The Uncoupling Protein 1 Gene (UCP1) Is Disrupted in the Pig Lineage: A Genetic Explanation for Poor Thermoregulation in Piglets

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Piglets appear to lack brown adipose tissue, a specific type of fat that is essential for nonshivering thermogenesis in mammals, and they rely on shivering as the main mechanism for thermoregulation. Here we provide a genetic explanation for the poor thermoregulation in pigs as we demonstrate that the gene for uncoupling protein 1 (UCP1) was disrupted in the pig lineage. UCP1 is exclusively expressed in brown adipose tissue and plays a crucial role for thermogenesis by uncoupling oxidative phosphorylation. We used long-range PCR and genome walking to determine the complete genome sequence of pig UCP1. An alignment with human UCP1 revealed that exons 3 to 5 were eliminated by a deletion in the pig sequence. The presence of this deletion was confirmed in all tested domestic pigs, as well as in European wild boars, Bornean bearded pigs, wart hogs, and red river hogs. Three additional disrupting mutations were detected in the remaining exons. Furthermore, the rate of nonsynonymous substitutions was clearly elevated in the pig sequence compared with the corresponding sequences in humans, cattle, and mice, and we used this increased rate to estimate that UCP1 was disrupted about 20 million years ago.

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Introduction

Pigs are unusual mammals in a number of ways: they are the only ungulates that (1) build nests for birth (Figure 1A), and (2) have large litters, where (3) each young is behaviorally well developed but with a poor thermoregulatory ability [1,2]. It is also striking that piglets appear to lack brown adipose tissue (BAT) [3] and rely on shivering as the main mechanism for thermoregulation [4,5]. BAT is a specific type of fat that is widely expressed in neonatal animals as well as in hibernating rodents [6]. Its physiological role is to generate heat by uncoupling oxidative phosphorylation. Uncoupling protein 1 (UCP1) is exclusively expressed in BAT and plays a crucial role for thermogenesis. UCP1 is located in the inner membrane of the mitochondria, where it catalyzes a regulated proton leakage across the membrane. The established energy is then released as heat [6]. It has been proposed that the acquisition of BAT and UCP1 gave the early mammals an evolutionary advantage by allowing them to be active during periods of nocturnal or hibernal cold and to survive the cold stress at birth [6].

No conclusive evidence for the presence of BAT [7] or for the expression of UCP1 [8,9] has yet been demonstrated in pigs, despite considerable efforts to study this subject. It has therefore been questioned whether pigs express this tissue, and it has been suggested that UCP1 expression may have been down-regulated during domestication because of strong selection for an efficient energy metabolism. Here we provide an explanation for poor thermoregulation in pigs and the evolution of the unique features in maternal behavior and piglet development. We demonstrate that UCP1 became disrupted in the pig lineage about 20 million years ago.

Results

We took advantage of the recent release of a partial pig genome sequence [9] to investigate the porcine UCP1 locus. The human transcript was blasted against the Sus scrofa trace archive (http://www.ncbi.nlm.nih.gov/Traces/trace.cgi) using discontiguous Mega BLAST (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/blast/tracemb.shtml). Two hits, pig trace sequences 81263277 and 782925243, corresponding to UCP1 exons 2 and 6, were obtained and used for primer design. The complete genome sequence of pig UCP1 was determined by long-range PCR and genome walking by using genomic DNA from a Large White domestic pig and the porcine bacterial artificial chromosome (BAC) clone PigE-117H8 [10] containing UCP1. An alignment with the corresponding human sequence revealed two gaps in the porcine sequence, reducing the total size from 10.1 kilobases in humans to 4.3 kilobases in the pig (Figure 1B). Alignments with cattle UCP1 showed that Gap1, located in intron 2, represents an insertion in the human lineage or a
**Synopsis**

Brown adipose tissue (BAT) is unique to mammals. It is rich in mitochondria and generates heat to maintain body temperature during cold stress, referred to as nonshivering thermogenesis. BAT is found in abundance in rodents as well as in newborns of larger mammals, including humans. Uncoupling protein 1 (UCP1) is exclusively expressed in BAT and is localized to the inner membrane of the mitochondria. Its physiological role is to uncouple oxidative phosphorylation so that most of the energy in fat stores is dissipated as heat rather than being converted to ATP.

