from medically terminated pregnancy tissues. The adult tissues (brain, kidney, heart, liver, and spleen) were collected from consenting patients who donated their healthy tissues to previous studies in the Cancer Genetics Laboratory (University of Otago, Dunedin, New Zealand). Human peripheral blood DNA from healthy individuals (aged 4–9 years) was obtained from a concurrent project in our laboratory.

DNA was extracted from tissues using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA, Cat. No. 51306) according to the manufacturer's protocol. The fetal sex of each terminated pregnancy sample was determined by using sex-chromosome-specific PCR to account for methylation differences due to X-chromosome inactivation in females. Of the 31 collected samples, 15 were from female fetuses and 16 were from male fetuses. Once fetal sex was determined, maternal contamination of villous samples was excluded by X-chromosome microsatellite analysis.

Methylated DNA immunoprecipitation (MeDIP) and promoter microarrays

DNA from 15 first-trimester female placenta samples was pooled into three groups based on gestational age: 35–41 days ($n = 4$), 42–48 days ($n = 5$), and 49–55 days ($n = 6$). Control DNA from female postnatal peripheral blood samples was pooled into four groups ($n = 12$ in each group). In each pooled group, 10 μg genomic DNA was digested by the NlaIII restriction enzyme (New England Biolabs, Ipswich, MA, USA, Cat. No. R0125L). Digested DNA was purified using the QIAquick PCR Purification Kit (Qiagen, Cat. No. 28104). Immunoprecipitation of methylated DNA was performed using 5 μg of the anti-5-methylcytosine antibody (Aviva Systems Biology, San Diego, CA, USA, Cat. No. AVAMM99021) according to the manufacturer's instructions. The Aviva Systems Biology's DNA Methylation ChIP-DSL platform (version H20K 2.0) was used for the directed selection and ligation (DSL) of immunoprecipitated DNA and hybridisation to 20,000 gene promoter microarrays. A Lowess normalisation was performed on data from seven microarrays (three first-trimester placenta arrays, four peripheral blood control arrays) using the GeneSpring software (Simon Andrews,

Babraham Bioinformatics). Normalised intensity ratios were calculated by dividing the normalised red intensity by the normalised green intensity (i.e., MeDIP DNA intensity/input DNA intensity) and were used as a measure of gene methylation. Candidate genes were selected based on the largest differences between the normalised intensity ratios of first-trimester placenta and peripheral blood.

Sequenom MassARRAY EpiTYPER analysis

Approximately 1 μg of genomic DNA was bisulfite-converted using the EZ DNA Methylation Kit™ (Zymo Research Corp., Irvine, CA, USA, Cat. No. D5002). A modification to the manufacturer's protocol replaced the conversion incubation step with an alternative cycling protocol: 95°C for 30 s, 50°C for 15 min for 20 cycles. PCR was performed directly after the bisulfite conversion of genomic DNA according to the manufacturer's protocol (Sequenom, San Diego, CA, USA). Primers were designed using the EpiTyper software (Sequenom). Primer sequences are listed in Supplementary Table 1. Post-PCR reactions were performed according to the manufacturer's protocol (Sequenom). Data were analysed using the MassARRAY EpiTYPER software (Sequenom). Individual CpG-site methylation values were averaged to determine the mean promoter methylation for each amplicon.

Gene expression

All tissue examined by Sequenom were included in the expression assays. RNA was extracted from 50 mg of frozen human tissue samples using the PureLink Micro-to-Midi Total RNA Purification System (Invitrogen, Carlsbad, CA, USA, Cat. No. 12183-018). Complementary DNA was synthesised from approximately 2 μg of RNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Carlsbad, CA, USA, Cat. No. 4387406). Using primers that detect all transcripts of *KCNH5*, real-time PCR was performed in a PRISM 7900HT Sequence Detection Instrument (Applied Biosystems) using the TaqMan Gene Expression Assay for *KCNH5* (Applied Biosystems, Assay ID: Hs00544949_m1) according to the manufacturer's protocol. Relative gene expression was normalised to input RNA since reference gene expression was not consistent across the different tissues.

Immunohistochemistry

Tissue sections were obtained from paraffin-embedded blocks of first-trimester placental tissue ranging from 35 to 55 days gestation. The EnVision Dual Link Detection System (Dako, Glostrup, Denmark, Cat. No. K4065) using 3,3′-diaminobenzidine (DAB) substrate was performed using a rabbit polyclonal antibody for KCNH5 (Abcam, Cambridge, MA, USA, Cat. No. ab32975).

Results

Hypomethylation of *KCNH5* in the placenta is lineage-specific

To screen for differentially methylated genes between the human placenta and somatic tissues, DNA from placental villous cells of first-trimester female placentas were compared to whole blood DNA by using methylated DNA immunoprecipitation (MeDIP) followed by hybridisation to 20,000 gene promoter microarrays. Twenty-eight candidate genes were identified that showed the largest differences in measured MeDIP "methylation" between first-trimester placenta and peripheral blood (Supplementary Table 1). The differential methylation of these genes was then quantified by using Sequenom MassARRAY. Sequenom analysis was performed on the same first-trimester placenta and peripheral blood samples that were used in the MeDIP microarray experiment in addition to a selection of other placental, fetal somatic, and adult somatic tissues. Differential promoter methylation between first-trimester placenta and peripheral blood was confirmed in three genes: *AICDA*, *AKR1C4*, and *KCNH5* (Fig. 1).

Although these three genes showed low promoter methylation in first-trimester placenta and higher methylation in peripheral blood, the region of the potassium voltage-gated channel, subfamily H member 5 gene (*KCNH5*), examined by Sequenom (Fig. 2a), displayed striking patterns of promoter methylation when all tissues were compared (Fig. 2b). *KCNH5*, also known as *EAG2*, involved in the regulation of cell cycle and proliferation (Pardo et al. 1999), showed low promoter methylation in all tissues derived from the extra-embryonic lineages and

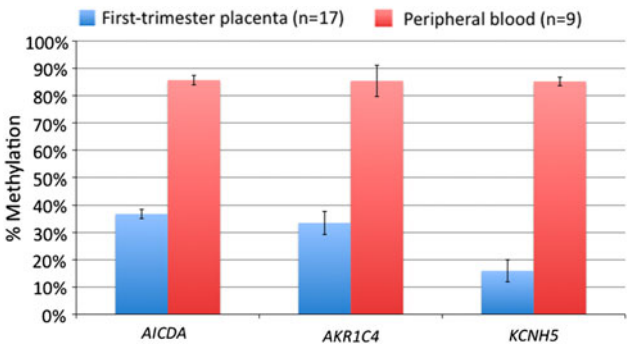

Fig. 1 Mean CpG methylation for the three candidate genes confirmed by Sequenom analysis. *Error bars* represent 95% CI of the mean

