Generation of human endometrial knockout cell lines with the CRISPR/Cas9 system confirms the prostaglandin F2α synthase activity of aldo-ketoreductase 1B1

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ABSTRACT: Prostaglandins (PGs) are important regulators of female reproductive function. The primary PGs produced in the endometrium are PGE2 and PGF2α. Relatively little is known about the biosynthetic pathways leading to the formation of PGF2α. We have described the role of aldo-ketoreductase (AKR)1B1 in increased PGF2α production by human endometrial cells following stimulation with interleukin-1β (IL-1β). However, alternate PGF synthases are expressed concurrently in endometrial cells. A definite proof of the role of AKR1B1 would require gene knockout; unfortunately, this gene has no direct equivalent in the mouse. Recently, an efficient genome-editing technology using RNA-guided DNase Cas9 and the clustered regularly interspaced short palindromic repeats (CRISPR) system has been developed. We have adapted this approach to knockout AKR1B1 gene expression in human endometrial cell lines. One clone (16-2) of stromal origin generated by the CRISPR/Cas9 system exhibited a complete loss of AKR1B1 protein and mRNA expression, whereas other clones presented with partial edition. The present report focuses on the characterization of clone 16-2 exhibiting deletion of 68 and 2 nucleotides, respectively, on each of the alleles. Cells from this clone lost their ability to produce PGF2α but maintained their original stromal cell (human endometrial stromal cells-2) phenotype including the capacity to decidualize in the presence of progesterone (medroxyprogesterone acetate) and 8-bromo-cAMP. Knockout cells also maintained their ability to increase PGE2 production in response to IL-1β. In summary, we demonstrate that the new genome editing CRISPR/Cas9 system can be used in human cells to generate stable knockout cell line models. Our results suggest that genome editing of human cell lines can be used to complement mouse KO models to validate the function of genes in differentiated tissues and cells. Our results also confirm that AKR1B1 is involved in the synthesis of PGF2α.

Key words: prostaglandins / gene expression / cell culture / endometrium / gene mutations

Introduction

Prostaglandins (PGs) are synthesized by virtually all nucleated cells of the body and represent a family of lipid mediators acting locally to maintain homeostasis through complementary and sometimes opposite actions (Poyser, 1995; Lim et al., 1997; Thatcher et al., 2001; Jabbour and Sales, 2004; Spencer et al., 2007; Smith et al., 2011). PGs are particularly important for normal female reproductive function and also contribute to pathological conditions in that system (Jabbour and Sales, 2004). Prostaglandin F2α (PGF2α) contribute to parturition and initiation of menstruation. Painful menstruation is attributed to an ischemic response to abnormally high levels of PGF2α. The first step in PG formation is the conversion of arachidonic acid (AA) liberated from membrane phospholipids into prostaglandin G2 (PGG2) and then prostaglandin H2 (PGH2) by one of two prostaglandin G/H synthases (PTGS-1 or -2) better known as cyclooxygenases (COX-1 or -2). After PGH2 formation, terminal synthases generate the bioactive PGs (Smith et al., 2011) (Fig. 1). Specific terminal synthases required for enzymatic production of PGs were identified for PGF2α (Tanikawa et al., 2002), thromboxane A2 (TxA2) (Tanabe and Ullrich, 1995), prostacyclin (PGI2) (Ullrich et al., 1981), prostaglandin D2 (PGD2) (Christ-Hazelhof and Nutergen, 1979) and more recently for PGF2α (Bresson et al., 2011). Non-steroidal
anti-inflammatory drugs such as aspirin, ibuprofen and naproxen aim at inhibiting PG biosynthesis at the level of PGHSs (Fig. 1). While PGF2α has not been studied as extensively as other PGs, it nevertheless plays important roles in the regulation of ocular pressure, renal absorption, adipocyte differentiation as well as cardio-vascular and female reproductive functions (Bresson et al., 2012). The immortalization of normal human endometrial cell lines (Chapdelaine et al., 2006) in our laboratory allowed us to study PG biosynthesis at the cellular and molecular levels and to highlight a new function of aldo-ketoreductase (AKR) 1B1 as a potent PGF2α synthase (Bresson et al., 2012). Indeed, we have observed that increased production of PGF2α in response to IL-1β was associated with up-regulation of AKR1B1 and COX-2 proteins (Bresson et al., 2012). Most prostaglandin F2α synthases (PGFS) identified to date are AKRs. We have demonstrated that in addition to AKR1C3, AKR1B1 was able to produce PGF2α in the endometrium (Bresson et al., 2012) and AKR1A1 may also play the same function (Lacroix Pepin et al., 2013). Since several proteins with putative PGF synthase (PGFS) activity are expressed concurrently in the same cells at the same time, identification of the primary PGF synthase involved under specific conditions would require complete and selective inhibition of individual synthases. Pharmacologic inhibitors do not exhibit the required selective action and siRNAs do not lead to complete inhibition of expression (Bresson et al., 2012). The mouse does not constitute an appropriate model for AKR1B1 because its activities are covered by two parent, but not identical enzymes; AKR1B3 and AKR1B7 (Kabututu et al., 2009; Pastel et al., 2012). In spite of that, null mutations for each of AKR1B3 and AKR1B7 were generated and lead to interesting observations regarding the physiological role of AKR1Bs (Lo et al., 2007; Kabututu et al., 2009). Unfortunately, only little consideration was given to PGFS activity for AKR1B7 and none for AKR1B3. Indeed loss of AKR1B7 was associated with a 50% decrease in PGF2α levels in adipose tissue and cells, while the remaining 50% could be attributed to AKR1B3 (Volat et al., 2012). Aldose-reductase-like enzymes are highly related to often co-expressed isozymes, making functional analysis of one isoform or the other a challenging task.