Piglets are sensitive to cold stress and rely on shivering as the main mechanism for thermoregulation. Furthermore, pigs are the only hoofed mammals that build nests for birth; in modern pig production, heat-producing lamps are used to keep the piglets warm. It is also striking that pigs appear to lack BAT.

Here the authors show that the UCP1 gene is disrupted in domestic pigs and their wild ancestors. The inactivation of UCP1 was estimated to have happened about 20 million years ago. The finding provides an explanation for the poor thermoregulation in piglets that may have led to the evolution of the unique maternal behavior in this species.

Deletion that occurred before the split of the pig and cattle lineages. Gap2 is unique to the pig and eliminates exons 3 to 5, implying that UCP1 is disrupted. The presence of this deletion was confirmed by PCR amplification of the deletion breakpoint in pigs representing many different breeds, as well as in European wild boars, Bornean bearded pig (S. barbatus), wart hog (Phacochoerus africanus), and red river hog (Potamochoerus porcus).

Southern blot analysis using a porcine probe revealed a single restriction fragment consistent with the presence of a single UCP1 copy in the pig genome (Figure S1). The similarity between the end sequences of the porcine UCP1 BAC clone and sequences flanking the human UCP1 locus supports the interpretation that we have analyzed the true UCP1 locus and not a defect duplicated version.

The UCP1 coding sequence in pigs contains three additional disrupting mutations: a two-base pair insertion in exon 1, a 16-base pair deletion in exon 2 (both causing frameshifts), and a nonsense mutation in exon 6 (Figure S2). In contrast, strong purifying selection is observed at the UCP1 locus in humans, cattle, and mice, as is evident from the 5- to 10-fold higher rate of synonymous (dS) versus nonsynonymous (dN) substitutions among these species (Figure 1C). As expected, dS is nearly identical when comparing humans with pigs and humans with cattle. In contrast, dN was markedly elevated in all pairwise comparisons involving the pig (Figure 1C). For example, human versus pig: dN(BAT) = 13.9 ± 2.4%, human versus cattle: dN(BAT) = 8.8 ± 1.9%, Z = 1.66, p < 0.05 in a one-sided test; dD = s(D) where D = dN(BAT) + dN(B-H), V(D) = V(dN(BAT)) + V(dN(B-H)), and s(D) = [V(D)]^1/2 [11]. This implies that porcine UCP1 was inactivated sometime subsequent to the divergence from the cattle lineage and that it has accumulated synonymous and nonsynonymous substitutions at the same rate since then. The inactivation of UCP1 was dated to have happened about 20 million years ago by using the estimated dN rate for the human/cattle comparison and the estimated dS rate for the pig/cattle comparison to explain the excess of nonsynonymous substitutions in the pig UCP1 sequence; the estimated time since the divergence of the human and pig/cattle lineages (94 million years) and the pig and cattle lineages (60 million years) were from Springer et al. [12]. The result is consistent with the observation that UCP1 is disrupted in several pig species.

**Discussion**

We have shown that UCP1 was inactivated in the pig lineage about 20 million years ago. UCP1 knockout mice show only a mild phenotype; they are cold sensitive but fully viable [13]. Furthermore, these UCP1-null mice develop BAT, which implies that the disruption of porcine UCP1 cannot by itself explain the absence of BAT. A possible evolutionary scenario is that a pig ancestor lost UCP1 function and the ability to use BAT for thermoregulation because of no or only weak selection for this mechanism in a warm climate; the wild boar is the only porcine species that has adapted to temperate climates, whereas all other Suidae live in tropical or subtropical environments. The wild boar has then evolved compensatory mechanisms to adapt to a cold environment. Newborn pigs use shivering for thermogenesis [4,5], and the wild boar is the only ungulate that builds a thermoprotective nest for giving birth (Figure 1A). A temperature around +20 °C has been estimated in winter farrowing nests of free-ranging domestic pigs despite outside temperatures down to −20 °C [14]. In modern pig production, heat-lamps are used to facilitate thermocontrol in piglets.