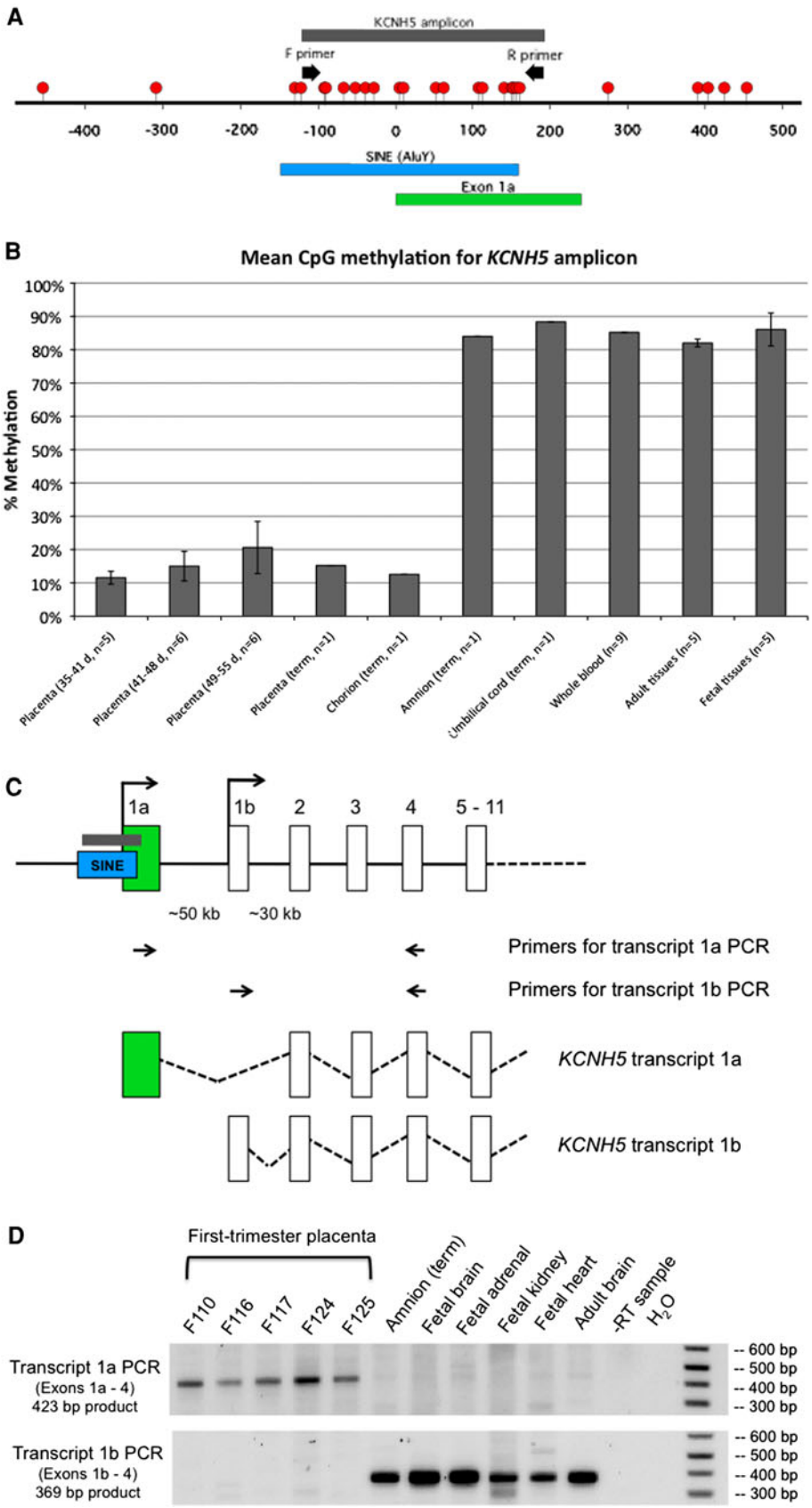

Fig. 2 Lineage-specific methylation of *KCNH5* regulates the expression of alternative gene transcripts. **a** The region of *KCNH5* that was analysed by Sequenom. Coordinates refer to the genomic location with respect to the transcription start site. Forward and reverse primers are shown in *black*. *Red circles* represent CpG sites. The 313-bp amplified product for *KCNH5* is shown in *grey*, which included 17 CpG sites for methylation analysis. **b** Mean promoter methylation for *KCNH5* transcript 1a that was quantified by Sequenom analysis. *Error bars* represent 95% CI of the mean. **c** Genomic arrangement of *KCNH5*'s alternative promoters and first exons. The SINE retrotransposon (*AluY* element) that gave rise to the exon 1a promoter is shown in *blue*. The *green box* represents the alternative exon 1a and *white boxes* represent other exons. The *grey bar* represents the region of differential methylation, which spans the entire SINE element. Transcript-specific PCR primers are represented by *black arrows*. **d** End-point RT-PCR results to distinguish *KCNH5* transcripts. The top gel contains PCR products from the transcript 1a PCR and the bottom gel contains PCR products from the transcript 1b PCR. −RT, no reverse transcriptase control, first-trimester placenta

high promoter methylation in all tissues derived from the embryonic lineage (Fig. 2b). In particular, the contrasting level of methylation between the adjacent and adherent chorion (13%) and amnion (86%) membranes indicated that *KCNH5* hypomethylation is not specific to the placenta but is a feature of tissues derived from the extra-embryonic lineages. *AICDA* and *AKR1C4* showed low promoter methylation in first-trimester placenta and higher methylation in peripheral blood. However, when first-trimester placenta and term placenta were compared to the other somatic tissues, the difference in methylation was less pronounced since these tissues showed lower levels of methylation than peripheral blood (Supplementary Fig. 1).

Methylation regulates lineage-specific expression of alternative *KCNH5* transcripts

Gene expression was assessed using RT-PCR to determine whether the differential promoter methylation was inversely correlated with gene expression. It was expected that these three hypomethylated genes would be expressed in placenta; therefore, the absence of *AKR1C4* expression in placenta excluded this gene from further analysis (Supplementary Fig. 2A). Expression of *AICDA* was detected in placenta but also in peripheral blood—a tissue with high levels of methylation (Supplementary Fig. 2B). Since the predicted inverse relationship was not confirmed in most somatic tissues, and there is no evidence for an alternative start site for *AICDA* on the UCSC Genome Browser, methylation is not likely to be regulating *AICDA* expression so this gene was excluded from further analysis. Expression of *KCNH5* was detected in the placenta and the expected inverse correlation was observed between promoter methylation and expression in tissues derived from the extra-embryonic lineages (first-trimester and term placenta) (Supplementary Fig. 2C). The variable levels of *KCNH5* expression in the highly methylated epiblast-derived (embryonic) tissues combined with the presence of an alternative start site for *KCNH5* on the UCSC Genome Browser (Kent et al. 2002) suggested that expression of *KCNH5* in epiblast-derived tissues might be regulated by an alternative promoter.

