Generation of porcine fibroblasts overexpressing 11β-HSD1 with adipose tissue-specific aP2 promoter as a porcine model of metabolic syndrome

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Abstract. Metabolic syndrome arises from a combination of disorders that increase the risk of cardiovascular disease and diabetes. In previous studies, it was observed that overexpression of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) induced obesity and the insulin resistance that accompanies metabolic syndrome in rodent adipose tissue. Based on these observations, it was hypothesized that overexpression of 11β-HSD1 may be suitable for the generation of a porcine model of metabolic syndrome. It was evaluated that promoter activities of the porcine adipose fatty acid-binding protein (aP2) gene generates adipose tissue-specific 11β-HSD1 expression. In adipose tissue, the maximum promoter activity (-2,826 to +51 nt) of aP2 was 200-fold higher than that of a promoterless construct. In addition, 11β-HSD1 transcriptional levels were significantly increased following the introduction of the aP2 promoter into 3T3-L1 adipocytes. These observations indicate that the aP2 promoter may facilitate 11β-HSD1 overexpression in porcine adipose tissue. Transgenic fibroblasts were generated containing 11β-HSD1 cDNA controlled by the aP2 promoter with two screening markers, green fluorescence protein and a neomycin-resistance gene. It was hypothesized that transgenic fibroblasts may be useful for generating a porcine model of metabolic syndrome.

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Introduction

Metabolic syndrome caused by insulin resistance and abnormal adipose tissue deposition is associated with various risk factors, including coronary artery disease, stroke, fatty liver development and type 2 diabetes (1,2). This syndrome is also known as cardiometabolic syndrome, metabolic syndrome X, syndrome X, insulin resistance syndrome and Reaven's syndrome. The majority of patients with this disorder are older, obese and insulin-resistant, and have a sedentary lifestyle (3). Clinical symptoms of metabolic syndrome include hypertension, hyperglycemia, hypertriglyceridemia, decreased high-density lipoprotein levels, increased triglyceride levels and abdominal obesity (4,5); however, the etiology of this syndrome is not yet clearly understood.

Numerous mouse models, established through spontaneous mutations or genetic modification, have been employed to investigate metabolic syndrome. Leptin-deficient [$Lep^{(ob/ob)}$] (6), leptin receptor-deficient [$LepR^{(db/db)}$] (7), lethal yellow agouti (A^y/a) (8), melanocortin 3 receptor-deficient (9), melanocortin 4 receptor-deficient (10), low-density lipoprotein receptor-deficient (11) and apolipoprotein E-deficient mice have been generated (12,13). These animal models develop clinical symptoms similar to those observed in humans, including increased obesity and insulin resistance (14,15).

11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) is an NADPH-dependent enzyme that is highly expressed in key metabolic tissues, including liver, adipose tissue and the central nervous system (16). In metabolic tissue, including liver and adipose tissue, 11β-HSD1 converts cortisone into the active hormone, cortisol (17,18). 11β-HSD1 transgenic mice develop abdominal obesity, hyperglycemia, insulin resistance, hyperphagia, hyperleptinemia and increased intra-adipose and portal levels, but not systemic corticosterone levels (19). In addition, the adipose tissue of obese humans has been observed to exhibit elevated 11β-HSD1 activity (20).

Genetically modified mice are an attractive tool as they are small in size, easy to handle and possess short generation intervals. However, the phenotypic, genetic, physiological

and anatomical characteristics of mice are not similar to humans. As pigs are physiologically similar to humans (21), in the present study, genetically modified porcine transgenic fibroblasts were produced to induce metabolic syndrome-like symptoms in a porcine model. An 11β-HSD1 cDNA construct conjugated to the pig adipose fatty acid-binding protein (aP2) promoter was generated to facilitate 11β-HSD1 gene expression in porcine adipose tissue. These transgenic fibroblasts may serve as a source for somatic cell nuclear transfer (SCNT) to produce a porcine model of metabolic syndrome.

