MicroRNA-134 suppresses endometrial cancer stem cells by targeting POGLUT1 and Notch pathway proteins

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A B S T R A C T

We aimed to ascertain the role of microRNAs (miRNAs) in regulating human endometrial cancer stem cells (HuECSCs). The expression level of miRNA-134 (miR-134), a member of the DLK1-DIO3 genomic imprinted miRNA cluster, differed significantly between HuECSCs and human endometrial cancer cells (HuECCs). miR-134 inhibited HuECSCs proliferation and migration by targeting protein O-glucosyltransferase 1 (POGLUT1) expression. Exogenous miR-134 overexpression downregulated POGLUT1 and Notch pathway proteins in HuECSCs in vitro. miR-134 overexpression affected the G2/M phase of HuECSCs and suppressed the growth of xenograft tumours formed. Thus, endogenous miR-134 regulation in HuECSCs may suppress tumorigenesis in human endometrial carcinoma. © 2014 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

1. Introduction

Type II endometrial carcinoma (EC) is a common gynaecologic malignancy having a poor prognosis; it is oestrogen receptor-negative, poorly differentiated, and of high grade [1–3]. CD44+/CD133+ carcinoma stem cells from human type II EC cell lines KLE and AN3CA play a crucial role in proliferation, metastasis, recurrence, and development of chemotherapy resistance [4]. However, the potential molecular mechanisms involved proliferation and migration of CD44+/CD133+ carcinoma stem cells are poorly understood. MicroRNAs (miRNAs) are 19–23-nucleotide (nt) non-coding RNAs regulating biological processes essential for cancer initiation and progression, leading to affect the stability of their target genes by the binding of specific target mRNAs [5–7]. miRNAs can function as oncogenes or tumour suppressors [8–11]. In previous studies, Dong et al. reported that some microRNA influenced epithelial–mesenchymal transition, cancer stem cell “stemness” of human endometrial cancer by targeting the PTEN-P13K-akt-mTOR axis, EGFR/HER2 or VEGF-related pathway [12,13]. On the other hand, many miRNAs are part of a unique group termed the DLK1-DIO3 genomic imprinted miRNA cluster [14–18]. In humans, this region contains paternally expressed genes delta-like 1 homolog (DLK1), retrotransposon-like 1 (RTL1), and iodothyronine deiodinase 3 (DIO3), as well as maternally expressed genes MEG3, MEG8, and antisense RTL1 [14]. The DLK1-DIO3 region has undergone important evolutionary changes, the most important being the acquisition of the imprinting control region. It hosts 53 miRNAs on the forward strand and 1 miRNA on the reverse strand [14]. In the DLK1-DIO3 region, the 5’-end of the domain harbours DLK1, while DIO3 lies at the 3’-end [15]. Lethality and developmental abnormalities observed in conceptuses with maternal and paternal uniparental chromosome 14q32 disomy demonstrate the need for the correct dosage of imprinted genes in this domain [15]. Some miRNAs in the DLK1-DIO3 miRNA cluster regulate carcinoma proliferation and invasion [16–18]. Coordinated upregulation of the DLK1-DIO3 miRNA cluster at human chromosome 14q32.2 might define a novel molecular (stem cell-like) subtype of hepatocellular carcinoma associated with poor survival [16]. Inhibiting endogenous miRNA-134

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(miR-134), a part of the DLK1-DIO3 miRNA cluster, partially reverses the suppressive effects of hepatocyte nuclear factor 4x on Kirsten rat sarcoma viral oncogene homolog (KRAS) expression and hepatocellular carcinoma malignancy [17]. Several miRNAs in the DLK1-DIO3 miRNA cluster repress epithelial–mesenchymal transition in breast cancer by targeting the twist family bHLH transcription factor 1 (TWIST1) gene signalling network [18].

Protein O-glucosyltransferase 1 (POGLUT1) was initially identified in CD34+ cells of patients with acute myeloid leukaemia transformed from myelodysplastic syndrome [19–21]. The C-terminus of POGLUT1 contains a highly conserved domain (CAP10) and an endoplasmic reticulum (ER) retention signal motif (KT6); the N-terminus contains a hydrophobic signal peptide [21]. POGLUT1 is overexpressed in several leukaemia cell lines [19–21] and is an essential regulator of Notch signalling [19,21].