An interesting topic for future research will be to study UCP1 in more distantly related species to date more precisely the disruption of this gene in the pig lineage. It will be of considerable interest to investigate if the loss of gene expression closely correlates with changes in behavior and litter size among species. Our study does not resolve whether the disruption of UCP1 was the primary event leading to the loss of BAT or whether it was a secondary event. This question could possibly be resolved by a comparative study of different Suidae species, but it cannot be answered by analyzing sequence data from one species only, because of the poor precision in dating mutation events that happened 20 million years ago. Another important question is whether the loss of UCP1 and BAT is associated with other genetic changes during the evolution of the pig lineage. For instance, it is possible that the disruption of UCP1 has led to an upregulation of UCP2 expression, as observed in the UCP1 knockout mice [13].

**Materials and Methods**

**Animals and BAC resources.** We used genomic DNA representing several different breeds of domestic pigs and samples from European wild boar, wart hog (P. africanus), Bornean bearded pig (S. barbatus), and red river hog (P. porcus). The pig BAC PigE-117H8 DNA [10] was also used, because its end sequences showed homology to sequences flanking UCP1 in the human genome (http://www.sanger.ac.uk/cgi-bin/Projects/S_scorpia/WebFPcreport.cgi?mode=wfcreport&name=PigE-117H8).

**PCR analysis.** All primers used for PCR and sequence analysis are listed in Table S1. PCR products were excised from agarose gels and purified using an EZ.N.A. Gel Extraction Kit (Omega Bio-tek, Doraville, Georgia, United States).

**Long-range PCR.** Forward and reverse primers were located in exon 2 and 6 (primer pair UCP1_1–6). PCR was performed using Expand Long Template PCR system MixI (Roche Diagnostics GmbH, Mannheim, Germany) with the following modifications: 400 µM dNTP, 0.10 M betain, and a final volume of 25 µl. The PCR was run using 1–2 µg of genomic DNA and BAC PigE-117H8 DNA.

**GenomeWalker.** The GenomeWalker universal kit (BD Biosciences, Palo Alto, California, United States) was used to make libraries of...
Large White pig genomic DNA and BAC PigE-117H8 DNA. PCR was performed using either Expand Long Template PCR system (Roche Diagnostics GmbH) or the AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, California, United States).

**UCP1 exon 1.** The pig trace sequence 848676505 was used to design a forward primer for amplification of the entire exon 1 of UCP1. The consensus sequence from the long-range PCR together with the sequences from GenomeWalker were used to design the reverse primer (UCP1_L_B).

**UCP1 amplicon spanning Gap2.** A PCR amplicon of about 200 base pairs spanning Gap2 was amplified with the Gap2 primers (Table S1). This amplicon included intron 2 and 5 sequences, showing significant similarity to the corresponding human sequences to ensure that the amplicon spanned the deletion breakpoint.

**Sequencing.** Purified PCR products were sequenced using the forward and reverse PCR primers plus four additional primers for the long-range PCR product: UCP1seq forward and reverse and UCP1seq2 forward and reverse. Sequencing products were generated using the DYE Magnetic FL dye terminator kit (Amersham Biosciences, Uppsala, Sweden) and separated on a MegaBACE 1000 capillary instrument (Amersham Biosciences). Sequences were aligned using Sequencer 3.1 software (Gene Codes, Ann Arbor, Michigan, United States). Some PCR products were cloned into a pCR 2.1-TOPO vector (Invitrogen, Paisley, United Kingdom) to facilitate sequencing.