Materials and methods

Cell culture. Mouse embryonic 3T3-L1 fibroblast adipose-like cells were purchased from the Korean Cell Line Bank (Seoul, South Korea). 3T3-L1 preadipocytes were incubated, unless otherwise indicated, at 37˚C and, unless otherwise indicated, all cell culture materials were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). On day 30 of pregnancy, porcine fibroblasts were obtained from a miniature pig fetus (Yucatan pig; Optifarm Solution Inc., Gyeonggi-do, South Korea). Fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; WelGENE, Daegu, South Korea), 50 U/ml penicillin and 50 μg/ml streptomycin in a humidified 5% CO_2 atmosphere.

Genomic DNA extraction and PCR. Genomic DNA was isolated from the fibroblasts using a G-DEX™ IIc genomic DNA extraction kit (Intron Biotechnology, Seoul, South Korea). All experimental procedures and animal use were approved by the Ethics Committee of the Chungbuk National University. Genomic DNA (1 μg) was amplified in a 20 μl PCR containing 1 unit LA-*Taq* polymerase (Takara Bio, Inc., Shiga, Japan) for long-range PCR or 1 unit i-star *Taq* polymerase (Intron Biotechnology), 2 mM dNTPs (Takara Bio, Inc.) and 10 pmol each specific primer. All primers are presented in Table I. PCR conditions were as follows: denaturation at 95˚C for 30 sec, annealing at 62˚C for 30 sec and extension at 72˚C for 1 or 2 min. PCR products were separated on a 0.7% agarose gel, stained with ethidium bromide, imaged under UV illumination and processed for cloning. The gel image was scanned using Gel Doc EQ (Bio-Rad, Hercules, CA, USA).

RNA preparation and semi-quantitative PCR. Total RNA was extracted from 3T3-L1 cells or pig liver (Yucatan pig; Optifarm Solution Inc.) using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. Total RNA concentration was determined by measuring absorbance at 260 nm (Epoch microplate spectrophotometer; BioTek, Winooski, VT, USA). First strand cDNA was prepared by subjecting total RNA (1 μg) to reverse transcription using M-MLV reverse transcriptase (Invitrogen Life Technologies) and random primers (9-mers; Takara Bio Inc.). Optimal conditions for logarithmic phase PCR amplification of the target cDNA were determined by amplifying aliquots of total cDNA (1 μg) using a different number of cycles. The cytochrome *c* oxidase subunit 1 (1A) gene was used as an internal control to eliminate the possibility of RNA degradation and to account for variation in mRNA concentration. A linear correlation between the PCR product band visibility and the number of amplification cycles was observed in the target mRNA products. The 1A gene and target gene, insulin, were quantified using 28 and 30 cycles, respectively. PCR conditions were as follows: denaturation at 95˚C for 30 sec, annealing at 58˚C for 30 sec and extension at 72˚C for 30 sec. PCR products were separated in a 2.3% agarose gel, stained with ethidium bromide and imaged under UV illumination. The image was scanned and band densities were analyzed using Gel Doc EQ (Bio-Rad).

Vector construction. Restriction enzymes were obtained from Takara Bio, Inc. Specific regions of the aP2 promoter were prepared by long-range PCR using porcine genomic DNA as a template and specific primers containing restriction enzyme sites (*Mlu*I at 5' end or *Bgl*II at 3' end). Amplified fragments were digested with *Mlu*I and *Bgl*II and ligated into the promoterless pGL3-Basic luciferase expression plasmid (Promega Corporation, Madison, WI, USA). The 11β-HSD1 expression cassette plasmid was produced in several steps. 11β-HSD1 cDNA was prepared by PCR using cDNA from pig liver as a template. The amplified fragments were inserted into the recombinant pGL3 construct containing the pig aP2 promoter region (-2,826 to +51 nt). For the selection cassettes, the enhanced green fluorescent protein (EGFP) gene was amplified from the pIRES2-EGFP plasmid (Clontech Laboratories Inc., Mountain View, CA, USA), digested with *Eco*RV and *Bam*HI and inserted into the pIRESneo plasmid (Clontech Laboratories Inc.). The EGFP and neomycin-resistant (Neor) genes were amplified by PCR, digested with *Sal*I and ligated into the recombinant pGL3 vector encoding pig 11β-HSD1 cDNA controlled by the porcine aP2 promoter. Finally, sequences of the targeting vector were confirmed by nucleotide sequencing (Genotech Corporation, Daejeon, South Korea).

