Diversity of Epstein–Barr virus BamHI-A rightward transcripts and their expression patterns in lytic and latent infections

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INTRODUCTION

Epstein–Barr virus (EBV), a potentially oncogenic human herpesvirus, infects more than 90 % of adult populations worldwide. Although the vast majority of individuals harbouring EBV spend their lives as asymptomatic carriers, latent infections may induce endemic Burkitt’s lymphoma, nasopharyngeal carcinoma (NPC), gastric carcinoma (GC), NK/T-cell lymphoma, pyothorax-associated lymphoma (PAL), chronic active EBV infection (CAEBV) and so on (Rickinson & Kieff, 2001; Imai et al., 1998; Takakuwa et al., 2003). The latency-associated genes encode six nuclear antigens (EBNAs) and three latent membrane proteins (LMPs). In addition to EBV-encoded small RNAs (EBERs), large amounts of gene transcripts designated BamHI-A rightward transcripts (BARTs) or the complementary strand transcripts (CSTs) were first reported in the NPC C15 cDNA library in 1989 as a 4.8 kb product by Northern blot analysis (Hitt et al., 1989). Later, the expression of BARTs was reported in EBV-positive Burkitt’s lymphoma cell lines, and latent and lytic-stage EBV infections as well as NPC (Brooks et al., 1993; Chen et al., 1999; Kienzle et al., 1998; Zhang & Ooka, 1995). So far, at least six BART splicing forms have been reported: BARF0, RK-BARF0, RPMS1A, A73 and RB3. Functional analyses have demonstrated that the BARTs might serve as regulatory RNAs to limit lytic gene expression, and help ensure maintenance of the viral latency program (Karran et al., 1992). The six BARTs are also assumed to possess their own ORFs, and their functions have been reported (Zhang et al., 2001; Smith et al., 2000; Fries et al., 1997). Thus, the presence of BART proteins has been assumed in EBV-infected cells, although not all the proteins have been identified in vivo. Furthermore, a large number of microRNAs (miRNAs) are encoded in the intron of the BART gene (Cai et al., 2006). The levels of expression of the miRNAs are linked in part to the transcription of the BART mRNAs (Edwards et al., 2008). The reported sequences of BART splicing forms have differed, and no conclusion has yet been gained. We designed this study to clarify the sequences of the BART splicing forms and their expression.
METHODS

Cell line cell-culture conditions. A total of 36 cell lines were used in this study. They included 17 samples of Burkitt’s lymphoma, three of PAL, seven B-lymphoblastoid cell lines (LCLs) and nine T/NK-cell lines, including CAEBV, NK- and T-cell lymphomas (Imai et al., 1998). B-cell lines were grown in RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum (FCS) (Hyclone) and antibiotics. T- and NK-cell-type cell lines were grown in RPMI 1640 medium with 10% FCS or 10% pooled human AB type serum, recombinant human interleukin-2 (rIL-2; Takara, 50–100 U ml\(^{-1}\)), 2 mmol l-glutamine l\(^{-1}\), 1 mmol sodium pyruvate l\(^{-1}\), 0.1 mmol nonessential amino acids l\(^{-1}\) (Gibco-BRL), 2 mmol HEPES l\(^{-1}\), 50 \(\mu\)mol 2-mercaptoethanol l\(^{-1}\) and antibiotics. The cells were cultured in humidified air with 5% CO\(_2\) at 37 °C.

Preparation of frozen tissues. Two samples of PAL, three of nasal lymphoma (NK/T-cell type), three of nasal-type lymphoma (NK-cell type), three of NPC and two of GC were used in this study. All samples were stored at \(-80\) °C or in liquid nitrogen and were positive for EBV by checking with \(\text{BamHI}\) W region PCR or EBER in situ hybridization. All tissue materials were obtained for diagnostic or therapeutic purposes and utilized for the present study with approval of the ethical committee in Okayama University Hospital (no. 993).

RT-PCR. Total RNA was extracted from the samples with TRIzol reagent (Invitrogen), and cDNA was generated with a random hexamer (Takara) in the presence of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL). The cDNA samples were amplified by PCR using specific primer sets for BART-splicing forms (Fig. 1, Table 1). The integrity of the RNA was checked by the parallel amplification of \(\beta\)-actin mRNA. The PCR products were visualized by UV fluorescence after staining with ethidium bromide, subsequent to electrophoresis in 2% agarose gels.

