

Generation of pig primary fibroblast cells harboring defective *MC4R* genes by *N*-ethyl-*N*-nitrosourea mutagenesis: A gene-driven, nontransgenic approach to pig improvement

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ABSTRACT

Transgenic pigs have been produced with the aim of further improving pigs in terms of economic and environmental traits, but these animals have not been allowed to enter the food chain. As an alternative approach to generating pigs with novel traits of economic importance that cannot be introduced by conventional breeding, we propose a strategy for combining *in vitro* mutagenesis of pig primary cells with *N*-ethyl-*N*-nitrosourea (ENU) and somatic-cell nuclear transfer (SCNT) technology. To explore the feasibility of this strategy, we treated pig primary fibroblast cells with ENU, estimated the per-base mutation frequency induced by the mutagen, clonally cultured about 4000 of the mutagenized cells, and screened them for mutation within the coding region of the melanocortin-4 receptor (*MC4R*) gene, a key gene in energy homeostasis. Through this screening, we obtained 14 cell clones, each harboring a heterozygous base change within the coding region for *MC4R*. Of the mutant cell clones, each of two contained a mutant allele encoding *MC4R* with greatly reduced receptor activity. By SCNT using these cell clones as donors, pigs harboring mutated *MC4R* alleles with reduced receptor activity can be produced. Our strategy for generating pigs with novel genetic traits likely will be more acceptable to the public than is the use of transgenic technology.

Keywords: Pig; Fibroblasts; *N*-Ethyl-*N*-Nitrosourea; Mutagenesis

1. INTRODUCTION

Thus far, improvement of commercial pigs has been achieved only by conventional breeding. A number of transgenic pigs have been developed with enhanced growth and production traits, or yielding a product that has been altered to make it more environmentally safe, but none of these pigs has been approved to enter the human food chain [1]. In plants, mutagenesis with chemical mutagens or radiation followed by phenotypic screening has been used by breeders to generate many improved commercial crops, and recently mutagenesis followed by gene-driven reverse genetic screening has been proposed as an important tool in crop improvement [2].

Mutant mice derived from embryonic stem (ES) cells mutagenized with chemical mutagens such as *N*-ethyl-*N*-nitrosourea (ENU) *in vitro* have been produced [3-7]. Somatic cell nuclear transfer (SCNT) of pigs has been developed, and many gene-knockout pigs have been generated by exploiting this technology [1,8]. Thus, generation of pigs with novel genetic traits by combining SCNT technology and *in vitro* mutagenesis of pig fibroblast cells with ENU would be an intriguing strategy for improving commercial pigs without the legislative regulations imposed on and social opposition to genetically modified organisms (GMOs). ENU mutagenesis is a conventional and safe method of altering the genetic traits of organisms, and foods derived from livestock produced by the use of SCNT technology and their progeny have been approved as safe in many countries, including the United States and Japan [9].

The melanocortin-4 receptor (*MC4R*) is a G-protein-coupled receptor and is positively coupled to adenylate cyclase via stimulatory heteromeric G proteins [10].

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MC4R is produced predominantly in the brain and is a key regulator of energy homeostasis. *MC4R* activation leads to decreased food intake and increased energy expenditure. When *MC4R* was inactivated through gene targeting, the knockout mice had severe obesity, together with increased body length [11]. Moreover, mice harboring a fully or partially defective *MC4R* have been produced through *in vivo* mutagenesis with ENU and have varying degrees of obesity [12,13]. Human genetic studies have confirmed that the *MC4R* is indispensable for human energy homeostasis. It is well accepted that mutation in human *MC4R* is the most common monogenic form of obesity, with more than 170 distinct mutations identified so far [14,15]. Two non-synonymous variants of pig *MC4R* with apparently normal receptor activity, R236H and D298N, have been reported, but the association between the presence of these variants and traits of economic importance such as growth and fatness remains unclear [16-18].

Here, we developed procedures that enabled the isolation of pig primary cell clones harboring ENU-induced mutations in *MC4R* gene. We obtained two cell clones, each harboring an allele encoding an *MC4R* mutant with greatly reduced receptor activity. Our results illustrate the feasibility of producing cloned pigs via SCNT of cells with mutations of interest induced by ENU mutagenesis.

2. MATERIALS AND METHODS

2.1. Fibroblast Cells

Pig fibroblast cells were prepared from a male fetus of Landrace × Large White at embryonic day 17, as previously described [8]. The sex of the fetus was verified according to a procedure previously described [19]. The cells were cultured at 38°C in an atmosphere of 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (Thermo Fischer Scientific, Waltham, MA, USA), 100 units/ml penicillin, 100 mg/ml streptomycin, 0.25 mg/ml amphotericin (Sigma-Aldrich, St. Louis, MI, USA), and 2 mM GlutaMAX (Life Technologies, Carlsbad, CA, USA). This medium will be referred to here as DME10. The cells were expanded to the third passage and stored at -190°C in Cellbanker 1 medium (Nihon Zenyaku Kougyou, Kouriyama, Fukushima, Japan) until use. Cells of this strain were used throughout the study and will be referred to as M6 cells.