Many studies have been demonstrated that Notch pathway regulates cell fate of proliferation and growth in mammal [19,20,22–26]. Core components of Notch signalling are transmembrane ligands (Delta and Serrate/jagged) and receptors (Notch 1–4) and transcription factor CSL (C-promoter-binding factor) [19,20,22–26]. There are four types Notch receptors are single pass transmembrane glycoproteins, Notch extracellular domains (NECDs) 8 contain 29–36 tandemly organized N-terminal epidermal growth factor (EGF)-like repeats in mammalian. Upon translocation to the nucleus, Notch intracellular domain binds to CSL (CBF1/Su(H)/Lag-1), a transcriptional repressor that recruits the co-activator Mastermind, and the complex activates the expression of Notch target genes [19,20,22–26]. Previous reports revealed that Notch pathway could be regulated by glycosylation [7,11,12,18]. Because the O-fucosylation and O-glucosylation were two different special modification ways of NECDs [19,20,22–26]. The O-fucosylation induced ligand binding to Notch-1 receptors, while the O-glucosylation induced ligand cleaving Notch1 receptors [19,20,22–26]. To date, POGLUT1 has been demonstrated as an O-glucosyltransferase to be able to hydrolyze UDP-Glc in vitro [19,20,22–26]. Moreover, O-glucosyltransferase could regulate folding and/or trafficking of Notch receptor and allow signalling at the cell membrane [19,20,22–26].

In here, we discovered in this study that miR-134 directly suppresses POGLUT1 and that miR-134 overexpression inhibits human EC stem cell (HuECCs) proliferation and migration both in vitro and in vivo. Our results indicate that increased expression of exogenous mature miR-134 may inhibit HuECCs growth by reducing POGLUT1 expression. Thus, POGLUT1 might be an important new therapeutic target for human EC.

2. Materials and Methods

2.1. Isolation and in vitro expansion of CD44+/CD133+ tumour cells by fluorescence-activated cell sorting

EC tissues were collected from the International Peace Maternity and Child Health Hospital (Shanghai, China) between May 2012 and December 2012 (Supplement Table 1). CD44+/CD133+ cell subpopulations were isolated from tumour samples as previously described [4]. After isolation, single cells were plated at 1000 cells/mL in DMEM: F12 (HyClone) supplemented with 10 ng/mL basic fibroblast growth factor, 10 ng/mL epidermal growth factor, 5 µg/mL insulin, and 0.5% bovine serum albumin (all from Sigma–Aldrich). CD44+/CD133+ cells were cultured under the above conditions as non-adherent spherical clusters (HuECCs). CD44+–/CD133– cells were cultured under general conditions as adherent clusters (HuECCs). All cells were cultured under the same conditions until passage 4 before further experiments.

2.2. miR-134 overexpression vector construction

A miRNA expression plasmid was constructed as previously described [9]. Briefly, in the negative control plasmid, 9 nucleotides in the miR-134 seed sequence were mutated (GGTAGGCTG to GCTGAGGCA). HuECCs were seeded at 3 × 10^5 per well in diameter 10 cm cell petri dish and transfected with 30 µg wild-type (WT) or mutant (Mut) miR-134 vector using Lipofectamine 2000 (Invitrogen).

2.3. Luciferase report assay

NIH-3T3 mouse embryonic fibroblast cells and HuECCs were seeded at 3 × 10^5 per well in 48-well plates and co-transfected with 400 ng pRNAT-CMV32-miR-134, pRNAT-CMV32-miR-134-Mut, or pRNAT-CMV32 (empty vector); 20 ng pGL3 cm-Poglut1-3UTR or pGL3 cm-Poglut1-3UTR-Mut; and pRl-TK (Promega, Madison, USA) using Lipofectamine 2000. Luciferase activity was measured after 48 h using the Dual-Luciferase Reporter Assay System (Promega, Madison, USA) [9].

