Cloning, characterization, and expression analysis of the pig (Sus scrofa) C1q tumor necrosis factor-related protein-5 gene

Jeffrey R. Sommer,1 Venkata R.M. Chavali,2 Sean G. Simpson,1 Radha Ayyagari,2 Robert M. Petters1

(The first two authors contributed equally to this work)

1Department of Animal Science, North Carolina State University, Raleigh, NC; 2Department of Ophthalmology, University of California, San Diego, La Jolla, CA

Purpose: Autosomal dominant early-onset long anterior zonules (LAZs) and late-onset retinal degeneration (L-ORD) in humans are associated with the S163R mutation of the complement 1q-tumor necrosis factor related protein-5 (CTRP5) gene. For using the pig as an L-ORD model for the study of pathology, we cloned, characterized, and studied the expression profile of pig CTRP5 (pCTRP5).

Methods: The pCTRP5 was cloned and sequenced from porcine genomic DNA. Bioinformatic analysis was done to evaluate the functional domains present in the pCTRP5 using PROSITE tools. The V5 epitope-tagged constructs of pCTRP5 and the mammalian promoters, elongation factor 1-α (EF) promoter and 579 bp of the putative promoter located upstream to pCTRP5 DNA, were used for in vitro expression analysis. The pCTRP5 expression, protein size, and cellular localization were studied in transiently transfected Cos-7 or ARPE-19 cells by western blot analysis using anti-CTRP5 and anti-V5 epitope antibodies. Expression of pCTRP5 in the pig eye tissues was analyzed by western blot analysis, real-time PCR, and immunohistochemistry.

Results: As predicted, pCTRP5 showed a 92% DNA homology and 98% amino acid homology with human CTRP5 (hCTRP5). Bioinformatic analysis revealed the presence of an alternate in-frame translational start site upstream to the presumed initiator codon. The presence of a putative promoter region upstream to the pCTRP5 was identified. The putative pCTRP5 promoter was found to be functional by western blot analysis. The size of the pCTRP5 protein (pCTRP5) was consistent with its predicted molecular weight, indicating that the potential alternative start site was not used. Western blot and RT–PCR analyses showed that pCTRP5 was predominantly expressed in RPE, a pattern of expression consistent with that found in mouse and human eyes.

Conclusions: The sequence and genomic organization of pCTRP5 was found to be similar to the human homolog. The DNA and protein sequence of pCTRP5 are highly homologous to hCTRP5, indicating that they are highly conserved. A putative promoter region (579 bp) present upstream to pCTRP5 was found to be functional and was able to drive the expression of the pCTRP5 gene cloned downstream. The tissue distribution in the eye and the expression profile of pCTRP5 in transiently transfected cells is consistent with hCTRP5 expression. Immunohistochemistry analysis of the pig retinal sections revealed localization of pCTRP5 to the apical and basolateral regions on the RPE and in the ciliary body. The potential in-frame alternate start site was found to be nonfunctional by western blot analysis of transiently transfected cells. Similarities between human and pig CTRP5 and the presence of an area centralis region in the pig similar to the human macula, together with its large eyeball size, makes the domestic pig a good model for the study of LAZs and L-ORD.

Late onset retinal macular degeneration (L-ORD) is an autosomal dominant retinal degeneration that is characterized by bilateral vision loss, dark-adaptation abnormalities, drusenoid deposits, lens anterior zonules, retinal degeneration, and choroidal neovascularization in humans [1-3]. Patients with L-ORD often present symptoms indistinguishable from early-stage age-related macular degeneration (AMD) or from retinal degeneration (RD) in its later stages. However, anterior segment abnormalities with long anterior zonules (LAZs) are seen only in L-ORD patients and are not observed in AMD or RD. L-ORD is known to be caused by a single missense mutation, S163R, in a conserved domain of the C1q tumor necrosis factor-related protein 5 gene (CTRP5) [3]. Patients with the S163R heterozygous mutation showed abnormal lens zonules (in the second decade), dark adaptation abnormalities in the fourth decade, progressing to drusen and macular degeneration with choroidal neovascularization (fifth decade). Some of the patients developed iris atrophy and secondary glaucoma in their 60s [3-5].