KCNH5 contains two known promoters: the region of dichotomous methylation that was quantified by Sequenom was present at the upstream promoter as shown in Fig. 2c. This upstream promoter contained the probe sequence that was spotted on the MeDIP promoter microarray. Transcript-specific RT-PCRs were thus designed to distinguish the alternative transcripts of *KCNH5* (Fig. 2c; primer sequences in Supplementary Table 3). Transcript-specific RT-PCR showed exclusive *KCNH5* transcription from exon 1a in placentas, and exclusive expression from exon 1b in somatic tissues and amnion (Fig. 2d). Therefore, dichotomous *KCNH5* promoter methylation is consistent with the lineage-specific expression of alternative transcripts: in tissues derived from extra-embryonic lineages, non-methylation of the upstream promoter permits transcription from exon 1a, whereas in tissues derived the embryonic (epiblast) lineage, transcription occurs from exon 1b. The exon 1b promoter is unmethylated in all tissues (see Supplementary Fig. 3).

Hypomethylation of a SINE (*AluY*) retrotransposon initiates a placenta-specific transcript of *KCNH5*

Analysis of the region of differential *KCNH5* methylation revealed that 147 bp of the upstream promoter and the first 162 bp of exon 1a have recently evolved from a SINE retrotransposon (Fig. 1a) (GRCh37 assembly chr14:63,568,599-63,568,907). This SINE retrotransposon is a member of the *AluY* family; the youngest of the *Alu* elements that diverged from the two older families (*AluS* and *AluJ*) 25 million years ago (Batzer and Deininger 2002; Batzer et al. 1996). The *AluY* element in *KCNH5* transcript 1a is present only in humans, old world monkeys (rhesus macaque), and apes (orang-utan and chimpanzee) (UCSC Genome Browser) (Kent et al. 2002). This suggests that the transposition event for this *AluY* element occurred approximately 25–30 million years ago, which is consistent with the timing of the emergence of the *AluY* family (Fleagle 1999).

KCNH5 protein analysis confirms lineage-specific transcription of *KCNH5*

Although the function of KCNH5 protein encoded by transcripts that use exon 1b has been reported in the literature (Jeng et al. 2005; Ju and Wray 2002; Ludwig et al. 2000; Saganich et al. 1999), the function of the placenta-specific transcripts that use exon 1a is unknown. Therefore, the expression of KCNH5 protein was characterised using a specific antibody that detects all isoforms (the extra-embryonic isoforms of KCNH5 lack 58 residues at the N terminal). Expression of KCNH5 was restricted to the nonvascular stromal and endothelial cells of the placental villous core, which are known to comprise part of the placental villous mesenchyme (Fig. 3) (Benirschke et al. 2006; Castellucci and Kaufmann 1982; King 1987). The specificity of the antibody was confirmed by Western blot analysis, which showed clear background-free bands, the migration of which was consistent with the predicted molecular weights (Supplementary Fig. 4).

Hypomethylation is a feature of other retrotransposon-derived gene promoters in the placenta

Since it has been previously reported that some genes show placenta-specific expression and that a disproportionate

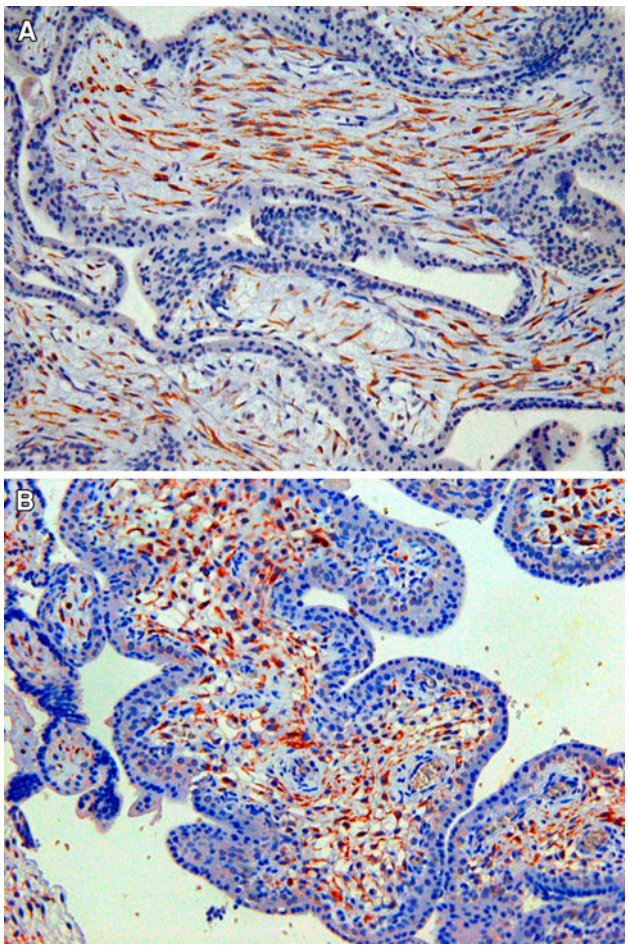

Fig. 3 Immunohistochemistry for KCNH5 in first-trimester placenta showed positivity for KCNH5 in the nonvascular stromal and endothelial cells of the placental villous core: **a** 5 weeks gestation and **b** 11 weeks gestation

number of these genes have retrotransposons in their regulatory regions (Bieche et al. 2003; Medstrand et al. 2005; Rawn and Cross 2008), we sought to determine whether other genes showed a methylation dichotomy between tissues that were derived from embryonic versus extra-embryonic lineages. Based on their reported placenta-specific expression (Bieche et al. 2003; Blond et al. 2000; Landry and Mager 2002, 2003; Medstrand et al. 2001; Millar et al. 2005; Rawn and Cross 2008; Reiss et al. 2007; Schulte et al. 1996), five genes with retrotransposon-derived promoters were selected for methylation analysis using Sequenom: *INSL4*, *ERVWE1*, *EDNRB*, *PTN*, and *MID1*. All five genes showed retrotransposon hypomethylation in the placenta (Figs. 4, 5, 6, 7, 8). Two of the five genes (*INSL4* and *ERVWE1*) displayed a lineage-specific methylation dichotomy that was similar to *KCNH5* (Figs. 4c, 5c). In particular, *INSL4* showed low promoter methylation in trophectoderm-derived chorion (13%) compared to the epiblast-derived amnion (93%) (Fig. 4c). Similarly, the *ERVWE1* promoter showed low methylation in extra-embryonic tissues (placenta and chorion) and higher methylation in all of the embryonic and adult tissues that were examined (Fig. 5c). Three genes (*EDNRB*, *PTN*, and *MID1*) displayed low methylation in placenta and chorion and higher methylation in fetal and adult somatic tissues (Figs. 6, 7, 8). The methylation in amnion was similar to the methylation levels of placenta and chorion. Each of these five genes displayed low placental methylation, which is consistent with the use of these retrotransposon-derived promoters as alternative gene transcription sites in the placenta.