Transient transfection and reporter gene assay. Transient transfection was performed using Lipofectamine™ 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. To account for varied transfection efficiencies of the various luciferase constructs, the Rous sarcoma virus (RSV)-lacZ plasmid was co-transfected with the luciferase constructs containing the pig aP2 promoter, as previously described (22,23). Briefly, $3x10^5$ cells were seeded in 6-well tissue culture plates (Invitrogen Life Technologies) 1 d prior to transfection. Constructs (4 μg) containing the aP2 promoter and RSV-lacZ plasmid (0.5 μg) were co-transfected into the cells with DMEM. Following an incubation period of 4 h, the media was replaced with DMEM containing 10% FBS and glucose (0.1 or 4 mM) and the cells were incubated for an additional 48 h at 37˚C. Cell lysates were assayed for luciferase activity using a luciferase assay system (Promega Corporation). Luminescence was measured using a GloMax 20/20 Luminometer (Promega Corporation). β-galactosidase activity was measured using a β-galactosidase enzyme assay system (Promega Corporation). Relative luciferase activity was calculated as luciferase activity/β-galactosidase activity (%).

Establishment of transgenic cell lines. Porcine fibroblasts were transfected with the linearized targeting vector using Lipofectamine 2000. Following 24 h transfection, the medium was replaced with DMEM supplemented with 10% FBS and 250 μg/ml G-418 (Roche Diagnostics, Indianapolis, IN, USA)

Table I. Primer sequences and restriction enzymes.

Name	Restriction enzyme	Direction	Sequences (5' to -3')
Pig aP2 promoter (-4,424)	*Mlu*I	Forward	ACGCGTTATGGGAAGTATGTTTTGGA
Pig aP2 promoter (-2,826)	*Mlu*I	Forward	ACGCGTGGACTTTAATGGACACCTCACC
Pig aP2 promoter (-658)	*Mlu*I	Forward	ACGCGTTACAACCCAACAGCAAAA AAGCC
Pig aP2 promoter (+51)	*Xho*I	Reverse	CTCGAGCCTTCAGGAAGGTGCAATGAC
Pig 11β-HSD cDNA	*Bgl*II	Forward	AGATCTATGGCTTTTATGAAAAAATATCTCCTCC
Pig 11β-HSD cDNA	*Xba*I	Reverse	TCTAGACTAGTTGTTTGTAAACCTTTCCATATTA
EGFP cDNA	*Eco*RV	Forward	GATATCCACAACCATGGTGAGCAAGGGCGA
EGFP cDNA	*Bam*HI	Reverse	GGATCCTTACTTGTACAGCTCGTCCATGCC
Confirming primer a		Forward	CCATGATAATAAGCCTGCTCTACTCCA
Confirming primer b		Reverse	GGAAGTCATGAAGGCCTGGGTGATG
Confirming primer c		Forward	CATGAAGCAGCACGACTTCT
Confirming primer d		Reverse	CCTAGGAATGCTCGTCAAGA
1A		Forward	CCAGGATTTGGAATTATTTC
1A		Reverse	GAAAATAAAGCCTAAGGCTC
Pig 11β-HSD		Forward	CAACGTGTCAATCACGCTCT
Pig 11β-HSD		Reverse	TTCCTGGATTTTCCAACAGG

11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; EGFP, enhanced green fluorescent; 1A, c the ytochrome *c* oxidase subunit.