Southern blot hybridization. The agarose gels after electrophoresis were blotted onto Hybond-N (Amersham Biosciences) nucleic acid transfer membranes. The membranes were then washed briefly and subjected to prehybridization and hybridization for 6 h with DIG-labelled internal oligonucleotide probes (Table 1). After the membranes were washed, anti-DIG peroxidase-labelled antibody (Dako) was used. For detection of probes on a blot, the ECL Western blotting Detection System (Amersham Biosciences) was used and the chemiluminescent signals were exposed to X-ray film for 30 s.

Generation of plasmids and sequence analyses of PCR products. After the PCR products had been processed by phenol/chloroform and ethanol precipitation, DNA was digested by \(\text{XbaI}\) or \(\text{HindIII}\) and \(\text{BamHI}\). The PCR products were ligated into the pUC119 plasmid vector.

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**Fig. 1.** Diagram showing the position on the genome and structure of BART mRNAs, and PCR primers used for detection of alternately spliced \(\text{BamHI-I–A}\) mRNAs. BART mRNAs are distinguished by different combinations of exons (boxes) and introns (thin lines). BART ORFs are also shown as shaded boxes. The number of amino acids in each ORF is indicated. The designed RT-PCR specific primer pairs are shown by arrowheads, and the expected sizes of the PCR products are also indicated. Numbers refer to the end nucleotides present in the spliced RNA at exon boundaries and are based on the updated EBV DNA sequence (accession no. AJ507799) (de Jesus et al., 2003).
vector (Takara) using T4 DNA Ligase (New England Biolabs). This ligation mixture was transformed into Escherichia coli (DH5α), and the incorporated plasmid DNA was extracted. Sequencing reactions were conducted using the BigDye Terminator Sequencing kit (Applied Biosystems) and resolved on an ABI 310 automated DNA sequencer (BE Biosystems). Sequences were read in the reverse direction using the cloning vector primer 5'-GTTTTCCCAGTCAGCAGG-3'.

### Quantitative analysis of BART mRNAs.
Quantitative RT-PCR was performed for BARF0, RPMS1, RPMS1A and A73 mRNAs with a LightCycler PCR system (Roche Diagnostics), using SYBR Green I as a double-strand DNA-specific binding dye. By performing a melting curve analysis at the end of each PCR, the identity of the PCR product of unknown samples could be confirmed by reference to its documented melting temperature ($T_m$).

### Relationship between BART mRNAs and EBV reactivation.
EBV-infected B-cell lines Akata, P3HR1 and Namalwa were reactivated with 20 ng ml⁻¹ phorbol 12-myristate 13-acetate (PMA). The samples were collected at pre-stimulation (control sample), and at 8, 24, 48 and 72 h after induction. RNA collection and cDNA synthesis were as described above. Quantitative RT-PCR was performed for BZLF1 and RPMS1 mRNA with a LightCycler PCR system (Roche Diagnostics) (Table 1). The ratio of drug-treated samples to the control in BZLF1 or RPMS1 mRNA was calculated.

### BART splicing form in B95-8 strain.
It has been shown that the B95-8 strain has a deletion in the BamHI I region of the BARTs (Sadler & Raab-Traub, 1995). B95-8 strain-derived cell lines such as IB4 and CLL-LCL, and non-B95-8 strain cell lines such as Akata, Rael and Jijoye, were included in the experiment. Total RNA collection, Table 1. Oligonucleotides used in RT-PCR and Southern blotting

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer or probe</th>
<th>Nucleotide sequence (5’–3’)</th>
<th>Coordinates</th>
<th>Annealing temperature or purpose</th>
<th>Amplified product</th>
</tr>
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<tr>
<td>BARF0</td>
<td>F-VA/B</td>
<td>TCTTCTAGAGAAGCGATATCCTGAGGATCAGGAGG</td>
<td>155322–155349, 156527–156529, 159803–159781, 158751–158750</td>
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<td>486 bp</td>
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<tr>
<td></td>
<td>R-VIA/B</td>
<td>AGGGAGATCCGCCACCCCACTGGGGCGCATCCG</td>
<td>155661–155690, 159919–159894</td>
<td>65 °C</td>
<td>238 bp</td>
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<tr>
<td>RK-BARF0</td>
<td>F-VA'</td>
<td>TCTCTAGATGGGAGAGGGTGATCTTGTAGTGTCGCC</td>
<td>156500–156526</td>
<td>72 °C</td>
<td>488 bp</td>
</tr>
<tr>
<td></td>
<td>R-VII'B'</td>
<td>AGGGGATCTCTGGTGACCGGCTCCCGGGGG</td>
<td>158776–158752</td>
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<td>485 bp</td>
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<tr>
<td>RPMS1</td>
<td>F-V</td>
<td>GAAAAGCTTGGGATTAATGCGGACCTTACAGGACGATGGTACGCTACCACG</td>
<td>149683–149707</td>
<td>72 °C</td>
<td>344 bp, 555 bp</td>
</tr>
<tr>
<td>RPMS1A</td>
<td>F-VA/B</td>
<td>Ditto</td>
<td>158661–158638, 138381–138403</td>
<td>72 °C</td>
<td>For BARF0, RPMS1, RPMS1A, A73, RB3, B95-8 strain analysis</td>
</tr>
<tr>
<td>A73</td>
<td>F-V</td>
<td>Ditto</td>
<td>158661–158638, 138381–138403</td>
<td>72 °C</td>
<td>For RK-BARF0</td>
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<tr>
<td>RB3</td>
<td>F-III'</td>
<td>TCTTCTAGAAATGGGGCGTGCTGCTGGCC</td>
<td>159803–159781, 158751–158750</td>
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<td>For B95-8 strain analysis</td>
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<tr>
<td></td>
<td>R-VIIA'</td>
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<tr>
<td>Probe</td>
<td>5'DIG-VIIA</td>
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<td>158644–158625</td>
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<td>F-BZLF1</td>
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<td>90045–90050, 90135–90148</td>
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<td>For B95-8 strain analysis</td>
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Table 2. Coordinates of mapped BART exons