Discussion

In this study, the differential methylation of *KCNH5* led to the discovery of a novel placenta-specific transcript that was derived from the insertion of a retrotransposon. The vast majority of genes that were predicted to show a difference in methylation by MeDIP were either not subsequently validated or the difference in methylation between placenta and several somatic tissues was not likely to be biologically meaningful. In accord with other groups, we found a low rate of successful candidate gene validation (Farthing et al. 2008; Nair et al. 2011; Rajendram et al. 2011).

In tissues derived from the extra-embryonic lineages (placenta and chorion), we show that the *KCNH5* promoter 1a escapes methylation-induced silencing thus allowing expression of a novel placental *KCNH5* transcript. The hypomethylation of this promoter is especially notable since the sequence lies within a SINE (*AluY*) element, and this is one of very few examples of human transcripts initiated by a SINE element (Romanish et al. 2009). Our results show that the differential methylation of this recently evolved retrotransposon correlates with the lineage-specific expression of *KCNH5*'s alternative transcripts. Since retrotransposons are normally silenced by DNA methylation to prevent genome dysfunction (Goll and Bestor 2005; Yoder et al. 1997), the atypical avoidance of methylation by the retrotransposon-derived *KCNH5* promoter, combined with the exclusive expression of transcript 1a in the placenta, leads us to suggest that this *AluY*-derived transcript has a functional role in the placentas of humans and recent primates. We speculate that expression from exon 1a in the placenta blocks the exon 1b promoter by transcriptional interference (Shearwin et al. 2005). Alternatively, the placenta does not have appropriate tissue-specific transcription factors for exon 1b.

KCNH5 protein isoforms in the placenta differ from isoforms in epiblast-derived tissues in two ways. First, the placental isoform has 58 fewer amino acids at the N terminal since translation initiates at exon 2 rather than at

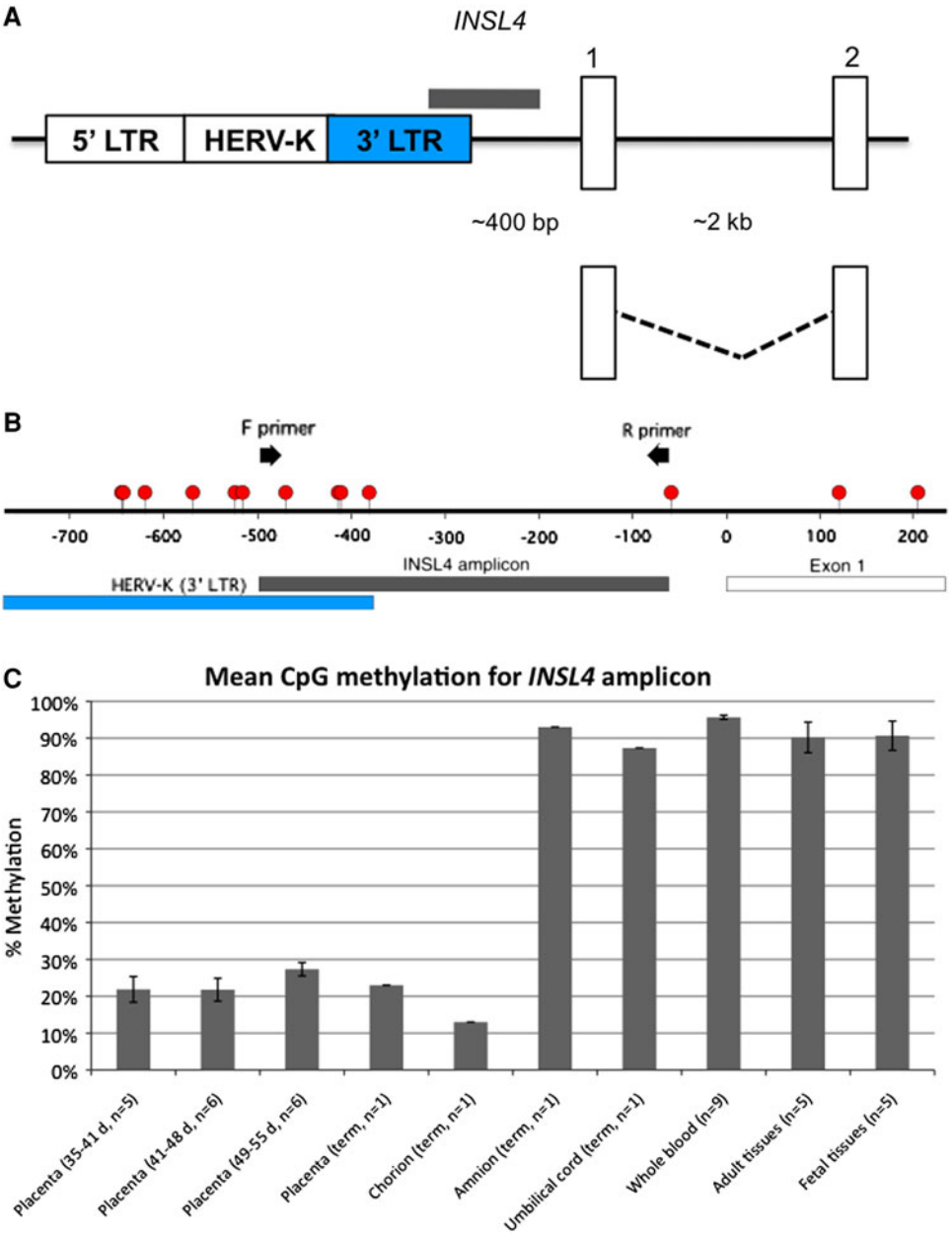

Fig. 4 Lineage-specific methylation of *INSL4*. **a** Genomic arrangement of *INSL4*. The HERV-K gives rise to the placenta-specific *INSL4* transcript (shown), the expression of which is regulated by a promoter derived from its 3′ LTR (in *blue*) (Bieche et al. 2003). *White boxes* represent gene exons. The *grey bar* shows the region of differential methylation that was quantified by Sequenom. **b** The region of *INSL4* that was analysed by Sequenom. Coordinates refer to the genomic location with respect to the transcription start site. Forward and reverse primers are shown in *black*. *Red circles* represent CpG sites. The 438-bp amplified product for *INSL4* is shown in *grey*, which included four CpG sites for methylation analysis. **c** Mean promoter methylation for *INSL4* that was quantified by Sequenom analysis. *Error bars* represent 95% CI of the mean

exon 1b. Second, the reported exon 1a transcripts (AK074484 and BC043409) truncate in exon 7. Based on the evolutionary conservation of this isoform over 30 million years of primate evolution, we expect that these structural differences between the placenta and epiblast-derived tissues suggest a functional role in the human placenta. Importantly, the predicted placental KCNH5 isoforms are consistent with that observed on the Western blot (Supplementary Fig. 4).