In order to provide a definite proof of AKR1B1 contribution to uterine PG production, genome editing of an endometrial cell line appeared as an elegant solution. Indeed, it would provide a functional model comparable to a conditional endometrial knockout in humans. The recently described Clustered Regularly Interspaced Short Palindromic Repeats combined with CAS9 (CRISPRs-Cas9) system appeared as a very potent approach to reach this goal.

Recent development in genome editing brought new technologies such as zinc finger nucleases (ZFNases), transcription activator-like effector

Figure 1 Prostaglandin biosynthesis pathway. Phospholipase cPLA2 (PLA2G4) releases AA from membrane phospholipids and COX enzymes (PTGS1, PTGS2) convert it to PGG2 and PGH2, the common precursor for all PGs. PGH2 is then converted into one of the active PG by specific terminal synthases such as PGE synthases (PTGES, PTGES2, PTGES3), PGF synthases (AKR1B1, AKR1C3, AKR1A1), PGD synthase (PGDS) and thromboxane synthase 1 (TXAS1). CBR1 is able to convert PGE2 into PGF2α, while AKR1C3 can convert PGD2 into 9α,11β-PGF2. PG2 and PGF2α are inactivated into PGEM and PGFM by HPGD (15-PGDH). PGD2 converts spontaneously into bioactive PGJ2, whereas unstable PG2 and TXA2 convert into inactive 6K-PGF1α and TXB2.
nucleases (TALENs) and more recently the CRISPR/Cas9 system for the generation of null mutation of targeted genes in somatic cell lines (Carroll, 2012). In the present report, we have used the CRISPR/Cas9 system (Hwang et al., 2013; Mali et al., 2013) to generate null AKR1B1 human endometrial cell lines from immortalized human endometrial stromal cells (HIESC-2) previously developed in our laboratory (Chapdelaine et al., 2006). We present also an elaborate characterization of one resulting cell line lacking the expression of AKR1B1 protein.

Materials and Methods

Materials

Reagents were purchased from the following suppliers: RPMI without phenol red, superscript III reverse transcriptase, 1-kb DNA ladder, dithiothreitol, 5x first-strand buffer and TRIzol were from Life Technologies, Inc. (Burlington, ON, Canada). Random primer-pd(N)6, deoxy-NTPs, RNA Guard, rTaq DNA polymerase and PCR 10× buffer from GE healthcare Canada (Baie d’Urfe, QC, Canada). Plasmid pDrive (TA cloning kit) and DNA purification kits were from Qiagen (Missisauga, ON, Canada). All oligonucleotides were chemically synthesized and purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA). The other chemicals used were of molecular biology grade available from Laboratoire Mat or Fisher Biotech (Québec, QC, Canada). AA was from Cayman Chemical (Ann Arbor, MI); Interleukin-1β (IL-1β) was purchased from Research and diagnostic systems (Minneapolis, MN, USA).

Design and construction of single guide RNA (sgRNA) targeting the coding sequence of AKR1B1 exon I

The single guide RNA (sgRNA) production was based on the protocol described by Mali (Mali et al., 2013). Since NGG is the protospacer-adjacent motif (PAM) required in the target site of the sgRNA we first selected four sgRNA target sites (Fig. 2C) by seeking sequences corresponding to the 5′N6NGG genomic site around exon I of the AKR1B1 gene (ID: 231). The gBlock™ template purchased from IDT (Integrated DNA Technologies Inc. IA, US) was a 455-pb fragment containing the U6 promoter, n19 target site, sgRNA scaffold and a termination signal necessary for sgRNA expression. Our methodology uses pCR3.1 vector (Invitrogen) in which we have inserted a gBlock and which was described as Mali et al. (2013). This gBlock was amplified by PCR making a PCR fragment of ~455 pb with the following primers aagcaagaagcggcccgccgccgtTGATACAACAAAAGCGGCTTAAAAAG sens (BssHII) aagcaagaacccgctcggctcggcggctaATGCGAACAC TTTGTAGAGAAGGACGAAACACCG rev (Apai). After amplification, the gBlock PCR product was digested with BssHII and Apal1 and cloned in the pCR3.1 vector digested with the same restriction enzymes. The resulting recombinant vector containing the gBlock could then serve as a template to introduce different PCR fragments (containing variable targets), which will be digested by Ndel/Xbali and then directly cloned in the recombinant vector pCR3.1. This new process allowed quick generation of new gRNA by simple replacement of a 173 nt portion of the original gBlock containing alternate 19 nt targets as described below.