CTRP5 is a short chain collagen gene that encodes a 25 kDa secretory glycoprotein with three conserved domains: a signal peptide (residues 1–15), a collagen domain (residues

Correspondence to: Robert M. Petters, Department of Animal Science, North Carolina State University, Campus Box 7621, Raleigh, NC, 27695; Phone: (919) 515-4021; FAX: (919) 515-6884; email: bob_petters@ncsu.edu
cones [16–18], it will be a better model for studying macular
like region, the area centralis, with a high concentration of
lack both these features. Since the pig has a distinct macula-
is justified, rather than studying mouse models, since mice
addition, to understand the mechanism underlying L-ORD or
difference between humans and mice relates to ocular size. In
present in humans [15]. However, the greatest phenotypic
pathology is not known to date.

interact with CFH and MFRP, its function in disease
its 5′ region [11]. The protein and DNA sequence of
CTRP5 is also reported in serum, adipocytes, and other tissues
present in RPE and the ciliary epithelial layers in the eye [6]. Expression of
CTRP5 is also known to interact with protein and DNA sequence of
CTRP5 is highly conserved in mammals, birds, and zebra fish [9].
CTRP5 is known to express as a dicistronic transcript and is
located in the 3′ untranslated region of the membrane-type
frizzled-related protein (MFRP) gene [10]. A functional
promoter for human CTRP5 (hCTRP5) has been identified in
its 5′ region [11].

The CTRP5 protein has been shown to interact with complement factor H (CFH), which has been reported to be a major genetic factor associated with AMD and an early-onset recessive drusen phenotype (Figure 1) [12]. Apart from CFH, CTRP5 is also known to interact with membrane-type frizzled-related protein (MFRP) [13]. The C1q domain present in CTRP5 is known to strongly interact with the complement C1r/C1s, Uegf, Bmp1 (CUB) domains joined by
LDLα (together known as the CUBT domain) in MFRP.
Mutations in MFRP are reported to cause an autosomal recessive syndrome of nanophthalmos, retinitis pigmentosa, foveoschisis, and optic disc drusen in human subjects and retinal degeneration in the rd6 mouse model [10,14]. The highest levels of CTRP5 and MFRP expression were detected in RPE and the ciliary body, the tissues that are involved in the disease pathology [3]. Even though CTRP5 is known to interact with CFH and MFRP, its function in disease pathology is not known to date.

There are many currently existing mouse models for studying AMD that mimic most of the phenotypic features present in humans [15]. However, the greatest phenotypic difference between humans and mice relates to ocular size. In addition, to understand the mechanism underlying L-ORD or AMD, studying an animal model having a foveal pit or macula is justified, rather than studying mouse models, since mice lack both these features. Since the pig has a distinct macula-like region, the area centralis, with a high concentration of cones [16-18], it will be a better model for studying macular
degeneration. Understanding the structure and expression of
CTRP5 in the pig will enable us to manipulate it and to make models to mimic the disease in human L-ORD patients. In the present study, we describe the cloning, sequencing, and characterization of CTRP5 in pigs (pCTRP5, in Sus scrofa) and its putative promoter. These studies will provide the background for the development of a pig model of LAZs and L-ORD for the investigation of therapeutic interventions.

METHODS

All animal protocols were conducted in accordance with state and federal guidelines and were approved by the Institutional Animal Care and Use Committee, North Carolina State University.