**Sequence alignments.** UCP1 sequences were aligned using the dotpath program (http://liv.bmc.uu.se:16080/cgi-bin/emboss/dotpath) (Gary Williams, MRC Rosalind Franklin Centre for Genomics Research, Cambridge, United Kingdom) in EMBOS explorer [15]. All pairwise alignments of pig, human, and cattle sequences were made. UCP1 exons 1, 2, and 6 from humans, mice, cattle, and pigs were aligned using MEGA 2.1 [16] and used to estimate \( d_s \) and \( d_a \). The time since the disruption of the porcine UCP1 gene was calculated as follows: 

\[
\frac{t}{94} = \frac{1}{2} \left( \frac{d_s(B. taurus)}{C_0} - \frac{d_s(Sus scrofa)}{C_0} + \frac{d_s(Sus scrofa)}{C_0} - \frac{d_s(Bos taurus)}{C_0} \right) = \frac{d_s(B. taurus) - d_s(Sus scrofa)}{C_0}
\]

The symbols have the following meanings: \( t \) is the number of years for which UCP1 has evolved as a pseudogene in the pig lineage; 60 million years is the estimated time since divergence of pig and cattle [12], \( d_s(B. taurus) \) is the estimate of \( d_s \) between cattle (Bos taurus) and pigs (Sus scrofa). We assumed that synonymous substitutions have accumulated at the same rate in the cattle and pig lineage, \( d_s(Bos taurus) \) is the estimate of \( d_s \) between cattle (Bos taurus) and humans (Homo sapiens); 94 million years is the estimated time since divergence of cattle and human [12]. Thus, the expression \( d_s(Bos taurus) \times 60/94 \) estimates the proportion of nonsynonymous substitutions that have accumulated in the cattle lineage subsequent to the split from the pig lineage 60 million years ago. We assumed that before UCP1 was disrupted in the pig lineage, it accumulated nonsynonymous substitutions at the same rate as in the cattle lineage. \( d_s(Sus scrofa) \) is the estimate of \( d_s \) between cattle and pigs.

**Supporting Information**

**Figure S1.** Southern Blot Analysis Using Genomic DNA Representing Different Pig Breeds

Southern blot analysis using genomic DNA representing different pig breeds shows a single UCP1 fragment. 10 μg of DNA from each individual was digested with HindIII and separated on a 0.7% agarose gel. UCP1 exon 1, 2, and 6 fragments were used as probes (PCR primers Exon 1, 2, and 6; Table S1); each fragment was labeled separately. No recognition site for HindIII is present in the pig sequence. Lanes 1 and 2, wild boars; lanes 3–11, Large White; lane 12, Duroc; lanes 13 and 14, Hampshire.

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**Figure S2.** Alignment of Exon 1, 2, and 6 of Human, Mouse, Cattle, and Pig UCP1, and the Corresponding Amino Acid Sequences

Identities to the master sequence (human) are marked with dots, and missing or insertions/gaps are indicated by dashes. (A) The alignment reveals a two-base pair insertion in exon 1 and a 16-base pair deletion in exon 6 of the pig sequence, indicated by stars. Arrows point out the first nucleotide in exon 2 and 6. (B) The insertion differences were
ignored when the pig sequence was translated. The translated sequence reveals a premature stop codon in the pig sequence. Found at DOI: 10.1371/journal.pgen.0020129.sg002 (76 KB TIF).

**Table S1.** A List of Primers Used to Amplify and Sequence the Porcine UCP1 Gene

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**Accession Numbers**

The pig sequence has been deposited in GenBank (http://www.ncbi.nlm.nih.gov/Genbank) under accession number DQ372918. The Ensembl (http://www.ensembl.org/index.html) accession numbers for the genes and transcripts in this paper are cattle gene (ENSBTAG00000006467), cattle transcript (ENSBTAT00000006097), human gene (ENSG00000109124), human transcript (ENST00000262999), and mouse transcript (ENSMUST0000034146).

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**Author contributions.** LA conceived and supervised this study. FB and LA designed the experiments. FB and UG performed the experiments. FB and LA analyzed the data and wrote the paper.

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**Competing interests.** The authors have declared that no competing interests exist.

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