2.2. Mutagenesis of Pig Fibroblast Cells with ENU

Ten days before mutagen treatment, one vial of M6 cells was thawed and placed in DMEM containing 20% fetal calf serum, the antibiotics listed above, and 2 mM GlutaMAX. This medium will be referred to as DME20. In

subsequent cultures, the M6 cells and their mutants were maintained in DME20 at 38°C in an atmosphere of 5% CO₂. The thawed M6 cells were subcultured (1:5) 8 days before mutagen treatment. The cells were confluent 3 days before mutagen treatment; they were refed with fresh medium on this day and the next day. One day before mutagenesis, the cells were replated in appropriate numbers of 10-cm dishes so that the number of cells per dish was 1.1×10^6 . Sixteen hours after the replatings, the culture medium was removed, and the cells were incubated at 38°C for 1 h with DMEM containing 0 mM, 0.5 mM, 1.0 mM, or 1.5 mM ENU (Sigma-Aldrich) and 10 mM HEPES (pH 7.4). The cells were then washed with PBS and cultured in DME20. The colony-forming ability of the cells in one dish for each dose of ENU was assayed immediately after the ENU treatment. Two days after ENU treatment, most of the cells treated with 1.0 mM or 1.5 mM ENU were trypsinized and frozen until use in clonal culture, as described below. The remaining cells for each dose of ENU were seeded in six 10-cm dishes (3×10^5 cells/dish) to examine mutation frequency. The next day, the culture medium was replaced with DME20 containing 0.3 μM ouabain (Sigma-Aldrich). Six days after the addition of ouabain, the cells were refed with DME containing fresh 0.3 μM ouabain. Thirteen days after the start of ouabain selection, the cells were stained with methylene blue to count the colonies. At the start of ouabain selection, a portion of the cells was diluted and plated at cloning density in medium lacking ouabain to determine the cloning efficiency at the time of selection. This value was used to determine the number of ouabain-resistant (Ouar) colonies per total number of clonable cells.

2.3. Isolation of Independent Mutant Cell Clones Resistant to Ouabain

M6 cells were treated with 1.5 mM ENU as described above, and, at the end of the treatment, the cells were washed with PBS, trypsinized, and seeded onto 24-well plates at a density of 5000 cells/well. They were then cultured so that each mutant clone was independently isolated. After 3 days, the medium was replaced with DME20 containing 0.3 μM ouabain. After another 6 days, the cells were refed with fresh DME20 containing 0.3 μM ouabain. 7 days later, colonies growing in the wells (no more than one colony per well) were separately replated into the wells of 24-well plates and maintained. In subsequent cultures, all isolated cell clones were maintained in the presence of 0.3 μM ouabain.

2.4. Nucleotide Sequence Analysis of Cell Clones Resistant to Ouabain

Total RNA was prepared from M6 cells and each of the Ouar^r cell clones described above by using an RNeasy

Mini Kit (Qiagen, Venio, The Netherlands). cDNA was synthesized with a ReverTra Ace- α -cDNA synthesis kit (Toyobo, Osaka, Osaka, Japan) and used as a template in PCR with the primers GGGACACAGACCACCGCCAC TATG (forward) and GTCGTTCCCACAGACGGATGT TTCTC (reverse) to amplify the entire coding region of the pig Na⁺/K⁺-ATPase α 1 subunit gene (ATP1A1) [20]. PCRs were performed in 50- μ l reactions by using KOD FX DNA polymerase (Toyobo), with the following cycling parameters: 94°C for 2 min; five cycles of 98°C for 10 s and 74°C for 3 min; five cycles of 98°C for 10 s and 72°C for 3 min; five cycles of 98°C for 10 s and 70°C for 3 min; 21 cycles of 98°C for 10 s and 68°C for 3 min; and 68°C for 7 min. Amplified DNA was purified by using a Wizard SV Gel and PCR Clean-up System (Promega, Fitchburg, WI, USA), and the nucleotide sequence of the DNA was determined by using external and internal primers on a DNA sequencer (Applied Biosystems Model 3100; Life Technologies). Nucleotide sequences were analyzed with DNASIS software (Hitachi, Hitachi, Ibaraki, Japan).