Cloning pCTRP5 and constructing the expression vector: PCR primers, CTRP5 6up (5′-GGG ACA CCA CGA GGT ATT TCC CTG-3′) and 2082lp (5′-AGC CCT CCC TTC TGC CTG AAC AC-3′), were used to amplify 2,146 bp of CTRP5 from the pig genomic DNA using Phusion Hot Start High-fidelity DNA polymerase (New England BioLabs, Ipswich, MA). The PCR amplicon was agarose gel purified and annealed with “A” overhangs before cloning into the mammalian expression vector pEF6/V5-His-TOPO (Invitrogen, Carlsbad, CA). The positive clones containing pCTRP5 were confirmed by PCR amplification using the nested primer set CTRP up/lp (5′-AGC GCT ACC TGG TGC CGG GCG TGG CTT GGG C-3′), which flanks the Ser163 codon in exon 2 of pCTRP5. This vector was called pEF CTRP5 wt. An Arg163 mutation was introduced by site-directed mutagenesis into the pEF CTRP5 wt plasmid using the 5′ phosphorylated primers CTRP5 AGG mut F (5′-P[CTCT]TTG GAG TCT GGG AGG AGC A-3′) and CTRP5 AGG mut R (5′-P[CTCT]GTC ACG AAT GGC GAG TCC AT-3′) for amplification and then recircularization to make the pEF CTRP5 mt plasmid. Restriction endonuclease digestion analysis with Nhel was used to screen for the mutation because the C→G mutation eliminates the Nhel site. Positive clones containing the mutant CTRP5 were confirmed by sequencing. A possible promoter and alternative in-frame ATG start site found in the 5′ upstream sequence of the pig
genomic DNA was removed from with the 5′ phosphorylated primer set: ATG out up (5′[P]-GAG GCC GGG AGC GAG GTG TGT C-3′) and ATG out lp (5′[P]-ACT GGT GTG GCA AGG GCA ATT C-3′). The resulting amplicon was recircularized. We called this version of the CTRP5 “S” for short (pEF CTRP5 S).

For ease of detection of protein expression in transient transfection assays, a 3′ in-frame fusion was made with the V5 epitope of the pEF6/V5-His-TOPO cloning vector. The abbreviation “T” (for “tagged”) was used to denote plasmids that were V5 epitope-tagged (e.g., pEF CTRP5 wt T). To investigate the promoter activity of the CTRP5 upstream sequence, we made constructs with EF promoter and putative pCTRP5 promoter sequences cloned upstream to pCTRP5. These constructs were transiently transfected into ARPE-19 cells or Cos-7 cells to study the pCTRP5 (protein) expression by western blot and immunocytochemistry analysis.

5′ RACE analysis of pCTRP5: To determine the transcription start site of pCTRP5, a 5′ RACE was performed using the RNA isolated from pig RPE tissue using an RNaseqy Mini isolation kit (Qiagen, Valencia, CA). Ten micrograms of the total RNA was used with the First Choice RLM RACE kit (Invitrogen). The cDNA was synthesized using an Enhanced Avian RT first-strand synthesis kit (Sigma-Aldrich, St. Louis, MO) at 65 °C using a primer specific to the CTRP5 coding sequence (Primer Race1: 5′-CCC TCG CTT TCT TCT CCC-3′). PCR was performed with NEB’s Long Amp DNA polymerase using these primers: 5′ RACE Outer Primer (5′-CGC GGA TCC 72 °C for 40 s. Primers 5′-GTC CAT CGC CTC TTT CTT 95 °C for 10 s, annealing at 56 °C for 30 s, and elongation at 72 °C for 40 s. Primers 5′-GTG CAT CTC CTC TTT CTT CC-3′ (forward primer) and 5′-ATG CCA ATG TAG TCA CCC AC-3′ (reverse primer) were used for amplification of pCTRP5. The expression of pCTRP5 was determined in pig eye tissues (cornea, ciliary body-iris [CB-I], lens, retina, and RPE). In addition, four housekeeping genes—Gapdh, HPRT, β-actin (Actb), and RpL19—were also analyzed as controls to normalize the expression of pCTRP5. Expression levels (=standard error of the mean [SEM]) were calculated by analyzing at least three independent samples with replicate reactions and presented on an arbitrary scale that represented its expression over the housekeeping gene. The data was presented using the HPRT gene for normalization.

Immunohistochemistry: For immunolocalization of antigens, eyes from 7.4-month-old pigs were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer. Eyes in fixative were thoroughly washed with 1X PBS, cryoprotected in 30% sucrose, and embedded in OCT before sectioning. To localize pCTRP5 in the eye tissue, immunohistochemistry (IHC) was performed using anti-CTRP5 polyclonal antibody, as described earlier [6,11]. Sections were mounted in mounting medium containing DAPI, they were observed with a Zeiss confocal microscope, and images were captured.

**RESULTS**

Characterization of pCTRP5: A 2146 bp fragment of pig genomic DNA containing the presumed coding region for CTRP5 (Figure 2A) was cloned into the mammalian
expression vector pEF6/V5-His-TOPO (referred to as pEF CTRP5 wt). Amplification with nested CTRP5-specific PCR primers and subsequent sequencing confirmed the presence of the pCTRP5 coding sequence (Figure 2A).