KCNH5 transcript 1a is not the only retrotransposon-derived gene that is specifically expressed in the placenta. The expanding list of active retrotransposons in the placenta has led some to suggest that the evolution of placenta-specific promoters by ancient retroviral infection has facilitated the evolution of the uniquely invasive phenotype of the primate hemochorial placenta (Bieche et al. 2003; Cohen and Bischof 2007; Rawn and Cross 2008) through functions ranging from prevention of apoptosis to induction of cell proliferation and migration (Brandt et al. 2005; Heiss et al. 2008; Magarinos et al. 2007). Currently, it is unclear whether the globally hypomethylated state of the placenta has permitted widespread expression from retrotransposon-derived promoters, or whether these promoters in the placenta reflect specific selection of transcripts that enhance placental function and evolution.

Early placental insulin-like 4 (*INSL4*) is a primate-specific gene that encodes an insulin-like hormone peptide that is believed to be important for placental apoptosis

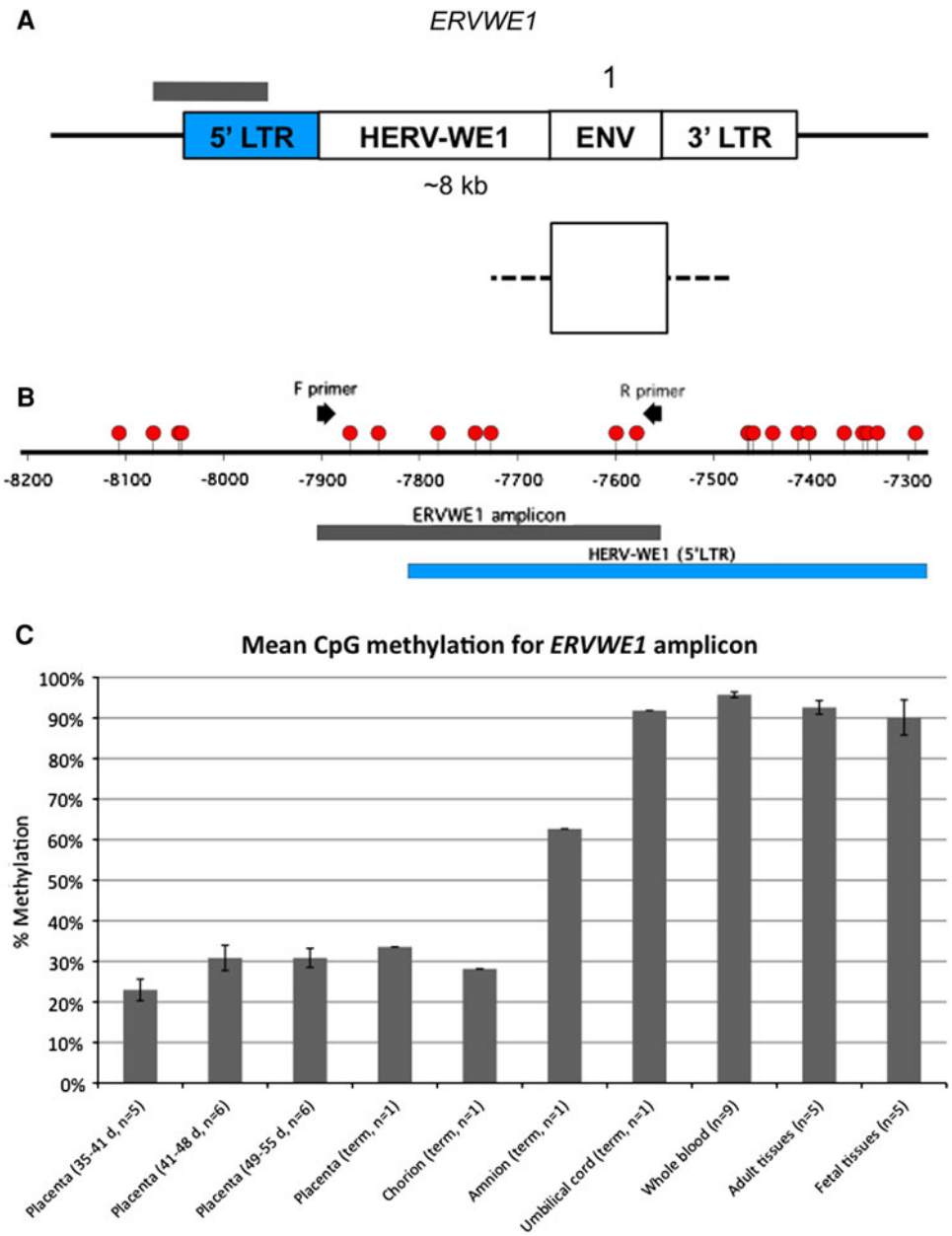

Fig. 5 Lineage-specific methylation of *ERVWE1*. **a** Genomic arrangement of *ERVWE1*. The envelope (ENV) sequence gives rise to *ERVWE1* (the syncytin-1 gene), the expression of which is regulated by a promoter derived from the 5′ LTR (in *blue*) of this HERV-WE1 (Blond et al. 2000; Cheng et al. 2004; Mi et al. 2000). The *grey bar* shows the region of differential methylation that was quantified by Sequenom. **b** The region of *ERVWE1* that was analysed by Sequenom. Coordinates refer to the genomic location with respect to the transcription start site. Forward and reverse primers are shown in *black*. *Red circles* represent CpG sites. The 352-bp amplified product for *ERVWE1* is shown in *grey*, which included 7 CpG sites for methylation analysis. **c** Mean promoter methylation for *ERVWE1*. *Error bars* represent 95% CI of the mean

(Bellet et al. 1997; Chassin et al. 1995; Millar et al. 2005). The promoter of *INSL4* contains an endogenous retrovirus (HERV) element that is postulated to mediate its placenta-specific expression in humans and recent primates (Fig. 4a) (Bieche et al. 2003). There are no reported alternative *INSL4* promoters or transcripts. *INSL4* is predominantly expressed in placenta; however, low levels of expression have also been detected in the maternal decidua at term (Millar et al. 2005).