2.4. RNA extraction and quantitative real-time PCR analysis

Total RNA was isolated from each cell type using TRIzol Reagent (Invitrogen). RNA samples were treated with DNase I (Sigma–Aldrich), quantified, and reverse-transcribed into cDNA using the ReverTra Ace-α First Strand cDNA Synthesis Kit (Toyobo). Quantitative real-time PCR (qRT-PCR) was conducted using a RealPlex4 real-time PCR detection system (Eppendorf, Germany) with SYBR Green Realtime PCR Master Mix (Toyobo) at 40 cycles of denaturation at 95 °C for 15 s and annealing at 58 °C for 45 s. Target cDNA was measured by relative quantification. A comparative threshold cycle (Ct) was used to determine relative gene expression normalized to 18S rRNA. For each sample, Ct values were normalized using the formula ΔCt = Ct–genes – Ct_18S RNA. Relative expression levels were calculated using the formula ΔΔCt = ΔCt_all_groups – ΔCt_blankcontrol_group. Values applied to plot relative gene expression were calculated using the expression 2^-ΔΔCt. Primers used for cDNA amplification were as previously described [20].

2.5. Cell proliferation assay

Cells were seeded at 2 × 10^3 per well in 96-well plates and cultured in DMEM supplemented with 10% foetal bovine serum (FBS) at 37 °C under 5% CO2 until 85% confluence. Methyl thiazolyl tetrazolium (MTT; Sigma Chemicals) (5 mg/mL) was added at different time points and incubated for a further 4 h. The reaction was terminated by adding 150 µL/well dimethyl sulphoxide (Sigma Chemicals). Cells were lysed for 15 min, and plates were gently shaken for 5 min. Absorbance at 490 nm was determined using a Model 680 Microplate Reader (Bio-Rad).

2.6. miRNA microarray analysis

RNA labelling and hybridization on miRNA microarray chips were conducted as previously described [27,28]. Total RNA (50 µg) was purified using the mirVANA miRNA isolation kit (Ambion, Austin, TX) to enrich the small RNA fraction. Purified RNA was labelled with fluorescein, and hybridization was carried out on the Agilent Mammalian miRNA Array (Shanghai genomePi- lot Technology, Inc., Shanghai, China) containing oligonucleotide probes for 2844 mature miRNA genes in triplicate (1823 human, 648 mouse, and 373 rat sequences). RNA samples were analysed on an individual chip. Hybridization signals were detected, and
scanner images were quantified. These quantified signal intensity values were normalized to per-chip mean values.

2.7. Northern blotting

For all cell treatment groups, 20 µg high-quality total RNA was analysed on a denaturing gel (7.5 M urea, 12% PAA) and transferred to a Hybond N+ nylon membrane (Amersham, Freiburg, Germany). Membranes were cross-linked using ultraviolet light for 30 s at 1200 mJ/cm² and hybridized to the miR-134 antisense Starfire probe, 5'-TTGGTGACTAGGTGGCCCACAGG-3' (IDT, Coralville, IA) to detect 22-nt miR-134 fragments. Membranes were washed and exposed to Kodak XAR-5 film for 20–40 h (Sigma–Aldrich). A human U6 snRNA probe (5'-GCCAGGCCCAGCTTAATCTTCTCGTATTC-3') was used as a positive control (exposure time, 15–30 min) [10].

2.8. Western blotting

Total protein extracts of each cell treatment group were resolved by 12% SDS–PAGE and transferred onto polyvinylidene difluoride (Millipore) membranes. Membranes were blocked and then washed 4 times for 15 min with Tris-buffered saline containing 0.3% Tween-20 at room temperature and then incubated with primary polyclonal antibodies (Supplement Table 3) [10]. Membranes were washed and incubated at room temperature with secondary peroxidase-linked goat anti-rabbit IgG (1:1000 dilution; Santa Cruz Biotechnology) for 1 h. Protein bands were visualized by enhanced chemiluminescence (ECL kit; Pierce Biotechnology) and autoradiography.

2.9. Flow cytometric analysis of cell cycle by propidium iodide staining

Cells of each group were seeded at 3 × 10⁵ per well in 6-well plates and cultured until 85% confluence. The cells were then washed with PBS thrice and collected by centrifugation (Allegra X-22R, Beckman Coulter) at 1000×g for 5 min. Cell pellets were resuspended in 1 mL PBS, fixed in 70% ice-cold ethanol, and kept in a freezer for more than 48 h. Before flow cytometry, the fixed cells were centrifuged, washed twice with PBS, and resuspended in propidium iodide (PI) staining solution (50 µL/mL PI and 250 µg/mL RNase A; Sigma–Aldrich). The cell suspension was incubated for 30 min at 4 °C and analysed by FACS (Quanta SC FlowCytometry, Beckman Coulter Commercial Enterprise (China) Co. Ltd., Shanghai, China). Using CellQuest software, 20000 events were acquired for analysis.