Generation of custom gRNA targeting AKR1B1

The gBlock inside of the recombinant pCR3.1 contains typical restriction enzyme sites where we were able to introduce different targets coming from DNA fragments obtained by PCR amplification. We only needed to synthesize one oligonucleotide containing the 19 nucleotides (N) corresponding to the different targets. For each one, we designed a single oligonucleotide (59 nt) containing 19 selected nucleotides (N, Fig. 2) presenting the following form: 5′GTGAAAGGACGAAACCGNNNNNNNNNNNNNNNNNNTTGGAAAGCTGTAAGATGC3′ (primer 1) complementary to the following primers: ACTTGAAAGTTTCTGACTTTGCTTTA- TATATCTTGTTGAAGAAGCGAGAAGTCTTTAATTTATGCTATTCTAGCTTAAAC (Rev const). The ‘N’s represents the 19nts genomic target without PAM (NGG).

The specific-target sgRNAs were produced by double PCR amplification with Phusion DNA polymerase (New England Biolabs) in Phusion HF buffer as follows: the three oligonucleotides were mixed together in the following proportions for the first short PCR (50 µl): N (Fw const), 20 ng (Rec const) and 5 ng (T) and a short PCR program was applied: 98°C 1 min (1 cycle), 98°C 5 s, 55°C 10 s, 72°C 10 s (5 cycles), 98°C 5 s, 60°C 10 s, 72°C 10 s (15 cycles), and stand at 4°C. A second PCR amplification was done from an aliquot (2 µl) of the first short PCR with the following primers bearing restriction enzyme sites at their ends: GACGCTGATCATATGCTTTACCCGTAAACTTGGTTTCTGATTCGTTTCTG (53 nt) (Fw const NdeI) and GAAAGCTGTTGGCTTAAAAAGACCCGACCTGCGTTCTCTTAAACCATGTTAAACG (59 nt) (Rev const XbaI). The second PCR reaction (100 µl) proceeded as follows: 200 ng of (Fw const, NdeI), 200 ng of (Rev const, XbaI), 2 µl aliquot of the first short PCR round, all in the HF buffer with Phusion enzyme (New England Biolabs) and the program used was: 98°C 1 min (1× cycle), 98°C 5 s, 55°C 10 s, 72°C 10 s (5 cycles), 98°C 5 s, 60°C 10 s, 72°C 10 s (30 cycles), 72°C 2 min and stand at 4°C. At the end of the second PCR, purification of the PCR product (173 pb) was done with QIAquick PCR purification kit (Qiagen) followed by digestion with Ndel/XbaI done in NEB2 buffer for 1 h at 37°C followed by purification with QIAquick PCR purification kit. After purification, the digested PCR product was ligated with Quick DNA ligase (New England Biolabs) with a pCR3.1 vector (Invitrogen) containing a gBlock™ module (gRNA) (see Fig. 2C) digested also with Ndel/- XbaI. The inserted gBlock™ in pCR3.1 (see Fig. 2C) and double digestion with Ndel/XbaI allowed to insert the desired target by modification of the T primer by simple substitution of the 19N's corresponding to the genomic target (see Fig. 2B and C).

Co-transfection of endometrial Stromal cells with sgRNA and Cas9 plasmids

HIESC-2 (Chapdelaine et al., 2006) were cultured in 6-well plates and maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and penicillin–streptomycin 1× (complete medium) in a 37°C humidified incubator with 5% CO2. When the cells reached 80% confluence the complete RPMI medium of each well (3 ml) was replaced with 2 ml of fresh RPMI medium without antibiotic. Co-transfection of the cells was done as follows: for each well, 4 µg of plasmid expressing the different sgRNAs (identified as sgRNA target #14, 15, 16 and 17) (see Fig. 2B) and 2 µg of the Cas9 plasmid (Addgene, Cambridge, MA, USA cat no 48668) diluted in the Opti-MEM medium were mixed with 10 µl of lipofectamine diluted in Opti-MEM medium. The complexes were added to each well and incubated for 18 h at 37°C before medium replacement with complete RPMI. Three days after transfection, cells were harvested and genomic DNA was analyzed for the presence of neomycin using the Surveyor™ enzyme as described below. At the same time, since both plasmids carry the neomycin resistance gene, transfected cells were deemed overrepresented among resistant clones. A sample of the cells were cultured in the presence of neomycin (400 µg/ml) for 2 days followed by two additional days in complete medium without neomycin after which cells were trypsinized, diluted and plated in 6-well plates. After 10 days, individual colonies were isolated with O-rings, harvested, seeded in 24-well plates and grown for 2 weeks until

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Figure 2: Human AKR1B1 exon 1. Part of the genomic sequence of AKR1B1 is presented in (A). Primers used to sequence are highlighted in green. The first exon of AKR1B1 is shown in bold, underlined and blue font. Oligonucleotides synthesized are represented in (B) where the 19 selected nucleotides for each target replaced the ‘N’. Target sequences used for each sgRNAs are represented above exon 1 sequence and separately in (B). Target sequences derived from genomic sequence of AKR1B1 exon 1 (A) were added to a synthesized gBlock from IDT (Integrated DNA technologies, Inc.) containing the U6 promoter + Target sequence of the sgRNA + guide RNA scaffold + termination signal. Then, the gBlock was amplified by PCR and inserted in the pCR3.1 plasmid between the BssHII and the ApaI sites to replace the CMV promoter and create a pCR3.1 U6-sgRNA plasmid (C). The sgRNA inserted in pCR3.1 U6-sgRNA plasmid contains two unique restriction sites (NdeI and XbaI), allowing to exchange the sequence to generate different sgRNAs by PCR amplification.
confluence when they were transferred to T25 flasks. When the latter reached confluence, cells were trypsinized and an aliquot was kept for protein analysis by western blot. The remaining cells were frozen or expanded in T75 flasks for additional analyses.