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<th>Exon</th>
<th>Coordinates</th>
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<tr>
<td>I</td>
<td>138352–138480</td>
</tr>
<tr>
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<td>159844–159861</td>
</tr>
<tr>
<td>Ib</td>
<td>149923–150077</td>
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<td>II</td>
<td>146233–146334</td>
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<tr>
<td>III</td>
<td>149581–150077</td>
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<tr>
<td>IIIa</td>
<td>149581–149712</td>
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<tr>
<td>IIIb</td>
<td>149923–150077</td>
</tr>
<tr>
<td>IV</td>
<td>150237–150348</td>
</tr>
<tr>
<td>V</td>
<td>155267–156737</td>
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<tr>
<td>VA</td>
<td>155267–155349</td>
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<tr>
<td>VA'</td>
<td>155267–155808</td>
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<tr>
<td>VB</td>
<td>155267–155808</td>
</tr>
<tr>
<td>VI</td>
<td>156846–156928</td>
</tr>
<tr>
<td>VII</td>
<td>158625–160531</td>
</tr>
<tr>
<td>VIIA</td>
<td>158625–158751</td>
</tr>
<tr>
<td>VIIB</td>
<td>159781–160531</td>
</tr>
<tr>
<td>VIIB'</td>
<td>159844–160531</td>
</tr>
<tr>
<td>VIID</td>
<td>158625–159609</td>
</tr>
</tbody>
</table>
cDNA synthesis, PCR amplification, Southern blot hybridization and sequence analyses of the PCR products are the same as described above. The sequences of the primers and the annealing temperatures for PCR amplifications are indicated in Table 1.

RESULTS

Diagram of BART splicing forms

Six types of BART splicing forms have been reported to date (Chen et al., 1999; Smith et al., 1993, 2000; Sadler & Raab-Traub, 1995; Smith, 2001) (Fig. 1, Table 2). Two TATA-less promoter regions, designated P1 and P2, have been clarified. The P1 and P2 initiation position is detected at 138357 and 138081, respectively (Chen et al., 2005). Exon I is the major start of BART mRNAs, but the entire structures of Exons I and II have not yet been confirmed (de Jesus et al., 2003; Smith et al., 1993). All transcripts utilize the same polyadenylation signal at 160506 (Sadler & Raab-Traub, 1995; Chen et al., 1992). Exon III (497 nt) is known to be spliced into Exons IIIa/IIIb, and both forms exist in each BART. The 5' sequence of A73 Exon V has not been identified. RK-BARF0 has three derivatives with differentially spliced Exon VIIB' (Kienzle et al., 1999). ORFs for other BART members have also been proposed (Fig. 1). The BARF0 ORF consists of 174 aa. RK-BARF0, with 279 aa, constitutes the largest, and the three RK-BARF0 derivatives are 150, 177 and 190 aa in size. The

Fig. 2. Results of BART RT-PCR analyses. X-ray film was exposed to chemiluminescent signals for 30 s (upper panel), and for 3 min in RK-BARF0 and 5 min in RB3 (lower panel). Asterisk sign: indicated cell lines are derived from the B95-8 strain, which lacks the RB3 band because of a genome deletion (Fig. 1).
RPMS1, RPMS1A and A73 ORFs consist of 103, 40 and 126 aa, respectively. The RB3 ORF is assumed to have 51 and 69 aa, but a 51 aa ORF cannot be expressed when spliced into Exon IIIa and IIIb.