2.10. Soft agar colony-formation assay

Soft agar assays were set up in 6-well plates. The base layer of each well had 2 mL of 1 × medium (DMEM + 10% FBS) and 0.6% low-melting agarose. Plates were chilled at 4 °C until solid. A 1-mL growth agar layer comprising 1 × 10⁴ cells suspended in 1 × medium and 0.3% low-melting agarose was poured over this. Plates were again chilled at 4 °C until the growth layer congealed. Additional 1 mL 1 × medium without agarose was added over the growth layer on day 0 and again on day 15 of growth. Cells were allowed to grow at 37 °C for 1 month and total colonies counted. Assays were repeated thrice. Results were analysed by paired t-test using PRISM Graphpad [9].

2.11. Transwell migration assay

Cells (2 × 10⁵) were resuspended in 200 µL serum-free medium and seeded on the top chamber of 8.0-µm pore, 6.5-mm polycarbonate Transwell filters (Corning). Medium (600 µL) containing 10% FBS was added to the bottom chamber. Cells were allowed to migrate for 24 h at 37 °C in a humidified incubator with 5% CO₂. Cells attached to the lower surface of the membrane were fixed in 4% paraformaldehyde at room temperature for 30 min and stained with crystal violet (Beyotime Institute of Biotechnology, China). Cells on the lower surface of the filters were counted using a microscope. Five fields were counted for each Transwell filter [9].

2.12. In vivo xenograft experiments

Logarithmically growing HuECSCs (1 × 10⁵) were inoculated into BALB/c nude mice. Each experimental group comprised 4 mice. After 12 weeks, the mice were sacrificed and tumours surgically excised [4]. Tumours were weighed, and the volume was calculated as follows: tumour volume (mm³) = (ab²)/2, where a represents the longest axis (mm) and b the shortest axis (mm).

2.13. Statistical analysis

Each experiment was performed as least 3 times; data are presented as mean ± SE where applicable. Differences were evaluated using Student’s t-tests. P values < 0.05 were considered statistically significant.

3. Results

3.1. Mature miR-134 in the DLK1-DIO3 miRNA cluster was downregulated in CD44+/CD133+ HuECSCs

The HuECSCs CD44+/CD133+ subpopulation in both KLE and AN3CA is relatively small [4]. Therefore, we used magnetic activated cell sorting to isolate and enrich the CD44+ and CD133+ subpopulations from the tissues of 4 EC patients [4]. Then, we used miRNA microarray analyses to identify differentially expressed miRNAs in CD44+/CD133+ HuECSCs and CD44+/CD133–HuECSCs. The expression of 1 miRNA decreased by >2-fold in HuECSCs compared to that in HuECCs, and the expression of 9 miRNAs increased by >2-fold (Supplement Table 2). miR-134 was expressed at significantly lower levels in HuECSCs than in HuECCs (Fig. 1A). To confirm this finding, northern blotting and miRNA-specific quantitative reverse transcription-PCR was used to measure mature miR-134 expression in HuECSCs and HuECCs. miR-134 hybridization signals were weaker in HuECSCs extracts than in HuECC extracts, revealing that miR-134 was downregulated in HuECSCs (Fig. 1B). miR-134 expression levels were found to be significantly lower in HuECSCs than in HuECCs (Fig. 1C).