### Genomic DNA extraction from cultured endometrial cells

Genomic DNA extraction was performed as follows: cells from individual wells were trypsinized (300 μl 1 x trypsin EDTA) diluted with complete culture medium (1 ml), and recovered by centrifugation at 3000 rpm. The cell pellet was washed three times with 1 ml PBS 1 x. A 25-μl lysis solution composed of EDTA 0.5M/10% Sarkosyl (11:1) was added to the cell pellet, supplemented with 2 μl protease K (10 mg/ml) and the mixture was heated at 50°C for 5 min. 150 μl TE 1 x (Tris–HCL 10 mM pH8.0, EDTA 1 mM) and 2 μl RNase A (1 μg/μl) were then added to the lysed cells and heated at 50°C for 10 min. DNA was isolated and purified by sequential extraction with phenol/chloroform/isoamyl alcohol (25:24:1) followed by chloroform–isoamyl alcohol (24:1) and after, 2 serial extraction with phenol/chloroform/isoamyl alcohol (25:24:1) followed by chloroform–isoamyl alcohol (24:1) and after, 2

### Surveyor nuclease assay for confirmation of genome modification

Genomic DNA (100 to 200 ng) of endometrial cells was extracted 72 h after transfection as described above. The genomic regions flanking the sgRNA target sites for exon 1 of AKR1B1 gene (Fig. 2A) were PCR amplified with a forward primer (pos) 5'-CACCGTTCTGGAAGCAGCTTTCC-3' and a reverse primer (pos) 5'-GCTCAACTGGGGTTTGACACC-3' using Phusion DNA Polymerase (New England Biolabs) in the presence of 2% DMSO: initial denaturation at 98°C (10 s), 30 cycles of denaturation at 98°C (10 s), annealing at 58°C (20 s) and extension at 72°C (30 s) for 35 x cycles, final extension 72°C (5 min). At the end of the PCR amplification, the products were purified using QiaQuick Spin Column (QIAGEN). A total of 400–600 ng (14 μl) of the purified PCR products were mixed with 4 μl of 5 x Phusion HF Buffer (NEB) and subjected to annealing to generate heteroduplexes: 95°C for 5 min; 95°C to 85°C ramping at –2°C/s; 85°C–25°C at –0.25°C/s and 25°C hold for 1 min and finally hold at 4°C. After annealing, products were treated with Surveyor nuclease and Surveyor enhancer 5' (Transgenomics, Inc., Omaha, NE, USA). All Surveyor analysis were done on 2% agarose gels containing RedSafe™ Nucleic Acid staining solution (Froggobio, Inc., Toronto, ON, Canada).

### Sequencing analysis of AKR1B1 gene and transcript to detect insertion or deletion (INDEL)

Genomic DNA corresponding to exon 1 of AKR1B1 of each clone was amplified by PCR using Phusion DNA polymerase in the presence of DMSO as described above but in the final extension step TAQ DNA polymerase (NEB) was added (1 μl) for an extension of 10 min at 68°C. The PCR products were purified using QiAquick gel extraction kit (Qiagen) from agarose gels and then cloned in the pDrive vector (TA cloning vector, Qiagen) using QIAGEN PCR cloning kit (Qiagen). The ligated PCR products were transformed with DH5α and the next day bacteria colonies were picked and seeded in LB medium and plasmids containing inserts were analyzed by sequencing. For mRNA analysis, total RNA of the different clones was extracted from T-75 flask with 1 ml TRIzol® Reagent (Life Technologies). The first strand of cDNA was synthesized at 42°C (50 min) from 2.5 μg of RNA using SuperScript III Reverse Transcriptase (RT, Life Technologies) with Random Hexamers (250 ng) (Life Technologies). Usually, aliquots (2 μl) of the first-strand cDNA were amplified with the following primers corresponding to exons 1, 2 and 3 of AKR1B1 mRNA: 20 pmol of each primer 5'-AGCCATGGAAAGCTTCTC-3' (forward) and 5'-GACCACTGCTTGAC-3' (reverse) were used in the PCR reaction using Taq DNA polymerase (NEB): initial denaturation at 95°C (1 min) for 1 cycle, denaturation at 95°C, annealing at 58°C (30 s), extension at 68°C (30 s) for 35X cycles and final extension at 68°C (10 min). The final PCR product (~220 bp) corresponding to the AKR1B1 mRNA transcript was gel purified, cloned in pDrive vector and processed for cDNA sequencing.