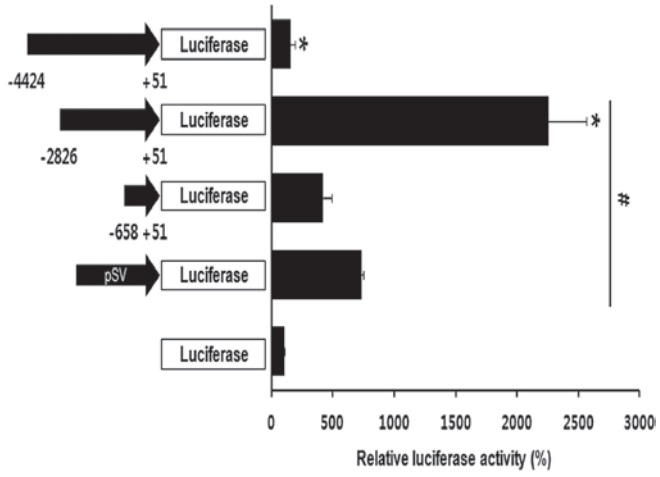

Figure 1. Luciferase reporter gene assay to evaluate aP2 gene promoter activity following transient transfection. 3T3-L1 cells were transiently transfected with designated constructs. Cells were co-transfected with a RSV-lacZ expression vector to normalize transfection efficiency. RLU was calculated as a percentage of the simian virus 40 promoter (pSV plasmid) RLU, which was set at 100%. Data are expressed as the mean ± SEM of three independent experiments conducted in triplicate. *P<0.05 vs. RLU of the promoterless plasmid; #P<0.05 vs. RLU of the -658 to +51 nt aP2 promoter. RLU, relative luciferase activity.

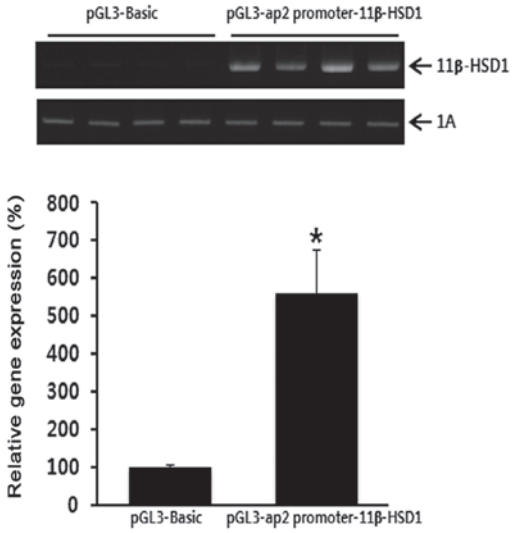

Figure 2. Overexpression of recombinant porcine 11β-HSD1 in 3T3-L1 cells. 3T3-L1 cells were transfected with the porcine 11β-HSD1 expression constructs containing the pig aP2 promoter. 11β-HSD1 mRNA expression was measured by semi-quantitative PCR. 11β-HSD1 mRNA expression was normalized against expression of the 1A gene as an internal control. *P<0.05 vs. the promoterless pGL3-basic. 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; 1A, cytochrome *c* oxidase subunit 1.

for 4 weeks. Antibiotic-resistant colonies were further selected according to EGFP expression observed with a fluorescence microscope (Nikon, Tokyo, Japan). Antibiotic and visually selected colonies were subjected to PCR-based genotyping and stored until required for SCNT.

Statistical analysis. Data are presented as the mean ± SEM. A statistical analysis was performed by Student's t-test for two-pair comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Functional analysis of the pig aP2 promoter. Prior to the generation of the 11β-HSD1 cDNA construct, a pig aP2 promoter that was hypothesized to be activated in adipocytes

Table II. Transfection efficiencies of the pig fibroblasts.

Transfection trials, n	G418-resistant colonies, n	EGFP-positive colonies, n	PCR-positive colonies, n
11	37	33	33

EGFP, enhanced green fluorescent.