2.5. Clonal Culture of Mutagenized Cells

A vial of cells treated with 1.0 mM or 1.5 mM ENU was thawed, seeded, and cultured in a 10-cm dish for 3 days until the cells became confluent. The cells were then trypsinized and suspended in 5 ml Hanks's balanced salt solution (Life Technologies) containing 2% fetal calf serum, 20 mM HEPES (pH 7.4), and 1 mM isopropyl iodide (Sigma-Aldrich). The cells were delivered to forty 96-well plates (each containing 150 μ l DME20) with a flow cytometer (EPICS ALTRA, Beckmann-Coulter, Brea, CA, USA), such that each well contained one living cell. The 40-plate set of mutagenized cells was allowed to grow for 1 week, after which the cells in each plate were replicated into two 96-well plates. The cells in these two sets of 96-well plates were allowed to grow for 2 weeks, during which time the cells were refed with DME20 once a week. Twenty-four to 26 days after the cell sorting, one set of 96-well plates was inspected under an inverted microscope to identify wells containing more than about 1×10^4 cells. The cells in these wells were then subjected to screening for mutation, as will be described below. Cells in another set of 96-well plates for use as master plates were cultured further until wells containing cell clones with mutations within *MC4R* gene were identified as will be described below, after which the mutant cell clones in the master plates were expanded and frozen until use.

2.6. Screening for Mutations Following Clonal Culture

Genomic DNA was prepared from the cells in the 96-well plates by using Proteinase K and DirectPCR cell

reagent (45 μ l/well) (Viagen-Biotech, Los Angeles, CA, USA). An aliquot of the DNA solution (2 μ l) was used as a template in PCR with the primers GGAGAAAAT CGCTGAGGCTA (forward) and TGTCCAGACTGTG TAAGGAGAA (reverse) to amplify the entire coding region for *MC4R* [18]. The PCRs were performed in 20- μ l reactions using TaKaRa Ex Taq Hot Start Version DNA polymerase (Takara Bio, Ohtsu, Shiga, Japan), with the following cycling parameters: 98°C for 10 s; 35 cycles of 94°C for 30 s, 63°C for 30 s, and 72°C for 1 min; and 72°C for 7 min. Amplified DNA was subjected to sequencing on a DNA sequencer (Applied Biosystems 3130 \times 1 Genetic Analyzer, Life Technologies). The nucleotide sequences of the cell clones were compared with that of M6 cells to identify mutations within the coding region for *MC4R*.

2.7. Construction of Plasmids Encoding Myc-Tagged *MC4R* Mutants

A pcDNA3.1-based expression vector producing *MC4R* was constructed essentially as described previously [21], after PCR amplification of the coding region for *MC4R* by using genomic DNA from M6 cells as a template. For immunologic detection purposes, the construct was tagged with an Myc-derived epitope (EQKLISEEDL) downstream of the initiation codon by using PCR-based site-directed mutagenesis and restriction fragment replacement strategy. Expression plasmids, each harboring a nucleotide change corresponding to that found in the mutagenized cell clones, were constructed by using a Quick-Change site-directed mutagenesis kit (Stratagene, LaJolla, CA, USA). All PCR-derived constructs were verified by DNA sequencing.

2.8. Transfection and cAMP Assays

COS-7 cells were grown in DME10 at 38°C in an atmosphere of 5% CO₂. Cells were seeded into six-well plates (2×10^5 cells/well), and 24 h later they were transiently transfected with plasmids encoding wild-type and mutant *MC4Rs* (2.0 μ g/well), along with pCBR-Control vector (0.5 μ g/well) (Promega), by using Lipofectamine LTX reagent (Life Technologies). One day after transfection, the cells were seeded into a 96-well plate (4×10^4 cells/well) precoated with polyethyleneimine (Sigma) and cultured in Waymouth's MB 752/1 medium (Life Technologies) containing 1% bovine serum albumin (Waymouth-BSA). The remaining cells were seeded into a 96-well plate for chemiluminescence measurement (GreinerBio One, Frickenhausen, Germany) and cultured in Waymouth-BSA at 38°C in an atmosphere of 5% CO₂. The next day, cyclic adenosine monophosphate (cAMP) assay and chemiluminescence measurement were performed. Cells were first washed once with Waymouth-BSA

and then incubated with 0.5 mM isobutylmethylxanthine (IBMX) (Sigma-Aldrich) in Waymouth-BSA for 15 min at 38°C. The medium was removed and replaced with Waymouth-BSA with 0.5 mM IBMX and increasing concentrations of alpha-melanocyte stimulating hormone (α -MSH) (Peptide Institute, Osaka, Osaka, Japan), and the cells were incubated at 38°C for 1 h. Intracellular cAMP was measured with a cAMP-Screen assay kit (Life Technologies). Chemiluminescence from the cells was measured by using Steady-Glo luciferase assay substrate (Promega). cAMP values were normalized against luciferase data to correct for variations in transfection efficiency and were then analyzed with GraphPad Prism 5 software (Graphpad Software, San Diego, CA, USA).