The primate placenta-specific syncytin-1 gene (*ERVWE1*) is derived from the envelope protein (ENV) of a HERV and regulates the differentiation of cytotrophoblast into syncytiotrophoblast (Blond et al. 2000; Frendo et al. 2003; Mi et al. 2000; Voisset et al. 1999). *ERVWE1* expression is regulated by the 5′ LTR of this HERV, which is located approximately 8 kb upstream of the ENV sequence (Fig. 5a) (Cheng et al. 2004). *ERVWE1* methylation was quantified by Sequenom using previously reported primers (Matouskova et al. 2006) (Fig. 5b, primer sequences listed in Supplementary Table 2).

The endothelin B receptor gene (*EDNRB*) encodes a G-protein coupled receptor for endothelin (Agapitov and Haynes 2002; Arai et al. 1993). In the placenta, endothelins are important for maintaining fetal-maternal blood circulation (Handwerger 1995). The placenta-specific transcript of *EDNRB* is regulated by a promoter derived from the 5′ LTR of an HERV-E located approximately 57.5 kb upstream of the native first exon (Fig. 6a) (Medstrand et al. 2001; Reiss et al. 2007). This LTR-derived promoter

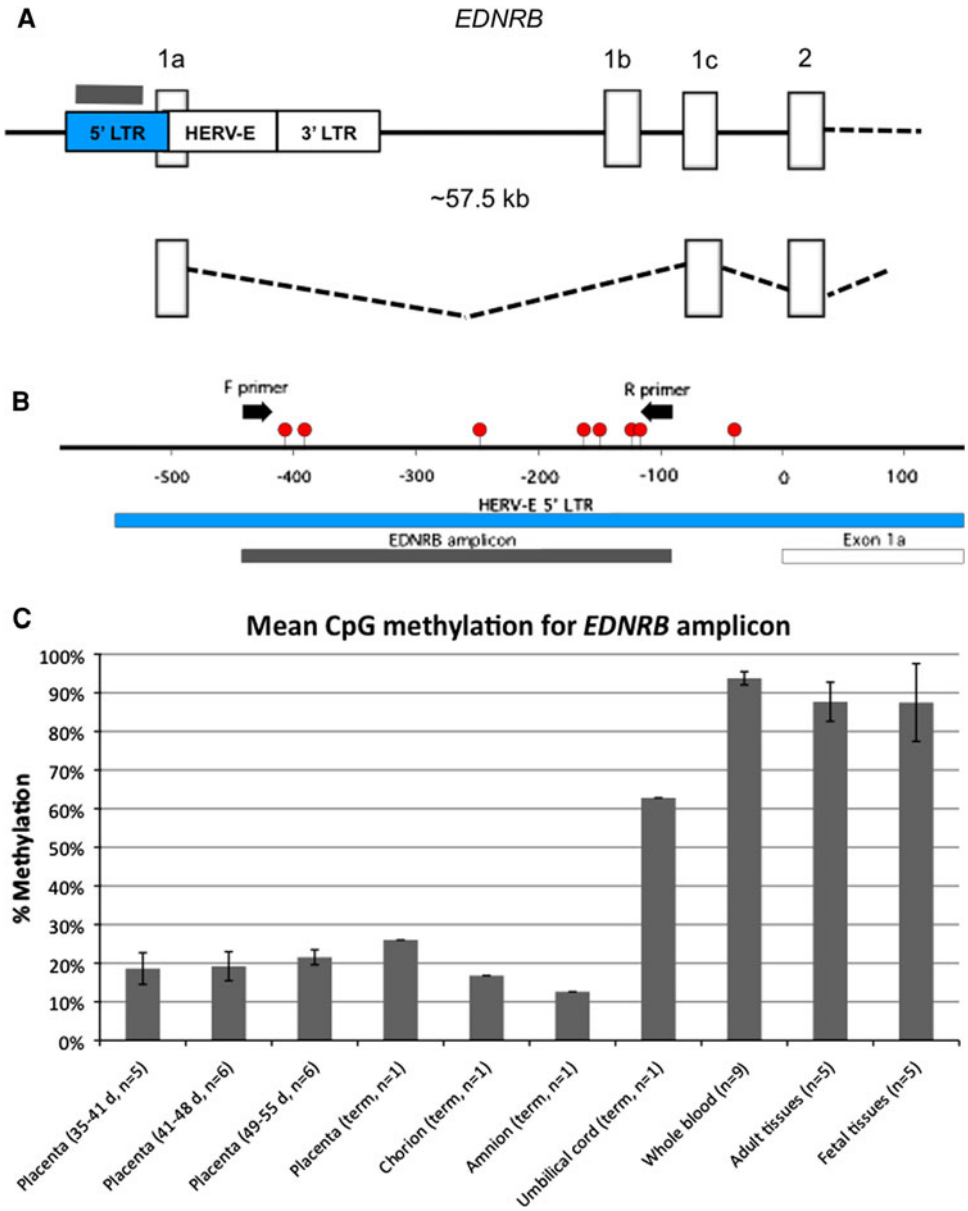

Fig. 6 Placental hypomethylation of *EDNRB*. **a** Genomic arrangement of *EDNRB* adapted from Reiss et al. (2007). The HERV-E located ~57.5 kb upstream of the ubiquitously expressed exons 1b, 1c, and 2 gives rise to a placenta-specific *EDNRB* transcript (shown), the expression of which is regulated by a promoter derived from its 5′ LTR (in *blue*) (Medstrand et al. 2001). The *grey bar* shows the region of differential methylation that was quantified by Sequenom. **b** The region of *EDNRB* that was analysed by Sequenom. Coordinates refer to the genomic location with respect to the transcription start site. Forward and reverse primers are shown in *black*. *Red circles* represent CpG sites. The 352-bp amplified product for *EDNRB* is shown in *grey*, which included seven CpG sites for methylation analysis. **c** Mean promoter methylation for *EDNRB*. *Error bars* represent 95% CI of the mean

regulates the expression of approximately 15% of *EDNRB* transcripts in the placenta, whereas *EDNRB* expression is initiated from downstream exons in all other tissues (Fig. 6a) (Landry and Mager 2003).

The pleiotrophin gene (*PTN*) encodes a heparin-binding cytokine protein that is involved in a wide range of functions, including cell differentiation and angiogenesis (Deuel et al. 2002). There are two *PTN* transcripts, one of which is expressed specifically in the placenta using a promoter derived from the 5′ LTR of an HERV-E (Fig. 7a) (Schulte et al. 1996). However, the *PTN* transcript derived from the non-LTR promoter is expressed in somatic tissues and has also been identified in placenta (Benson et al. 2009; Reiss et al. 2007).