### Detection of transcripts for alternate PGF synthases by RT–PCR

The expression of alternate enzymes potentially contributing to the generation of PGF2α was investigated by RT–PCR analysis of candidate genes. Specific primers for each candidate gene were those used during our previous collaboration with Phillips et al., 2010. Briefly: for carbonyl reductase (CBRI, NM_0011757), forward 5'-CCTGGACGTCTGGTCAACA-3' and reverse 5'-ACGGTTCAACACTCTTGGTTG-3' for a PCR product size of ~164 bp; for hydroxyprostaglandin dehydrogenase(HPGD 15-(NAD)), NM_000860 forward 5'-CTGGACATCGATGGTAACG-3' and reverse 5'-AAGTGTCTCCAGTGGTGGTG-3' for a PCR product size of 229 bp; for AKR1C3 (NM_003739), forward 5'-CAGCAAATGATACCGGAATGG-3' and reverse 5'-CCTACCTGGCTTATCTGACAC-3' for a product size of ~195 bp. As positive control, human β-actin (NM_001101) was used as forward 5'-ACGGCTGCAGTTCCCCCTCC-3' and reverse 5'-AGGCCATGCAATTTCACTGTT-3' primers for a PCR product of ~524 bp.

### Characterization of HIESC-2/CRISPR clones

Stromal clones were grown to 40% confluence in six-well plates, fixed with methanol at 4°C and processed as previously described (Kang et al., 2005). A monoclonal antibody coupled with Cy3 was used against vimentin (Sigma) (1/500). A mAb against cytokeratin (OK1, Dako, Mississauga, ON, Canada) was used at a 1/500 dilution and a secondary goat anti-mouse conjugated to Alexa Fluor® 488 (Molecular Probes, Inc., Eugene, OR, USA) was used. The fluorescence was visualized using a Zeiss Axiovert 100-Inverted microscope (Zeiss, Germany), and images were captured and integrated using the Northern Exposure program (Empix Imaging, Inc., ON, Canada). Induction of decidualization was performed as described for our HIESC-2 cell line (Kang et al., 2005; Chapdelaine et al., 2006). Preliminary studies (not shown) confirmed optimal decidualization at 6 days. Briefly, cells were grown to confluence in six-well plates containing RPMI without phenol-red (Life technologies) containing 2% dextran-charcoal-treated FBS (DC-FBS) and 1 x penicillin–streptomycin in the presence or absence of 0.5 mM 8-bromo-CAMP (Sigma, St. Louis, MO, USA) and 10–6 M medroxyprogesterone acetate (MPA) (Pharmacia Canada, Mississauga, ON, Canada). The culture medium was changed every other day and supernatants were collected after 6 days for prolactin (PRL) assay. PRL measurement was done by ELISA using the ADVIA Centaur® immunoassay system (Bayer HealthCare LLC, Tarrytown, NY, USA). The lower detection limit was 0.8 μg/l.

### Western blot analysis

Protein extraction and quantification for western blot analysis were performed as described previously (Arosh et al., 2002). For each sample, aliquots of 5–10 μg protein were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis 10% (SDS-PAGE). Proteins were then electro-transferred onto 0.45-μm nitrocellulose membranes (Bio-Rad Laboratories, Inc., Mississauga, ON, Canada). The membranes were blocked overnight at 4°C in phosphate-buffered saline (PBS) 0.05% Tween 20 (PBS-T)
containing 5% (wt/vol) fat-free dry milk (BLOTTO). Then, the membranes were incubated in PBS-T/5% BLOTTO with the following primary antibodies: anti-COX-2 (Merck 243) and anti-COX-1 (Merck 241) dilution 1/3000 were kindly provided by Dr S Kargman (Merck, QUE, Canada). The anti-microsomal PGE synthase 1 (anti-mPGES1) SC-166308 (1/500), AKR1A1 was probed with a monoclonal mouse anti-human AKR1A1 (1/500), CBR1 was detected with sc-70212 antibody (1/250), IGFBP-1 with sc-6072 antibody at a 1/100 dilution, 17β-HSD3 with sc-66415 antibody at a 1/200 dilution all from (Santa-Cruz Biotechnology, Dallas, TX, USA). The anti-AKR1B1 (PAS-29718, Thermo Scientific) was used at a 1/2000 dilution. Both the anti-AKR1B1 C-terminal probing amino acids 287–316 of AKR1B1 (AP12363PU-N) (1/1000) and anti-AKR1C3 antibody probing amino acids 17–44 used at a 1/1000 dilution (AP50133PU-N) came from ACRIS antibody GmbH. Finally, the anti-β-Actin (1/1000) (Sigma) was used as an internal standard at a 1/5000 dilution. Following incubation with the primary antibodies, the membranes were washed three times during 10 min in PBS-T. The membranes were then incubated in PBS-T/5% BLOTTO with the following appropriate secondary antibody either a goat anti-rabbit or anti-mouse conjugated with horseradish peroxidase (Jackson laboratories, West Grove, PA, USA). This secondary antibody was used at 1/10 000 for 1 h at room temperature followed by 3 washes of 10 min in PBS-T. The chemiluminescence signal was analyzed with an autoradiography film after treatment of the membrane with Renaissance reagent (NEN, Perkin Elmer, Boston, MA, USA). The signal intensity of the immunoreactions was quantified by densitometry using a Multimage Light Cabinet equipped with Alphalmager 2000 software (Cell Biosciences, Santa Clara, CA, USA).