3. RESULTS

3.1. ENU Mutagenesis and Estimation of Per-Base Mutation Frequency

To our knowledge, no previous report of mutagenesis of primary pig fibroblasts with chemical mutagens has been published. We first sought to determine appropriate conditions for ENU mutagenesis of primary pig fibroblasts, in which we exploited ouabain selection to monitor the frequency of induced mutations. Ouabain selection of human and rodent cells after mutagenesis has been used to monitor the mutation frequency in such cells [22-25]. Cellular resistance to ouabain results from specific missense mutations in the ATP1A1 gene ([26] and references therein).

The percent survival and the frequency of mutation induced by ENU of M6 cells after ENU treatment were shown in **Figure 1**. The mutation frequency, expressed as the frequency of Ouar cells in the total number of clonable cells, increased with increasing ENU concentration, reaching $1.15 \pm 0.16 \times 10^{-4}$ at an ENU concentration of 1.5 mM (**Figure 1(b)**). The frequency of Ouar cells in mock-treated cells was less than 1×10^{-7} (not shown).

In studies of mutagenesis of mouse ES cells and subsequent generation of mutant mice, the mutation frequency in ES cells was monitored by exploiting the X-linked hypoxanthine phosphoribosyl transferase (hpRT) locus to determine appropriate concentrations of ENU for mutagenesis [3,5-7], and the relationship between the frequency of HPRT-deficient cells and the per-base mutation frequency in the mouse genome were inferred from sequence analysis of randomly isolated mutagenized cell clones [3,7]. However, for unknown reasons, we were not able to detect any pig fibroblast cells deficient in HPRT activity after treatment of the cells with ENU or ethyl methanesulfonate (data not shown). We needed to examine the relationship between the mutation frequency (determined as the frequency of Ouar cells) and the per-base mutation frequency in the swine genome. If the

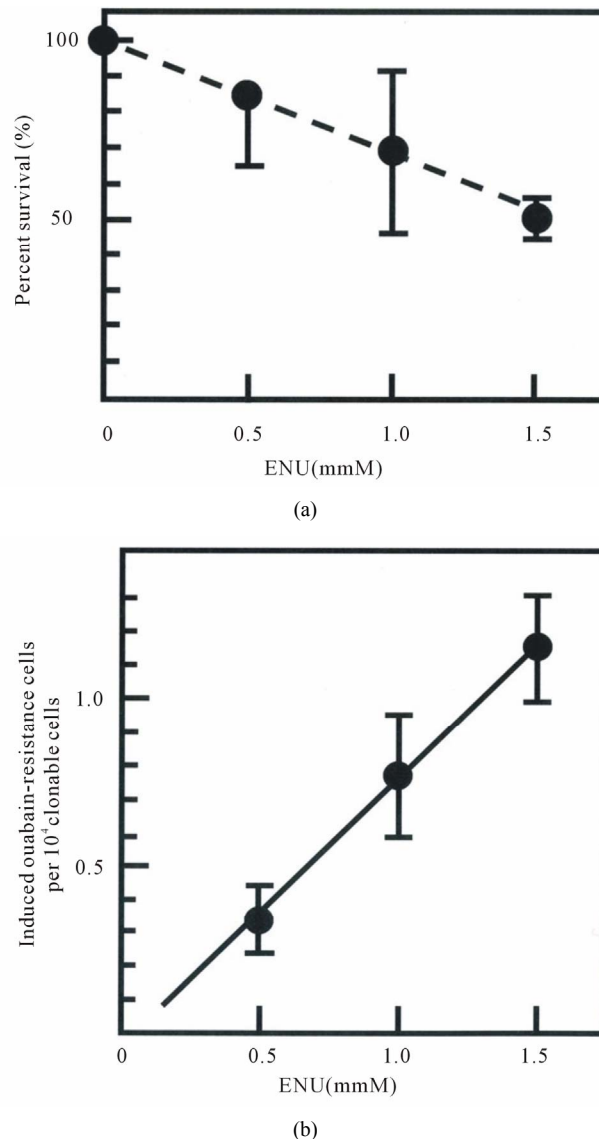


Figure 1. Cytotoxicity (a) and mutagenicity (b) induced by *N*-ethyl-*N*-nitrosourea (ENU) in pig fibroblasts. Percentage survival in panel A was calculated by dividing the cloning efficiency of cells treated with the indicated concentrations of ENU by that of mock-treated cells at the end of treatment. Cloning efficiency of the mock-treated cells ranged from 0.56 to 0.73. The frequency of induced Ouar^r cells (b) was calculated by dividing the number of Ouar^r colonies by the number of cells plated for selection, corrected by the cloning efficiency at the time of selection. Each point represents the mean of at least four experiments. Bars represent 1 SD.

per-base mutation frequency after mutagenesis were too low, we would have to screen too many clones of mutagenized cells to find one with a desired mutation, whereas if the frequency were too high, this high load of mutations in the genome would prevent the generation of fertile cloned animals after transfer of the nuclei of the mutant cells, just as has been pointed out in studies of mutant mice derived from mutagenized ES cells [3,7,27].