The midline 1 gene (*MID1*) encodes a microtubule-associated protein that is a member of the RING finger family (Schweiger et al. 1999). *MID1* has five alternative promoters to express tissue-specific gene transcripts, one of which is expressed specifically in the placenta and fetal kidney, another is expressed in only adipose tissue, and the remaining three promoters are used ubiquitously (Landry and Mager 2002; Reiss et al. 2007). The transcript expressed in placenta and fetal kidney uses a promoter that is derived from the 5′ LTR of an HERV-E (Fig. 8a). Of the fetal tissues summarised in Fig. 8c, fetal kidney showed the lowest level of methylation (21%), which is consistent with the use of this LTR-derived promoter for *MID1* expression in fetal kidney (Landry and Mager 2002).

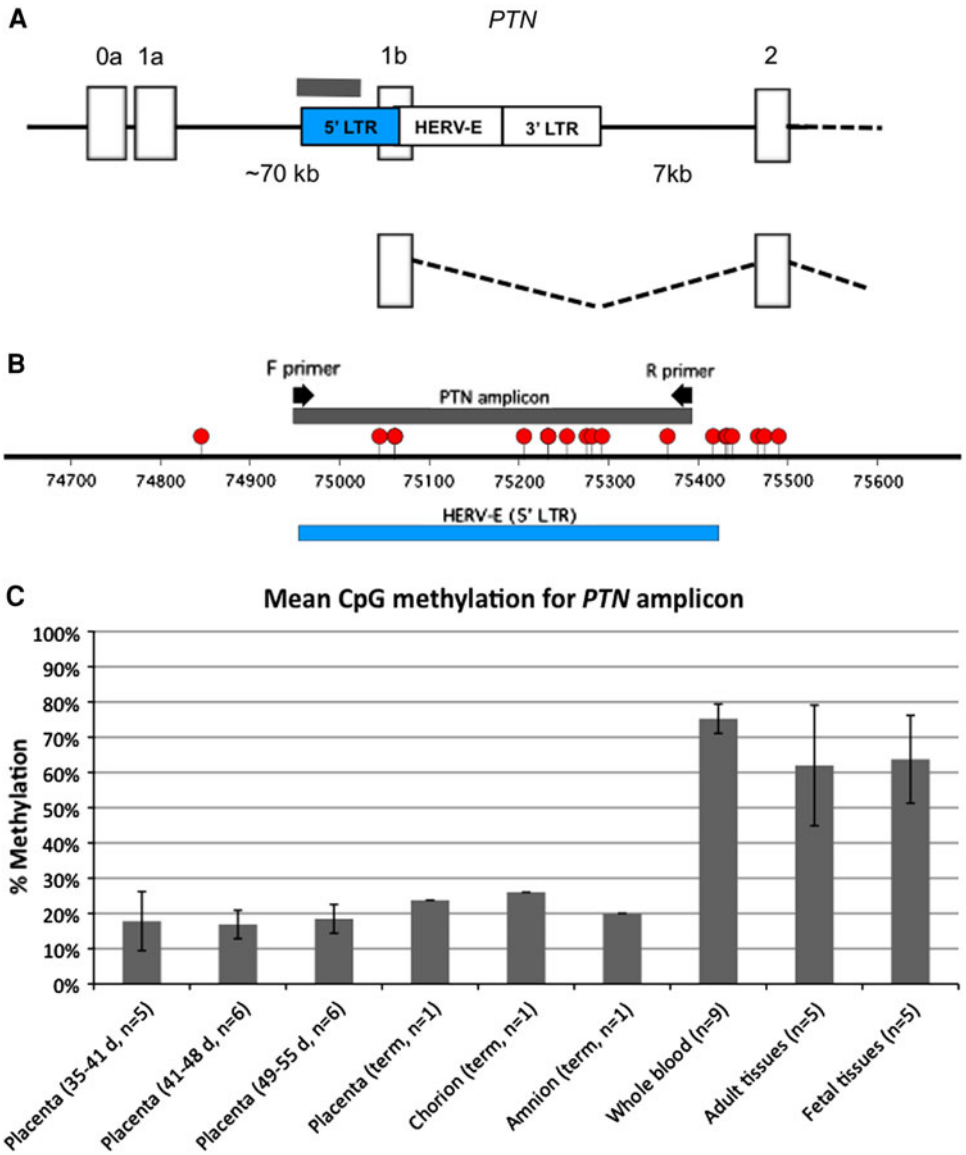

Fig. 7 Placental hypomethylation of *PTN*. **a** Genomic arrangement of *PTN* adapted from Reiss et al. (2007). The HERV-E gives rise to a placenta-specific *PTN* transcript (shown), the expression of which is regulated by a promoter derived from its 5′ LTR (in *blue*) (Schulte et al. 1996). The *grey bar* shows the region of differential methylation that was quantified by Sequenom. **b** The region of *PTN* that was analysed by Sequenom. Coordinates refer to the genomic location with respect to the transcription start site. Forward and reverse primers are shown in *black*. *Red circles* represent CpG sites. The 446-bp amplified product for *PTN* is shown in *grey*, which included nine CpG sites for methylation analysis. **c** Mean promoter methylation for *PTN*. *Error bars* represent 95% CI of the mean

In three of the retrotransposon-derived genes that we examined, i.e., *KCNH5*, *INSL4*, and *ERVWE1*, the level of promoter methylation in extra-embryonic tissues (including the chorion) is dichotomous with that in embryonic tissues (including the amnion). Differential expression of each of these genes presumably reflects the methylation-induced silencing of the exogenous DNA in epiblast-derived tissues and the escape from this mechanism in tissues derived from the extra-embryonic lineages. Given that retrotransposons are normally silenced by DNA methylation (an epigenetic modification that is assumed to be stable), these data provide evidence that the epigenetic switch that silences the expression of these retrotransposon-derived transcripts in the human embryo occurs during the earliest stages of cell lineage specification.