Figure 3 Use of CRISPRs/Cas9 system to silence AKR1B1 gene in HIESC. HIESC-2 cells were co-transfected with a plasmid containing Cas9 (Addgene, Church group, no 48668) and pCR3.1 U6-sgRNA plasmid using lipofectamine 2000 and maintained in culture for 3 days (A). After 2 days of neomycin selection, cells were harvested and seeded at low dilution for isolation of single clones (B). While single clone propagation was proceeding (2–3 weeks), an aliquot was used for DNA extraction and Surveyor digestion to test mutation efficiency (C). Finally, an aliquot of each clone was used to analyse AKR1B1 protein expression by western blot. Clones that achieved successful growth were also analyzed at the protein level to probe for AKR1B1.
**PGE2 and PGF2α immunoassay**

PGs were assayed by competitive EIA using acetylcholinesterase-linked PG tracers (Cayman, Ann Arbor, MI, USA) as described previously (Asselin et al., 1996).

**Statistical analysis**

Data were analyzed using GraphPad Prism 5 program. One-way ANOVA with Bonferroni as post hoc test with 95% confidence intervals was used for statistical significance. All numerical data are presented as the mean ± SEM, and differences were considered as statistically significant at the 95% confidence level (P < 0.05) Each experiment was repeated at least three times in triplicate.

**Results**

**Generation of null mutation of AKR1B1 gene in HIESC-2 cells**

Immortalized human endometrial stromal cells (HIESC-2, Chapdelaine et al., 2006) were transfected with four different sgRNA plasmids targeting various nucleotide sequences of the first exon of the AKR1B1 gene (Fig. 2). After 3 days, transfected cells were selected by incubation in the presence of neomycin and clones were propagated for 3 weeks (Fig. 3). Mutations of the genomic DNA were assessed by a Surveyor assay on DNA extracted from transfected cells and amplified with specific primers neighboring exon 1 of the AKR1B1 gene (Fig. 4). The Cas9 plasmid alone or in combination with a plasmid expressing GFP did not generate any fragment following Surveyor digestion. However, sgRNA plasmids corresponding to targets #14, 16 and 17 but not #15 generated fragments of predicted size indicating genomic modifications (see arrows Fig. 4A). Neomycin selection of the transfected cells increased the yield of mutated cells as seen for the gRNA #16 plasmid (Fig. 4A). Western blot analysis of AKR1B1 protein in global preparations of cells transfected cell clones. HIESC cells were transfected with lipofectamine 2000 with Cas9 and one of the four pCR3.1 U6-sgRNA plasmids (Fig. 2) coding for a sgRNA targeting exon 1 of the human AKR1B1 gene. The DNA was extracted from the cells after 72 h before and after neomycin selection (48 h). Part of the AKR1B1 gene was amplified by PCR and the presence of INDELs in the target sequence was detected by digestion with the Surveyor enzyme resulting in additional bands (A). After culture, the proteins were extracted and western blot for AKR1B1 was performed (B). Individual clones were isolated for cells treated with sgRNA #16 and western blot for the presence of AKR1B1 were performed (C).

**Characterization of clones obtained after CRISPR treatment**

The genomic DNA of clones 16-1, 16-2, and 16-4 was analyzed by PCR amplification with primers neighboring exon 1 of the AKR1B1 gene targeted by sgRNA#16. Figure 5C illustrates that clone 16-2 contained two different amplification products, whereas clones 16-1 and 16-4 contained only one amplification product. Sequencing of the two PCR bands from clone 16-2 for which the AKR1B1 protein was undetectable showed deletion of 68 nucleotides for the lower band and two nucleotides deletion for the upper PCR band (Fig. 5A). This result demonstrates that both alleles of clone 16-2 were mutated causing a frameshift of AKR1B1 transcript. Figure 5A shows that deletions correspond to the genomic area targeted by the sgRNA#16 construct. In contrast, sequencing of the single PCR band of clone 16-4 (Fig. 5B) shows that only one allele was mutated with a single nucleotide deletion, thus causing a frameshift while the other allele is intact. Clone 16-4 would thus represent haplotype mutation analogous to a (+/-) genotype for AKR1B1. As shown in Fig. 4C, clone 16-4 is able to express the AKR1B1 protein. The mRNAs were also analyzed to confirm the results at the protein level (not shown).

Further analysis on the association between AKR1B1 and PG synthesis activity was limited to wild-type (AKR1B1+/+) human endometrial stromal cells HIESC-2 (Chapdelaine et al., 2006), and clones 16-2...
AKR1B1 (−/−) and 16-4 AKR1B1 (−/+). Both clones presented with cell morphology similar to parental HIESC-2 cells with immunohistochemical positive for vimentin and negative for cytokeratin (Fig. 6A). Similarly, western blot analysis of alternate PGF synthases (Fig. 1) confirmed the absence of AKR1A1 and AKR1C3 (Fig. 6B) but also the presence of CBR1 protein in all cell lines. In contrast, wild-type endometrial epithelial cells (HIEEC) present with significant levels of all biosynthetic enzymes as we have shown previously (Bresson et al., 2012). RT–PCR analysis of RNA extracted from clones 16-2, 16-4, HIESC-2 and HIEEC revealed complete absence of HPGD transcript but presence of low levels of AKR1A1 and 1C3 transcripts in all stromal cell lines and high levels of CBR1 in all cell types (results not shown). The strong expression of the CBR1 transcript and protein (Fig. 6C) suggests that in the absence of AKR1B1, PGF2α could be produced from conversion of PGE2 present in the cells (Fig. 1).