**Detection of four major and two minor BART mRNAs and their splicing variants**

RT-PCR followed by Southern blot hybridization demonstrated that mRNA of four major BARTs was constantly expressed in all EBV-infected cells: a 486 bp band in BARF0, a 488 bp band in RPMS1, a 485 bp band in RPMS1A and a 487 bp band in A73. mRNA of two minor BARTs, RK-BARF0 (238 bp) and RB3 (555 and 344 bp), was detected much more weakly and less frequently in 13/49 (27%) and 35/46 (76%) of samples, respectively (Fig. 2). BART splicing variants were also frequently detected in a 403 bp band in BARF0, 596 and 405 bp bands in RPMS1, 593 and 402 bp bands in RPMS1A, and 595 and 404 bp bands in A73 mRNA, but these signals were much weaker than the major bands.

**Existence of two splicing variants in BARF0, RPMS1, RPMS1A and A73 mRNA**

Sequence analyses were performed on the 486 and 403 bp PCR products in BARF0; 488, 596 and 405 bp products in RPMS1; 485, 593 and 402 bp products in RPMS1A; 487, 595 and 404 bp products in A73; 238 and 752 bp products in RK-BARF0; and 344 and 555 bp products in RB3 (Fig. 2). The underlined PCR products are the equivalent of major and minor BART mRNAs.

The novel bands of 403 bp BARF0, 405 bp RPMS1, 402 bp RPMS1A and 404 bp A73 proved to be missing Exon VI. In addition, other novel bands of 596 bp RPMS1, 593 bp RPMS1A and 595 bp A73 had an unspliced exon structure between Exon V and VI. The sequence of the 752 bp RK-BARF0 novel band constituted a new splicing form: Exon VA''-VB-VI-VIIA-VIIB. The coordinate of Exon VA'' was terminated with 156286 at the 3' site (Fig. 3). Although the A73 ORF (126 aa) exists across from Exon V to VIIB, alternative splices of A73 represented by novel bands 595 and 404 bp could produce 33 and 32 aa proteins, respectively, due to the formation of new stop codons. Similarly, novel band 752 bp RK-BARF0 would encode an 87 aa protein, relative to the 279 aa length of the authentic RK-BARF0 ORF.

**The RPMS1A and RPMS1 splicing forms are abundantly expressed among BART mRNAs**

The mRNA levels varied greatly among the cell lines and BART splicing forms, but all EBV-positive cell lines and tissue samples revealed the four major transcripts. Overall,
the RPMS1A and RPMS1 splicing forms were abundantly expressed. Among the cell lines, the RPMS1A splicing form was the most abundantly expressed. LCLs showed higher levels of BART mRNA. Genome-integrated cell lines Raji, Namalwa, PAL1 and OPL2 had extremely low expression levels of the four major types of BARTs. In tissue samples, GC and NPC samples expressed the RPMS1 transcript at very high levels compared with other transcripts (Fig. 4).

**Enhancement of BART expression in the lytic infection cycle**

The RPMS1 expression level of Akata and P3HR1 cells changed in parallel with BZLF1 mRNA in the presence of PMA. On the other hand, the RPMS1 expression level of Namalwa cells did not change, regardless of PMA stimulation (Fig. 5). All terms of RPMS1 and BZLF1 mRNA expression showed the same level without PMA stimulation (data not shown). These data explain why BART mRNAs, or at least RPMS1 transcripts, are upregulated on EBV reactivation.

**Expression of BARTs without the usage of ordinary ORFs**

The BARTs start site is thought to be just upstream of Exon I, and all the transcripts utilize the same polyadenylation signal, almost at the end of Exon VII (Smith et al., 2000). The B95-8 strain has also been reported to generate BARTs, despite the lack of Exons II, III and IV, and our data were consistent with that result, except for the RB3 form (Sadler).
& Raab-Traub, 1995; Baer et al., 1984; Parker et al., 1990).

RT-PCR and sequencing methods were performed to analyse the B95-8 strain splicing form. RT-PCR and Southern blotting showed three dense bands at 514, 406 and 136 bp in the B95-8 strain (Fig. 6). In the sequencing analysis, we obtained three splicing forms: a 514 bp band derived from Exon I-VA-VB-VI-VII, a 136 bp band from Exon I-VII, and a 406 bp band that was an unusual form. These three forms lacked RPMS1, RPMS1A and RB3 ORFs. The B95-8 strain cell line showed these three bands at high density, but the same bands in the other cell lines (Akata, Rael and Jijoye cells) were weak. These results indicate that BARTs are formed between the deletion in the BamHI I region.