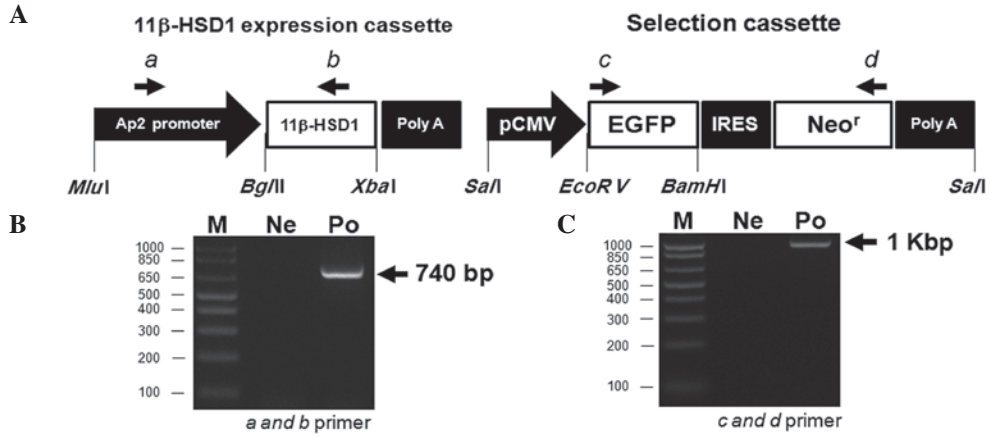

Figure 3. Schematic representation of constructs and PCR-based confirmation of the transgenic fibroblast identity. (A) 11β-HSD1 expression cassettes included an 11β-HSD1 gene with the aP2 promoter. The selection cassette contained EGFP and Neor genes linked by an IRES sequences. Integration of the constructs into fibroblast genomic DNA was confirmed by PCR using specific primers as indicated by the arrows. (B) Chromosomal insertion of the 11β-HSD1 expression cassettes was confirmed by PCR. (C) Transgenic fibroblasts containing the selection cassette were identified by PCR using specific primers. M, molecular marker; Ne, negative control without template; Po, a positive colony expressing the EGFP and Neor selection markers; EGFP, enhanced green fluorescent; Neor, protein neomycin resistant; 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1.

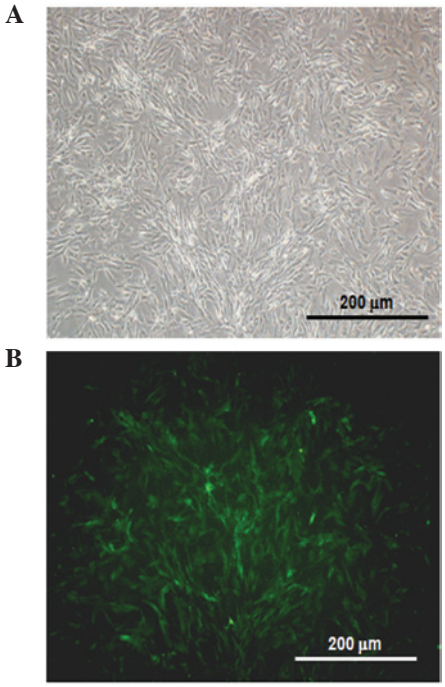

Figure 4. Morphology of the transgenic fibroblasts. Fetal porcine fibroblasts were transfected with targeting constructs using a liposomal-mediated gene delivery system and incubated with G-418 for 4 weeks. Fibroblasts stably expressing 11β-HSD1 were successfully produced. (A) G418-resistant cells were examined under light microscopy (magnification, x40) (B) EGFP expression was observed with fluorescent microscopy (magnification, x40). EGFP enhanced green fluorescent; Neor, protein neomycin resistant; 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1.

was produced. The aP2 gene is typically expressed in adipose tissue and the maximum promoter activity of aP2 in the adipose tissue was previously confirmed (24). A series of pig aP2 gene promoters with sequential deletions were inserted into the luciferase (Luc) gene in a promoterless pGL3-basic vector. The aP2 promoter constructs were introduced into the mouse embryonic fibroblast-adipose 3T3-L1 cells and the promoter activities were evaluated according to Luc expression. As presented in Fig. 1, the promoter region between -2,826 and +15 nt resulted in an ~20-fold increase of Luc activity compared with the promoterless construct. These observations indicate that -2,826 to +15 nt of the aP2 promoter was the best candidate adipocyte-specific expression promoter.

Overexpression of recombinant pig 11β-HSD1 in 3T3-L1 cells. Following verification that the aP2 promoter construct containing -2,826 to +15 nt had maximal activity, the construct was subcloned with porcine 11β-HSD1 cDNA. The resulting aP2/11β-HSD1 construct was capable of expressing 11β-HSD1 mRNA in an adipocyte-specific manner. 11β-HSD1 mRNA levels increased in the transiently-transfected 3T3-L1 cells (Fig. 2). These observations indicate that the recombinant aP2/11β-HSD1 construct was appropriate for expressing pig 11β-HSD1 in an adipose tissue-specific manner.