Decidualization response in wild-type and CRISPR mutated cells

As an additional test to confirm that stromal cell phenotype was maintained, cells were submitted to a decidualization protocol that was already tested for the parental stromal cell line (Chapdelaine et al., 2006; Kang et al., 2006). Both parental and mutated cells exhibited characteristic morphological changes following treatment with 8-bromo-cAMP and MPA (Fig. 7A) including increase in cell size and plurinucleation (results not shown). Prolactin production was measured as a functional marker and cell count was performed to normalize the results (ng prolactin /10^6 cells). Average cell number per well was 93, 8 and 20 × 10^6, respectively, for wild-type, 16-2 and 16-4 cells. Decidualization induced comparable prolactin increases of 18.7, 10.5 and 20.2-folds, respectively, for wild-type AKR1B1 (+/+), 16-2 AKR1B1 (−/−) and 16-4 AKR1B1 (−/+), as shown in Fig. 7B.

**Effect of AKR1B1 deletion on PGF2α production**

Following treatment of cells with IL-1β (1 ng/ml) for 24 h, both wild-type and clone 16-4 cells exhibited a similar response, but clone 16-2 did not respond with increased production of either PGF2α or PGE2 (Fig. 8B). At the protein level (Fig. 8A), IL-1β (1 ng/ml) induced an increase in mPGES-1 and COX-2 protein expression but in clone 16-2 the relative increase of Cox-2 protein was weaker. Side-by-side comparison of responses to IL-1β (1 ng/ml) AA (10 μM) in wild-type and clone 16-2 cells (Fig. 9) shows higher increase in COX-2 in the presence of IL-1 but otherwise comparable levels of enzymes involved in PG production (Fig. 9 A and B). The response of cell lines to AA in terms of PGF production was comparable but interestingly, PGE2 production was restored in clone 16-2 (Fig. 9 C and D).
Discussion

We demonstrate here the ability of the CRISPR/Cas9 system to modify the genome of immortalized endometrial cells and we confirm the importance of the AKR1B1 gene in the production of endometrial PGF2α.

PGF2α appears as the least studied member among PGs. The PGF2α/FP system is however clearly involved in endometrial pathologies including menstrual disorders and endometrial cancer (Milne and Jabbour, 2003; Jabbour and Sales, 2004; Sales et al., 2005; Catalano et al., 2011) and premature delivery (Olson and Ammann, 2007; Phillips et al., 2011).

Before our identification of AKR1B5 in the bovine (Madore et al., 2003), now bovine AKR1B1, all PGFS described were AKRs of the 1C family. AKRs are recognized as moonlighting enzymes able to partner with several types of substrates. The putative PGFS activity of AKR1B5 led us to investigate its human counterpart AKR1B1 well known for its role in the polyol pathway and contribution to diabetes complications. We (Bresson et al., 2011, 2012) and others (Kabututu et al., 2009; Nagata et al., 2011; Phillips et al., 2011) found that the human AKR1B1 was a functional PGF synthase in various tissues.

More recently, we evaluated the activity of another AKR, AKR1A1 as a potential alternate endometrial PGFS (Lacroix Pépin et al., 2013). With AKR1C3 already expressed, the presence of AKR1B1 and AKR1A1 as alternate PGF synthases complicates the picture as to how PGF2α production is regulated under normal or pathologic conditions in the human

Figure 6 Phenotypic characterization of clones 16-2 (−/−) and 16-4 (+/−). Cells of clones 16-2 and 16-4 were grown in parallel with wild-type stromal cells (HIESC-2) as control for 3 days and observed under phase contrast or following immunolabeling with vimentin or cytokeratin antibodies at 100× magnification (A). Photomicrographs show that normal and mutated cells exhibit similar morphology and expression of vimentin but not cytokeratin. Western blot analysis for the presence of alternate PGF synthases was performed (B and C). AKR1A1, AKR1B1 and AKR1C3 were assessed in HIEEC-22 (epithelial), HIESC-2 (stromal), clone 16-2 and clone 16-4. Recombinant Aldo-keto reductase proteins (rAKR) were used as internal standards (B). Western blot analysis of CBR1, IGFBP-1 and 17β-HSD3 is shown for the same cell lines (D). In all cases, β-actin was used as internal standard.
endometrium. Because abrogation of AKR1B1 by siRNA decreases AKR1B1 expression and PGF2α output only by 50% (Bresson et al., 2011), this approach did not appear sufficiently robust to address the issue. Complete knockout of individual genes involved in PGF2α appeared as the most logical solution. This was attempted in the mouse in spite of the absence of AKR1B1 in this species. In mice, two distinct genes, AKR1B3 and AKR1B7 for which null mutations were generated cover some but not all activities of AKR1B1. AKR1B3 knockout mice (Lo et al., 2007) appeared protected against cerebral ischemic injury, which is interesting given the documented ischemic responses to PGF2α (Norel, 2007), particularly in association with menstrual pain. Unfortunately, AKR1B3 null mice were not tested for PG production level and associated pathologies. AKR1B7 null mice exhibit excessive basal adiposity and greater sensitivity to diet-induced obesity not seen with the AKR1B3 knockout model.