DISCUSSION

We clarified the expression forms of the six BARTs encoded by the BamHI I–A region and their splicing variants in EBV-positive cell lines and tissue specimens. Of the six BARTs, mRNA of four major BARTs, including BARF0, RPMS1, RPMS1A and A73, was expressed in both lytic and latent EBV infections in all cell line cells and tissues examined. This observation is consistent with our study using crust samples obtained from patients with EBV-related skin disorders, and with previous reports (Brooks et al., 1993; Yamamoto et al., 2007). By contrast, the remaining two minor BARTs, RK-BARF0 and RB3 mRNA, were expressed less frequently, and when present, at much lower expression levels than those of the four major BARTs. Earlier investigators have reported that some patients harbouring EBV-positive tumour cells have antibodies and cytotoxic T-lymphocyte (CTL) responses to BARF0 and RK-BARF0, which may provide indirect evidence for their expression in vivo (Kienzle et al., 1999; Gilligan et al., 1991).

In the present study, the B95-8 strain was expected not to produce RPMS1, RPMS1A or RB3 mRNA because it lacks Exons II, III and IV, which contain ORFs for the above-mentioned transcripts (Fig. 1). The B95-8 strain-infected cells, however, expressed mRNA not only for BARF0 but also for RPMS1 and RPMS1A, by direct splicing of Exon I–V and Exon I–VII (Figs 2 and 6). This alternative transcription could be induced by frameshifts of the ORFs in the B95-8 strain-infected cells.

We detected at least two splicing variants of BARTs. Sequencing revealed that there were two common variants: a form of continuous sequence between Exons V and VI, and another form lacking Exon VI. These splicing variants were commonly detected in the four major BARTs (Fig. 3). These data suggest the possibility that there are unidentified splicing forms of BARTs and the related proteins in addition to the six known BARTs, as pointed out elsewhere (Chen et al., 1999; Smith et al., 1993, 2000; Sadler & Raab-Traub, 1995; Smith, 2001).

In quantitative analysis, the RPMS1A and RPMS1 splicing forms were abundantly expressed by any EBV-infected cell type. All the four major splicing forms, including BARF0, RPMS1, RPMS1A and A73 mRNAs, were detected at much lower levels when an EBV genome was integrated in the cellular DNA (Fig. 4). Cell line cells harbouring an integrated
EBV genome cannot readily be led to the lytic cycle, because lytic replication requires episomal viral DNA (Rickinson & Kieff, 2001; Lawrence et al., 1988; Chevalier-Greco et al., 1986; Daibata et al., 2002; Ooka et al., 1984). On the other hand, all four major transcripts were detected at much higher levels in LCLs. Furthermore, tissue samples from EBV-positive epithelial carcinoma cells such as NPC and GC were easily led to lytic cycle infection, thereby generating a great amount of RPM51 mRNA as compared with other transcripts (Hoshikawa et al., 2002; Chien et al., 2001; Lear et al., 1992).

The RPM51 mRNA was upregulated in association with the mRNA expression of a lytic infection-related, immediate early gene, BZLF1, in Akata and P3HR1 cells when these cells were stimulated with PMA (zur Hausen et al., 1978; Speck et al., 1997) (Fig. 5). Therefore, the high expression levels of the BART mRNA in epithelial carcinoma cells may depend on their susceptibility to induction of the lytic infection, because C/EBPα upregulates both the BZLF1 and BART P2 promoters, which are highly activated in epithelial cells (Chen et al., 2005). The four major BART mRNAs, including BARF0, RPM51, RPM51A and A73 mRNA, may be expressed vigorously by cells in the lytic infection cycles, but their expression was downregulated in the latent infection cycles, as observed in the cells with EBV genome integration.

Recently, several gene sequences related to miRNAs have been detected in the BamHI-A region of the EBV genome (BART-derived miRNAs) (Pfeffer et al., 2004). It has been postulated that the levels of BART-derived miRNAs are linked in part to the transcription of the BART mRNAs (Edwards et al., 2008). The mRNA-BART2 is located complementarily to BALF5 mRNA associated with lytic cycle infection. Therefore, the BART-derived miRNAs might control the maintenance of viral latency by blocking the expression of genes that lead to lytic cycle infection.

ACKNOWLEDGEMENTS

This work was supported by a Grant-in-Aid from the Japan Society for the Promotion of Science for Young Scientists (no. 19790787), and for Scientific Research (no. 23890238), and a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) for Scientific Research (C) (no. 25591653). We thank Dr Shosuke Imai (Toho Obstetrics and Gynaecology Hospital) for several cell lines and tissue samples.

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