To examine the relationship between the frequency of Ouar cells after ENU treatment and the per-base mutation frequency induced by this treatment, we first isolated 18 independent Ouar cell clones after mutagenesis with 1.5 mM ENU, and we determined the nucleotide sequence of the entire coding region of the pig ATP1A1 gene in these cells. Comparison of the sequences in the Ouar clones with that in M6 cells (GenBank Accession No. AB703104.1) unequivocally identified a heterozygous point mutation in each clone (**Table 1**). All of these mutations were missense mutations. T-to-A transversions were found most frequently among the mutations (38%); this was in accordance with the results of sequence analysis of HPRT-deficient mutants after mutagenesis of HFW human fibroblast cells with ENU [28]. Amino-acid residues at a total of eight positions were substituted in the Ouar clones: these were C104, Q111, D121, N122, I315, F316, L793, and T797.

A series of extensive random-mutagenesis studies of the sheep ATP1A1 gene has identified single amino-acid substitutions, each of which renders the enzyme resistant to ouabain ([26] and references therein). These amino-acid changes resulting from missense mutations occur at a total of 16 positions: C104, Y108, Q111, P118, D121, N122, Y308, L330, A331, T338, F786, L793, T797,

F863, R880, and F982. The amino-acid residues at these positions are assumed to be involved in binding between the enzyme and its inhibitor, ouabain; in fact, a recent X-ray crystallography study of a complex between pig ATP1A1 and ouabain has shown that all of the amino-acid residues mentioned above are included in the ouabain-binding pocket of the enzyme [29]. Notably, the amino-acid sequence is highly conserved between the sheep and pig ATP1A1 enzymes (99.3% identity), and all of the sheep enzyme amino-acid residues mentioned above are conserved at the same positions in the pig enzyme.

Of the eight positions where we found amino-acid substitutions in Ouar clones, six corresponded to the respective ones identified in the sheep enzyme as being involved in binding to ouabain, suggesting that each amino-acid substitution that we found altered the affinity between the enzyme and ouabain. The other two positions of amino-acid substitutions that we found—I315 and F316—had no counterparts in the positions identified in the sheep enzyme. These results suggest that not all of the amino-acid positions at which an amino-acid change makes the enzyme resistant to ouabain have been identified in the studies on the sheep enzyme; that the amino-acid substitutions at I315 and F316 have been missed in the studies of the sheep enzyme; and that I315 and F316 are in fact conserved in the sheep amino-acid sequence. We propose that the ratio between the number of amino-acid positions already identified in studies of the sheep enzyme and that of the positions not identified so far, including I315 and F316, can reasonably be estimated on the basis of our results as 6:2. This assumption gives a value of 21.3 ($16 \times [8/6]$) for the estimated total number of amino-acid positions at each of which an amino-acid change results in resistance of the enzyme to ouabain.

Taking into account the fact that an amino-acid residue is coded for by three nucleotides and the Ouar phenotype is dominant [26] and assuming that 70% of random nucleotide substitutions induced by ENU are missense mutations [30], the total number of nucleotide positions at each of which a nucleotide substitution would lead to generation of Ouar enzyme, and thus Ouar cells, would be 89.5 (*i.e.*, $21.3 \times 3 \times 0.7 \times 2$). This argument leads to an estimated per-base mutation frequency of (the frequency of Ouar cells/89.5). The frequency of Ouar cells induced by 1.5 mM ENU was 1.15×10^{-4} and that induced by 1.0 mM ENU was 0.79×10^{-4} (see **Figure 1(b)**); thus the estimated per-base mutation frequency was 1.3×10^{-6} (1 base change in 778 kilobases (kb)) with 1.5 mM ENU, and 0.9×10^{-6} (1 in 1132 kb) with 1.0 mM ENU. These results implied that, for example, one clone among 389 clones of the cells treated with 1.5 mM ENU would harbor a heterozygous base change in the coding region for *MC4R*.

Table 1. Mutations found in 18 independent Ouar^f cell clones.