Establishment of porcine fibroblast cell lines that overexpress 11β-HSD1. Our constructs were composed of two parts, an 11β-HSD1 expression cassette and a selection cassette (Fig. 3A).

The 11β-HSD1 expression cassette contained pig 11β-HSD1 cDNA with the porcine aP2 promoter region (-2,826 to +15 nt). The selection cassette contained EGFP and Neor genes, whose expression was regulated by a cytomegalic virus promoter. Expression of the EGFP and Neor genes enabled selection of the cells of interest using antibiotics and visual screening.

The targeting construct was linearized and used to transfect porcine fibroblasts isolated from Yucatan pigs. Fibroblasts were incubated in medium containing G-418 (250 μg/ml) for 4 weeks to select for the stable transfectants. Identification of Neor colonies as transgenic fibroblasts was confirmed by EGFP expression (Fig. 4). The clones were further identified by PCR-based methods using primers specific for the targeting constructs. Genomic DNA from clones expressing porcine 11β-HSD1 was analyzed with an a-b primer set (amplicon size, 740 bp; Fig. 3B) or with c-d primers (amplicon size, 1 kb; Fig. 3C). Transfection efficiency was ~89% (Table II). These observations indicate that the cell model may be useful for establishing a transgenic fibroblast system for creating porcine metabolic syndrome models by expressing adipose tissue-specific 11β-HSD1.

Discussion

Tissue-specific promoters generally contain *cis*-acting elements to which tissue-specific transcription factors bind and regulate tissue-specific expression of genes. Fatty acid-binding proteins are an intracellular hydrophobic ligand-binding protein family (25). aP2 (also known as fatty acid-binding protein 4) facilitates the intracellular solubilization and trafficking of lipids (26). This factor has been implicated as a strong candidate for adipose-specific genes representing the accumulation of lipids in pigs and cows (27,28). Previously, the aP2 promoter in humans and mice was analyzed to identify fat-specific regulatory regions (29). This promoter contains conserved transcription factor binding sites, including C/EBP, adapter primer-1, CAAT box, TATA box, direct repeat 1-type PPAR responsive element, short interspersed repetitive elements and another PPAR responsive element in humans, mice, cows, pigs and dogs (24). The -5.4 kb region upstream of the aP2 promoter is hypothesized to direct the adipose-specific expression of transgenes in mice (30). The aP2 promoter was utilized to overexpress 11β-HSD1 in adipose tissue. Activity of the porcine aP2 promoter in the 3T3-L1 adipose cells was examined. A specific portion of the porcine aP2 promoter (between -2,826 and +15 nt) was observed to possess the highest activity among the various tested promoter regions.

The 11β-HSD1 gene is highly expressed in the adipose tissue of obese humans (20). In the present study, a porcine fibroblast cell line was established expressing the pig 11β-HSD1 gene under the control of the porcine aP2 promoter. In addition, two selection markers, EGFP and the Neor gene, were used for genetic information of somatic cells for further SCNT-mediated cloning. The Neor and EGFP genes are used to select transgenic cell colonies to increase SCNT efficiency and identify transgenic piglets (31-33). Therefore, the use of these porcine fibroblasts cells is likely to enable us to achieve high somatic cell cloning efficiencies in mammals.

Although there are several animal models of metabolic syndrome, the majority are rodents, including mice and rats, which are relatively dissimilar to humans. A porcine model of metabolic syndrome has several advantages, including the physiological similarity between pigs and humans in terms of body fat distribution and fat cell size. Therefore, insight into lipogenesis, lipolysis and lipid mobilization gained from evaluating a porcine model may be useful for studying human obesity. In addition, numerous diagnostic and surgical techniques developed for humans are applicable in pigs, further indicating that the pig is a valuable model for investigating various human diseases.

In conclusion, a porcine fibroblast cell line containing constructs suitable for 11β-HSD1 overexpression was established. To assess adipose-specific expression of the 11β-HSD1 gene, an aP2 promoter controlling expression in adipose tissue was included in the construct. These porcine fibroblast cell lines may be a useful source for SCNT procedures to generate a porcine model of metabolic syndrome. Piglets derived from these cells may also provide critical information for understanding the pathophysiology of metabolic syndrome and developing novel diagnostic therapies for combating human metabolic syndrome.

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