The strong expression of KCNH5 in the stomal and endothelial cells of the placental villous mesenchyme (Fig. 2) may provide evidence for the origin of the mesenchyme—a question that has not yet been resolved. The trophectoderm was ruled out as the lineage origin of the villous mesenchyme when studies of mesenchymal development in the rhesus monkey showed that the trophectoderm gives rise only to subpopulations of the trophoblast (Benirschke et al. 2006). Some have proposed that the villous mesenchyme is epiblast-derived and develops from the caudal end of the primitive streak, a structure that forms during early mammalian development and that gives rise to the germ layers (Downs 2009; Luckett 1978). Others, however, suggest that villous mesenchyme is derived from the primitive endoderm after a two-step process, where the primitive endoderm first forms a network structure, or reticulum, which then differentiates into the villous mesenchymal cells of the placenta (Bianchi et al. 1993; Enders and King 1988). Overall, there is a paucity of observations

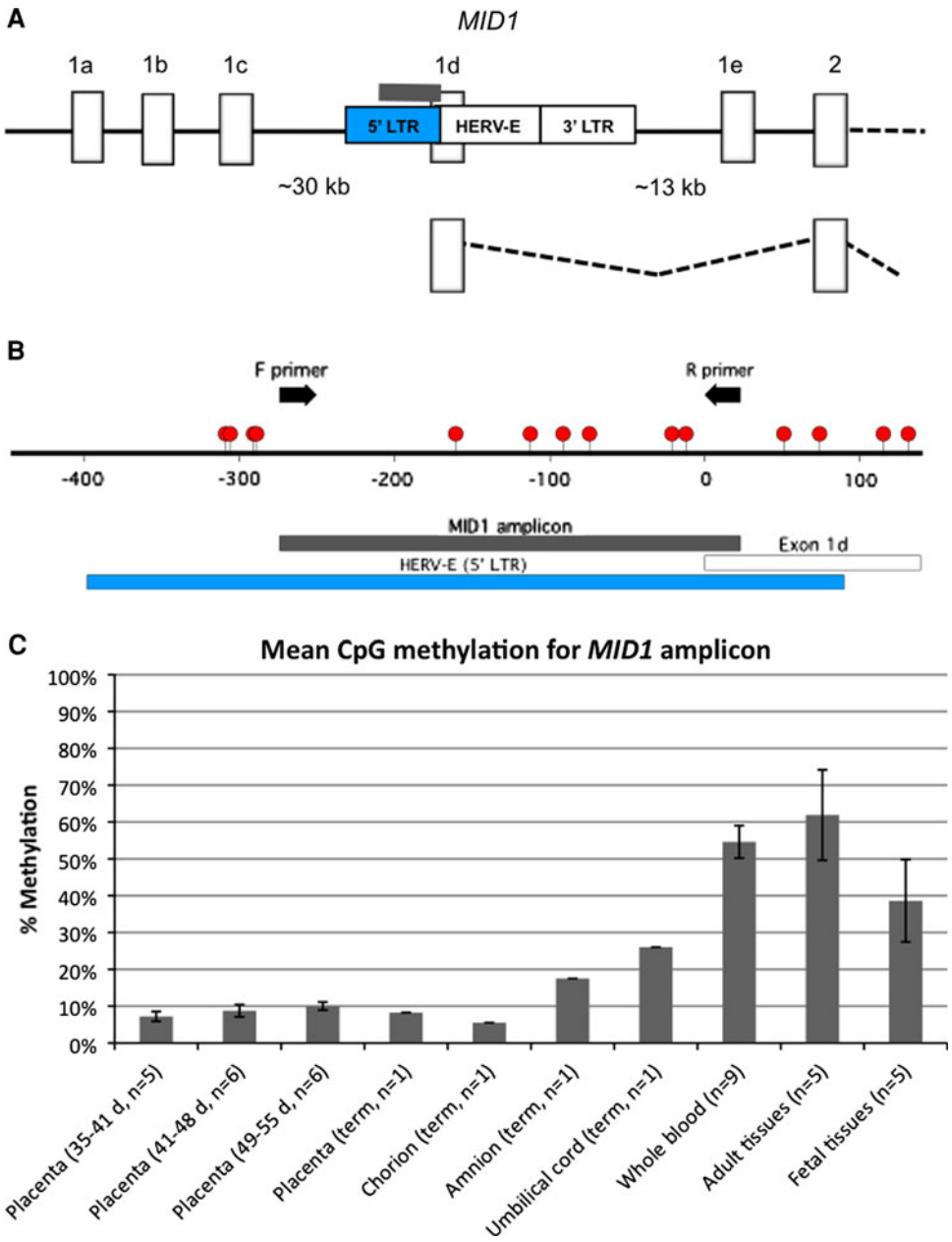

Fig. 8 Placental hypomethylation of *MID1*. **a** Genomic arrangement of *MID1* adapted from Reiss et al. (2007). The HERV-E gives rise to the placental *MID1* transcript (shown), the expression of which is regulated by a promoter that is derived from its 5′ LTR (in *blue*). This is one of five alternative promoters used to express *MID1* specifically in the placenta and fetal kidney (Landry and Mager 2002; Reiss et al. 2007). The *grey bar* shows the region of differential methylation that was quantified by Sequenom. **b** The region of *MID1* that was analysed by Sequenom. Coordinates refer to the genomic location with respect to the transcription start site. Forward and reverse primers are shown in *black*. *Red circles* represent CpG sites. The 298-bp amplified product for *MID1* is shown in *grey*, which included six CpG sites for methylation analysis. **c** Mean promoter methylation for *MID1*. *Error bars* represent 95% CI of the mean

supporting either the epiblast or the primitive endoderm as the lineage origin of the placental villous mesenchyme.

Given that total placental *KCNH5* transcription occurs exclusively from the unmethylated exon 1a promoter (Fig. 2d), we conclude that the expressing villous mesenchymal cells are unmethylated at exon 1a. With respect to the two possible sites of origin of the placental villous mesenchyme, there are two epigenetic scenarios. Since all tissues derived from the epiblast show dense methylation at the exon 1a promoter, the first scenario is that the placental mesenchyme demethylates *KCNH5* during transformation from the primitive streak to the mesenchyme. The second scenario is that the hypomethylation of *KCNH5* reflects the epigenetic state of its tissue of origin, thus favouring the primitive endoderm as its lineage origin. Although our conclusions are based on epigenetic and expression data for a single gene, our suggestion for primitive endoderm as the lineage origin of placental mesenchyme is supported by previously reported differences between the epigenetic states of the trophectoderm and primitive endoderm versus the epiblast. In comparison to embryonic tissues, both trophectoderm and primitive endoderm-derived tissues showed undermethylation of repetitive DNA sequences in the mouse (Chapman et al. 1984). This hypomethylation was not restricted to a certain class of repeat elements or a specific chromosomal location; instead, it was suggested to

be a feature of the entire genome in trophectoderm-derived and primitive endoderm-derived tissues (Chapman et al. 1984). Similarly, the epigenetic processes associated with X-chromosome inactivation in the mouse were reported to be different between the epiblast and the trophectoderm and primitive endoderm: X inactivation is random in the epiblast but imprinted in the trophectoderm and primitive endoderm (Mak et al. 2004; Monk and Harper 1979; Okamoto et al. 2004).

Importantly, hypomethylation of all five retrotransposon-derived genes reveals the epigenetic mechanism that permits the expression of these genes in the human placenta. Future work investigating the functional role of these unique genes may determine why the placenta allows these normally silenced repeat sequences to be actively expressed. Such findings may provide compelling evidence that retrotransposon function is not only necessary for the human placenta, but also that reduction in the level of retrotransposon expression, probably through gain of methylation, is detrimental to normal placental function.

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