3.2. Identification of miR-134 binding sites in the POGLUT1 3'-untranslated region

The miRBase Target database was used to analyse precursor miRNA sequences, mature miRNA sequences, chromosomal locations, miR-134 length, and potential target sites in POGLUT1. A putative 7-nt miRNA target site was identified in the human POGLUT1 mRNA 3'-untranslated region (3'-UTR) (Fig. 1D). To examine whether mature miR-134 regulated POGLUT1 expression, a luciferase reporter containing WT or Mut POGLUT1 3'-UTR miR-134 binding site, or an empty plasmid control was co-transfected with miR-134 expression vectors (WT-miR-134, Mut-miR-134, or empty vector control) into NIH-3T3 cells and HuECSCs. WT POGLUT1 3'-UTR luciferase reporter activity was significantly inhibited following miR-134 co-transfection; Mut POGLUT1 3'-UTR luciferase reporter activity was unaffected by the miR-134 co-transfection not only in NIH-3T3 cells, but also in HuECSCs (Fig. 1E). Thus, miR-134 targets POGLUT1 miRNA by specifically binding to its 3'-UTR.
3.3. miR-134 suppressed HuECSCs proliferation via cell cycle arrest

MTT assays revealed significant differences in the viability of HuECSCs transfected with WT or Mut miR-134. Compared to Mut miR-134-transfected cells, WT miR-134 overexpression significantly reduced HuECSCs proliferation after 24 and 48 h (Fig. 2A), indicating that exogenous miR-134 overexpression inhibits HuECSCs growth in vitro. Furthermore, flow cytometry revealed significant cell cycle arrest in the WT miR-134-transfected HuECSCs. Compared to the Mut miR-134-transfected HuECSCs, G2/M was arrested in WT miR-134-transfected HuECSCs, and the percentage of HuECSCs in the S phase significantly decreased at 48 h (Fig. 2D). Thus, exogenous miR-134 overexpression suppresses proliferation.

3.4. miR-134 weakened ability of HuECSCs to form sphere clone and multi-drug resistance

In order to evaluate whether exogenous miR-134 overexpression could suppress HuECSCs migration. In the soft agar colony-formation assay, miR-134-transfected cells plated at low density formed substantially fewer colonies (colony-formation efficiency: 16.86% ±

3.5. miR-134 suppressed HuECSCs migration

We examined whether exogenous miR-134 overexpression could suppress HuECSCs migration. In the soft agar colony-formation assay, miR-134-transfected cells plated at low density formed substantially fewer colonies (colony-formation efficiency: 16.86% ±
than Mut miR-134-transfected cells (colony-formation efficiency: 37.65% ± 5.76%; Fig. 2B).

In the Transwell migration assays, there were significantly fewer migrating WT miR-134-transfected cells/well (migrating cell numbers: 40 ± 7) than Mut miR-134-transfected cells/well (migrating cell numbers: 129 ± 18, Fig. 2C). These data indicate that exogenous miR-134 overexpression downregulates HuECSCs migration.

3.6. miR-134 overexpression in HuECSCs attenuated POGLUT1 and Notch pathway protein expression

We used qRT-PCR and Western blotting to evaluate the expression levels of POGLUT1 and Notch pathway proteins Notch-1, C promoter-binding factor 1 (CBF1), hes family bHLH transcription factor 1 (HES1), and p27 in WT and Mut miR-134-transfected HuECSCs. At 48 h, Poglut1, Cbf1, and Hes1 mRNA levels were all significantly lower in WT miR-134-transfected cells than in Mut-miR-134-transfected cells (Fig. 3A). Western blotting confirmed that the expression of POGLUT1 and Notch pathway proteins was significantly lower in WT miR-134-transfected HuECSCs compared to Mut-miR-134-transfected HuECSCs (Fig. 3B). However, both qRT-PCR and Western blotting showed that p27 expression was higher in WT miR-134-transfected HuECSCs than in Mut-miR-134-transfected HuECSCs (Fig. 3A and B). Therefore, exogenous miR-134 overexpression downregulates both endogenous POGLUT1 and Notch pathway proteins in HuECSCs.

3.7. miR-134 overexpression inhibited HuECSCs xenograft growth

The effect of miR-134 overexpression on tumour growth in vivo was investigated by subcutaneous inoculation of WT or Mut miR-134-transfected HuECSCs into nude mice. WT miR-134-transfected HuECSCs developed tumours. Although both experimental groups eventually developed tumours, the tumours formed by WT miR-134-transfected cells grew more slowly than tumours formed by Mut miR-134-transfected cells (Fig. 4B). Furthermore, when the mice were sacrificed 12 weeks after injection, the tumours from the Mut miR-134-transfected group were significantly heavier than those of the WT miR-134-transfected group (Fig. 4C).