The recent development of genomic edition with the CRISPR/Cas9 system now opens new avenues to explore gene function in differentiated human cells. Our initial approach was to create 4 sgRNAs that aimed at exon 1 of AKR1B1 genomic sequence (Fig. 2B). The sequences targeted by these sgRNA were then blasted to ensure minimal off-target potential. Considering the PAM sequence necessary for Cas9 digestion, target 16 (Fig. 2A) exhibited no obvious off-target (Mali et al., 2013), thus limiting the probability that other genes such as AKR1B10 and AKR1B exhibiting 68.2% homology with AKR1B1 would be affected. AKR1B10 showed only 8 out of 19 nucleotide correspondence with target 16 and lacked the PAM sequence that is essential for Cas9 activity. While the possibility for off-targets could not be eliminated completely, we believe that the process of clone generation and selection of mutated cells based on viability, morphology and PGs response kept this probability to a minimum.

The plasmids coding for the sgRNAs and Cas9 were co-transfected because the new plasmid permitting expression of both sgRNAs and Cas9 was not yet available (Cong et al., 2013). Of the four sgRNAs used, three successfully altered AKR1B1 sequence as evaluated by Surveyor (Fig. 4A). Among those, the two clones showing abrogation of AKR1B1 were obtained with the sgRNA #16. Sequencing of 16-2

Figure 7 Decidualization of wild-type HIESC-2 and clones 16-2 (−/−) and 16-4 (+/−). Decidualization was induced by a 6-day treatment with 8-bromo-cAMP (0.5 mM) and MPA (10−6 M) inducing morphological changes in stromal (HIESC-2) from a spindle to an ovoid shape (magnification 100 ×) (A). Decidualization stimulated prolactin secretion in all cell lines (B). After decidualization cell number ranged from 10 to 130 × 10^6 cells per well.
confirmed that no AKR1B1 protein could be produced from the isolated mRNA (results not shown). The parent clone 16-4 with normal or slightly diminished AKR1B1 production (Fig. 4C) showed normal sequence for one AKR1B1 allele and a single nucleotide deletion on the second at both DNA and mRNA levels (results not shown). All genomic mutations were located at the end of the nucleotide sequence targeted by sgRNA #16.

Clone 16-2 can be assimilated to a \((\sim / \sim)\), and clone 16-4 to a \((+ / \sim)\) genotype and both cell lines grew normally and exhibited morphologies comparable with wild-type \((+ / +)\) HIESC-2 cells (Fig. 6A). As expected, none of these cells of stromal origin expressed AKR1C3 (Bresson et al., 2012) and did not have significant levels of AKR1A1 either (Fig. 6B). These cells did not express mRNA for the PG catabolic enzyme HPGDH as we observed for primary stromal cell cultures (Parent et al., 2006) but CBR1, an enzyme able to convert PGE2 into PGF2α, could be detected by PCR and western blot but was not investigated further (Fig. 6C).

Clones and wild-type cells exhibited similar increases in PGE2 production (Fig. 8B) and COX-2 and MPGES-1 proteins (Fig. 8A). PGF2α production was highest in wild-type cells absent in clone 16-2 and intermediate in clone 16-4 in direct association with measured AKR1B1 protein level in the presence of IL-1β. When cells were
treated with AA, the absence of AKR1B1 did not have any impact on PGE2 production (Fig. 9C), whereas PGF2α was kept at its minimum (Fig. 9D). Under both IL-1β and AA, MPGES-1 was increased confirming that the PGE2 pathway was stimulated by IL-1β and AA in mutated cells (Fig. 9A and B). In clone 16-2, the presence of residual PGF2α in the absence of AKR1B1 might be due to accessory PGF synthase activity by enzymes such as CBR1 converting PGE2 into PGF2α (Fig. 6C).

Reduced PGE2 production associated with reduced AKR1B1-derived PGF2α production following IL-1β but not AA stimulation of PGs strengthens our hypothesis that a feedback loop between PGF2α and PGE2 may exist (Bresson et al., 2012). Results in Fig. 9 suggest that mechanisms upstream of COX-2 are responsible for increased PGE2 production in the presence of PGF2α. Since AA release by cPLA2 is dependent on Ca++ levels themselves elevated in response to FP prostanoid receptor activation by PGF2α (Jabbour and Sales, 2004) and (Krishnaswamy et al., 2009, 2010), this signal transduction pathway should be considered. PGE2 acting on EP2 prostanoid receptors and PGF2α acting through FP work as an opposing dyad in the reproductive system, in a way similar to TXA2 and PGI2 in the vascular system, thus allowing tight regulation of cell responses. Under the circumstances, the observed interactions between the PGE2 and PGF2α systems in endometrial cells are not only probable but necessary.

In summary, the ease and efficacy of editing the genome in differentiated human cells using the CRISPR/Cas9 system should have broad
implications for our ability to understand complex biological systems at the functional level. Our next goal will be to use the same approach to generate complementary and multiple knockout models where key enzymes of the PGE2 and PGF2α biosynthetic cascade will be invalidated. These models will allow us to conduct in human tissues, experiments that will validate and/or complement animal studies, especially when the genes or function involved are not identical.

**Authors’ roles**

N.L.-P., the first author, performed the experiments. P.C. involved in g block synthesis, contributed to writing of the paper and experimental design. J.P.T. involved in experimental design contribution, contributed to writing of the paper. M.A.F. involved in experimental design contribution, the corresponding author.

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**Conflict of interest**

The authors of this manuscript have nothing to declare.

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