Clone	Nucleotide	Amino acid	Amino acid
	change ^a	change ^b	change in sheep ^c
A2	T324G	C104W	C104F, A, Y
A3	T378A	N122K	N122D
A4	C2402T	T797I	T797A, V, N
A5	T375A	D121E	D121E, N, A, S
B1	T375A	D121E	D121E, N, A, S
B3	C2402T	T797I	T797A, V, N
B4	T378A	N122K	N122D
B5	T375G	D121E	D121E, N, A, S
B6	T378A	N122K	N122D
C4	T378G	N122K	N122D
2A4	T959A	F316Y	none
2B2	C2402T	T797I	T797A, V, N
2B3	T375G	D121E	D121E, N, A, S
2C3	C343G	Q111E	Q111R
2C4	T2390C	L793P	L793P, N, K
2C5	T956G	I315S	none
2C6	T2390C	L793P	L793P, N, K
2D3	T959A	F316Y	none

^aNucleotide numbering in GenBank accession No. AB703104; ^bFor the purpose of comparison with the data on sheep ATPase, amino-acid numbering starts from the fifth amino-acid residue G in the amino-acid sequence encoded by the nucleotide sequence in GenBank, above; ^cAmino-acid changes that are reported to render sheep ATPase resistant to ouabain and are located at positions corresponding to those found here are shown for comparison (see text).

3.2. Clonal Culture and Screening of Cell Clones

A total of 2100 clones of cells treated with 1.5 mM ENU and a total of 1893 clones of cells treated with 1.0 mM ENU were screened for the presence of mutation in eight separate clonal culture experiments (**Table 2**). The percentage of wells in 96-well plates that contained sufficient numbers of cells for DNA analysis at the time of inspection was 13.4%; this was lower than the cloning efficiency of the cells (0.56 - 0.73). Sorting of the cells on a flow cytometer at the beginning of the clonal culture experiments was indispensable, because once the cells had been grown at clonal densities and harvested, they could not grow when reseeded at clonal densities. For the screening, DNA including the entire coding region for *MC4R* was amplified by PCR using two primers, one just 5' and the other just 3' to the coding region, and its nucleotide sequence was determined by using the same primers: the coding region for pig *MC4R* is 999 base pairs long, and this region is continuous on the genome [18]. A total of 10 clones treated with 1.5 mM ENU and a total of four clones treated with 1.0 mM ENU were identified as each having a mutation within the coding region (**Table 3** and **Figure 2**). The cells found to contain mutations were picked from the corresponding wells in the master plates, expanded, and stored in liquid nitrogen. The nucleotide sequences of the *MC4R* coding regions in the expanded cells were determined after PCR amplifica-

Table 2. Summary of clonal culture experiments.

Experiment	No. of clones screened ^a	No. of mutations identified	No. of nonsilent mutations identified	Per-base mutation frequency ^b
1.5 mM ENU ^c				
1	359	0	0	0
2 ^d	239	0	0	0
3	584	3	1	2.6×10^{-6} (1 in 400 kb)
4	918	7	6	3.8×10^{-6} (1 in 263 kb)
Average ^e				1.6×10^{-6} (1 in 625 kb)
1.0 mM ENU ^c				
5	80	0	0	0
6	593	0	0	0
7	538	2	2	1.9×10^{-6} (1 in 538 kb)
8	682	2	1	1.5×10^{-6} (1 in 680 kb)
Average ^f				0.8×10^{-6} (1 in 1201 kb)

^aNumber of cell clones analyzed for the presence of mutation; ^bPer-base mutation frequency was calculated according to the following equation: Per-base mutation frequency = (No. of mutations identified)/[(No. of clones screened) × 999 × 2]. Note that the coding region for *MC4R* is 999 bp long, and two alleles of the gene were examined by sequencing of genomic DNA; ^cCells treated with 1.5 mM ENU were screened in experiments 1 through 4, and cells treated with 1.0 mM ENU were screened in experiments 5 through 8; ^dCells were seeded in thirty 96-well plates in this experiment; ^eArithmetic mean of per-base mutation frequency data in experiments 1 to 4; ^fArithmetic mean of per-base mutation frequency data in experiments 5 to 8.

Table 3. Mutants obtained in clonal cultures of fibroblast cells treated with ENU.

Mutant	Nucleotide change ^a	Amino acid change ^a
6D10 ^b	A554C	H7P
33H8 ^b	A616T	S28C
26G9 ^b	A663C	Q43H
34A9 ^b	G759T	silent
33B11 ^b	T804C	silent
15A7 ^b	T928C	S132P
26F4 ^b	T1088G	I185S
13G4 ^b	C1095T	silent
24C10 ^b	A1207G	R225G
36F5 ^b	A1480C	I316L
39A4 ^c	C744A	silent
3G6 ^c	C764T	S77L
24H11 ^c	A1012T	I160F
23C11 ^c	G1193T	R220I

^aNucleotide numbering and amino-acid numbering are those for GenBank accession no. NM_214173. The nucleotide sequence of the coding region for *MC4R* in M6 cells was identical to this GenBank sequence; ^bClones obtained from cells treated with 1.5 mM ENU; ^cClones obtained from cells treated with 1.0 mM ENU.