4. Discussion

Our team first reported the existence of a CD44+/CD133+ subpopulation in type II EC [4]. CD44+/CD133+ HuECSCs are highly proliferative, highly migratory, multidrug resistant, and highly tumourigenic [4]. We hypothesized that HuECSCs are the plausible root cause of metastasis or recurrence in EC; however, the mechanisms are still not understood. Using our previous miRNA research findings as a foundation, we focused on miRNA regulation to discover the regulatory factors and target genes underlying HuECSCs proliferation and migration to elucidate its mechanism. Here, we used miRNA microarray to analyse HuECSCs miRNA expression patterns. Interestingly, we found a unique miRNA group among the differentially expressed miRNAs: the DLK1-DIO3 miRNA cluster. Some HuECSCs and HuECC miRNAs had significantly different expression, and these were among the DLK1-DIO3 miRNA cluster. This cluster at 14q32.2 is closely related to tumour...
occurrence and development [14,16–18]. However, here, we found that HuECSCs miR-134 expression in the DLK1-DIO3 miRNA cluster was significantly decreased. Zha et al. used genome-wide screening and determined that miR-134 inhibited focal adhesion kinase phosphorylation and RhoA activation downstream of the b1 integrin pathway, thereby decreasing stress fibre formation and cell adhesion in hepatocellular carcinoma [29]. Li et al. reported that miR-134 inhibited transforming growth factor-β1-induced epithelial–mesenchymal transition by targeting forkhead box M1 in non-small cell lung cancer cells [30]. Yin et al. indicated that inhibiting endogenous miR-134 partially reversed the suppressive effects of hepatocyte nuclear factor 4a on KRAS expression and hepatocellular carcinoma malignancy [17]. Therefore, we hypothesized that miR-134 might be a suppressor of HuECSCs. Following bioinformatics analysis and firefly luciferase reporter assay, we identified POGLUT1 as a target gene of miR-134. Currently, some studies preliminarily elucidated relation between POGLUT1 and the Notch pathway [19,20]. Wang and Ma et al. showed that knockdown of POGLUT1 expression influenced ligand induced Notch activation, and weakened proliferation of leukemia U937 cell through CDK1-RB signalling pathway [19]. But also, they found that POGLUT1 did not affect the binding of cell surface Notch1 to either Delta1 or Jagged1, which might be related with different role of O-fucosylation and O-glucosylation in modulating Notch signalling (Because O-fucosylation inducing ligand binding to Notch1 receptors, but O-glucosylation inducing ligand cleaving Notch1 receptors) [19]. In addition, Chu and Wang et al. indicated that POGLUT1 was overexpressed in primary acute myelogenous leukaemia, T-acute lymphoblastic leukaemia samples and other leukaemia cell lines. And, POGLUT1 promoted Notch pathway activation through CDKI-RB signalling [20]. Thus, two studies on POGLUT1 suggested that its overexpression enhances Notch signalling pathway activation and stimulates cancer cell proliferation [19,20]. The Notch signalling pathway is established as being highly conserved, from Drosophila to mammals, and it regulates a wide range of cell fate decisions through direct cell–cell contact [19,20]. The Notch signalling pathway is regulated by many post-translational modifications, especially glycosylation [19,20]. Two unusual forms of modification of the Notch extracellular domain are O-fucosylation and O-glucosylation [19,20]. O-fucose is added by O-fucosyltransferase 1 (Ofut1/Pofut1 in Drosophila and mammals) and elongated by Fringe, and the roles of both in Notch signalling have been studied extensively [19,20]. However, the possible roles of miR-134, the related target gene Poglut1, and miR-134 regulation of the Notch pathway in HuECSCs are still not well elucidated.

The present study results demonstrate that miR-134 overexpression in HuECSCs downregulated the expression of POGLUT1 and Notch signalling pathway proteins NOTCH1, CBF1, and HES1 but upregulated the expression of the cell cycle-related gene p27. miR-134 overexpression also reduced HuECSCs proliferation and migration, both in vitro and in vivo. Thus, miR-134, which is a part of the DLK1-DIO3 miRNA cluster, has a potential ability to attenuate proliferation and migration of HuECSCs by targeting POGLUT1 and Notch pathway protein expression.
Conflict of interest statement

No conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.12.002.

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murine embryo and placenta by the DLK1-Dio3 imprinting control region. 