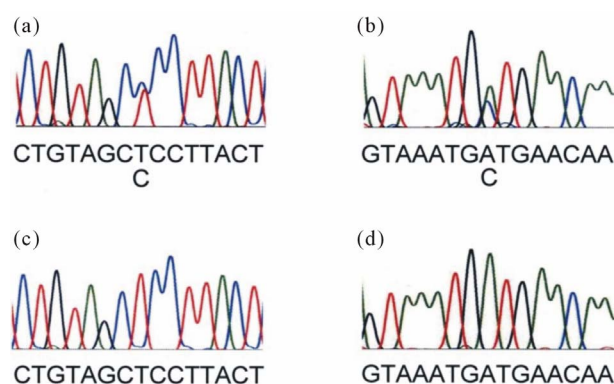


Figure 2. Sequencing traces of cell clones harboring *MC4R* mutants, S132P and I185S. Panel. (a) Sequencing trace (forward orientation) of the cell clone 15A7 harboring S132P. Panel; (b) Sequencing trace (reverse orientation) of the cell clone 26F4 harboring I185S. Panel; (c) Sequencing trace of wild-type cells which corresponds to panel A. Panel; (d) Sequencing trace of wild-type cells which corresponds to panel A. Nucleotide sequence is shown below each sequencing trace.

tion to verify the mutations.

All of the mutations were heterozygous base substitutions (**Table 3**). Ten were missense mutations, and the other four were silent. Average per-base mutation frequencies of cells treated with 1.5 mM ENU and 1.0 mM ENU, calculated on the basis of the sequence analysis, were 1.6×10^{-6} (1 in 625 kb) and 0.8×10^{-6} (1 in 1201 kb), respectively (**Table 2**). These average per-base mutation frequencies were in good agreement with the estimations of per-base mutation frequency based on the results of ouabain selection at the corresponding doses of

ENU. Moreover, the average per-base mutation frequency when the cells were treated with 1.5 mM ENU was comparable to the per-base mutation frequency estimated on the basis of the sequence analysis of ENU-treated mouse ES cells (2.2×10^{-6}), by which cells harboring mutations in Smad 2 and Smad 4 were obtained [7] This implies that pig fibroblast cells treated with ENU exhibited levels of mutability comparable to those reported for mouse ES cells.

3.3. Functional Analysis of Wild-Type and Mutant *MC4Rs*

α -MSH is considered to be an endogenous ligand of *MC4R* [10]. Wild-type pig *MC4R* and all the nonsynonymous mutants that we identified were examined for signal transduction activity upon stimulation with α -MSH in COS-7 cells transiently transfected with the receptor constructs. The responses of S132P and I185S were so greatly reduced that the maximum response (R_{\max}) and the half maximum effective concentration (EC_{50}) values of these mutants could not be determined (Table 4 and Figure 3). The R_{\max} values of S77L, R220I, R225G, and I316L were moderately reduced compared with that of the wild type, whereas H7P, S28C, Q43H, and I160F had R_{\max} levels similar to that of the wild type. The EC_{50} values of these active mutants were comparable to that of the wild type. Flow cytometry with an anti-myc antibody (Life Technologies) was used to analyze Cos-7 cells transiently transfected with plasmids encoding wild-type *MC4R*, I185S, and S132P and detected the expression of these encoded proteins on the surfaces of the transfected cells at levels comparable to each other, suggesting that the apparent deficiency in receptor activity shown with I185S and S132P was due

Table 4. cAMP-signaling activity of wild-type and mutant *MC4Rs*^a.

Mutation	EC_{50} (nM) ^a	R_{\max} (%WT) ^a
Wild type	2.3 ± 1.5	1.0
H7P	4.8 ± 1.5	1.03 ± 0.04
S28C	4.0 ± 1.3	1.15 ± 0.07
Q43H	4.3 ± 2.8	0.88 ± 0.03
S77L	3.0 ± 1.7	0.73 ± 0.04
S132P ^b	n.d.	n.d.
I160F	1.1 ± 0.4	0.87 ± 0.08
I185S ^b	n.d.	n.d.
R220I	4.9 ± 2.8	0.57 ± 0.04
R225G	6.5 ± 2.5	0.55 ± 0.11
I316L	1.7 ± 0.3	0.71 ± 0.1

^aMean and SD of three independent experiments in duplicate; ^bActivities of S132P and I185S were so reduced that the EC_{50} (half maximum effective concentration of alpha-melanocyte stimulating hormone) and R_{\max} (maximum response) values could not be determined.

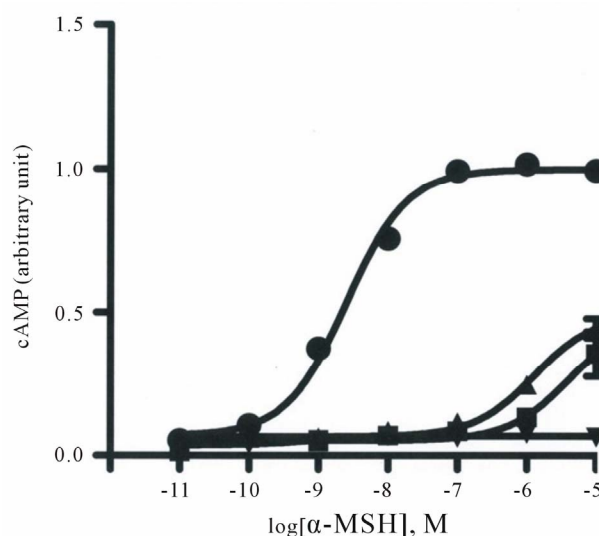


Figure 3. cAMP stimulation of wild-type (WT) *MC4R* and the mutants S132P and I185S in COS-7 cells with alpha-melanocyte stimulating hormone (α -MSH). The amounts of cAMP in the cells transfected with plasmids producing WT (\bullet), S132P (\blacktriangle), and I185S (\blacksquare) after α -MSH stimulation were normalized against luciferase data in each experiment (see Materials and Methods). Each point represents the mean of three experiments in duplicate. Bars represent 1 SD.

to intrinsic defects in these mutants (data not shown).

4. DISCUSSION

The generation of cell clones harboring I185S or S132P provides proof-of-principle of the feasibility of generating pig primary fibroblast cells harboring mutations of functional importance in genes of interest. The relationships that have been shown in previous studies between the functional activity of *MC4R* mutants and the phenotypes of humans or mice harboring *MC4R* mutants strongly suggest that cloned pigs produced via SCNT using cells harboring S132P or I185S as donors would have substantially increased appetite and decreased basal energy expenditure [11-14]. These pigs would be the founders of a generation of pigs with improved growth characteristics and would be valuable model animals for human obesity.

The estimated values for per-base mutation frequency based on ouabain selection were in good agreement with those based on the sequence analysis of randomly isolated mutagenized cell clones, suggesting that per-base mutation frequency in the genome can be reasonably well estimated by exploiting ouabain selection. Our results in this regard may also be applicable to large domestic animals, because all of the ouabain-binding sites in ATPase are conserved among mammals, with the exception of rodents [31]. It is pertinent to note here that the estimated values for per-base mutation frequency based on ouabain selection should be somewhat lower

than actual per-base mutation frequency in a sense that mutations other than nucleotide changes (deletions or insertions) are not represented in the estimated values.

Vivian *et al.* [7] treated CT129 ES cells with 1.7 mM ENU for 2 h, and five clones of the mutagenized cells yielded germ-line chimeras among the 13 clones tested. The per-base mutation frequency in the study by Vivian *et al.* [7] was comparable to ours (see Results). Greber *et al.* [5] treated E14.1 ES cells with 1.5 mM ENU for 90 min; one out of two mutant cell clones gave rise to germ-line chimeras. Chick *et al.* [4] mutagenized c7.1 ES cells with 4.3 mM ENU for 2 h, and chimera formation using two of three mutagenized cell clones resulted in germ-line transmission. These results suggest that pig fibroblast cell clones harboring S132P or I185S would give rise to cloned pigs upon SCNT, because the per-base mutation frequency introduced into these pig cells in the present study seems no higher than that introduced into the mutant ES cells mentioned above.

The ENU-generated mutant alleles of genes of interest in cloned pigs can be introgressed into commercial pigs by serial backcrosses: Introgression of alleles controlling coat color, such as the MC1R * 2 allele, has been performed in pig breeding [32]. Marker-assisted selection of founder animals in each generation of the backcross would facilitate the elimination of bystander mutations induced by ENU [33]. Total-genome sequence data [34] and high-density SNP panels [35] of the pig can now greatly facilitate this process.

Recently, gene-knockout pigs have been produced by using zinc-finger-nuclease (ZFN) or transcription activator-like effector nuclease (TALEN) [36,37]. In these studies, mutations were introduced into the genome of primary pig cells by use of ZFN or TALEN, and cells harboring the mutations were selected with the aid of marker genes and then subjected to SCNT. The resultant pigs are genetically modified owing to the presence of the marker genes, and they are therefore subject to the legislative regulations and social opposition surrounding GMOs. Moreover, licensing to produce and commercialize such pigs would be costly, because ZFN and TALEN are proprietary technologies [38,39], and the legislative question as to whether or not organisms engineered by the use of ZFN and TALEN—even when no exogenous DNA is stably integrated—are deemed non-GMO remains unsettled. Taking into consideration these uncertainties, we consider that our proposal for generating mutant pigs through *in vitro* ENU mutagenesis is currently the most feasible for generating commercial pigs with novel alleles of economical importance.

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