Sequencing analysis of the cloned pCTRP5 revealed it is 732 bp long with two exons that might encode for a 243 amino acid protein (molecular weight [MW] was nearly 25.5 kDa). DNA sequence’s homology with its human ortholog is 92% and amino acid homology is 98%; with variations at only 5 amino acid residues (Figure 2B). The sequence comparisons of pCTRP5 with other vertebrate and invertebrate sequences showed a high conservation of the C1q domain, which is the functionally active domain of CTRP5 [7,22]. PROSITE analysis showed the presence of the collagen domain to be from 36 to 95 amino acids and of the C1q domain to be from 105 to 232 amino acids in pCTRP5 protein. The region between 102 and 235 amino acids showed homology with the tumor necrosis factor-like protein family of proteins. Analysis of the genomic sequence indicated that a potential in-frame alternative start codon 141 bp upstream of the predicted pCTRP5 start codon, if translated, would result in an extra 47 amino acids at the N-terminus, to yield a protein of the predicted size of nearly 31 kDa (Figure 2A).

The CpG island search software detected the presence of a single CpG island of 578 bp with a GC content >70.6%. The observed CpG/expected CpG ratio in this region is greater than 0.65. Presence of a CpG island in the putative promoter region of pCTRP5 may help either to initiate DNA replication or transcription.

**Transient expression of CTRP5 in Cos-7 and ARPE-19 cells:**
To test whether the cloned pCTRP5 was capable of expressing pCTRP5, we cloned 2164 bp of pCTRP5 downstream to the human elongation factor 1α (EF) promoter (pEF CTRP5 wt). We used this vector to produce a series of expression vectors, including V5 epitope “tagged” (T) CTRP5 and “short” (S) versions that did not contain a 5′ upstream genomic sequence that included a putative pCTRP5 promoter and a potential alternative translational start site (pCTRP5 wt T and pCTRP5 wt S) expression vector. R163 mutant (mt) variations of each plasmid were also produced to test for their expression (Figure 3). The resulting plasmids were transiently transfected into Cos-7 cells and ARPE-19 cells independently, and the expression of pCTRP5 protein was studied using western blot analysis.

Western blot analysis with Cos-7 cells transfected with the plasmids pEF CTRP5 wt T and pEF CTRP5 mt T were incubated with an anti-V5 antibody to detect the CTRP5 fusion protein (Figure 4). The expression of the pCTRP5 V5 fusion protein indicated the functionality of the cloned pCTRP5 in producing CTRP5 (~31 kDa). The expression of pCTRP5 was comparable to the levels of CTRP5 produced by the V5 epitope-tagged hCTRP5 gene expressed under the control of the CMV promoters (pCMV CTRP5 wt T and pCMV CTRP5 mt T) that were used as controls.

**Promoter analysis of pCTRP5:**
Promoter identification software detected a TATA-less putative promoter region of an approximately 579 bp sequence upstream of the predicted pCTRP5 initiation codon. The Proscan Version 1.7 software tool predicted this promoter region to be in proper orientation to the start site, with a promoter score of 53.45 (promoter cutoff=53.0). The TSSG program, which searches for human PolIII promoter regions, predicted a promoter with a transcriptional start site at −245 bp from the start of translation, with a linear discriminant factor (LDF) score of 7.59 (threshold for LDF=4.00). Analysis of the putative pCTRP5 promoter region (579 bp) also identified several possible transcription-factor binding sites, including one site of NF-kB and four sites for the Sp1 binding site within the sequence (Table 1). The presence of a TATA box or GATA box was not detected, indicating that the pCTRP5 promoter might be a TATA-less promoter.

To test the functionality of the predicted promoter, the putative promoter region (579 bp) was cloned upstream to the start site of pCTRP5 and analyzed for its ability to express pCTRP5. Immunocytochemistry analysis was done to analyze protein expression in ARPE-19 cells transfected with the pCTRP5 wt T and pCTRP5 mt T constructs. The 579 bp pCTRP5 promoter was capable of expressing pCTRP5, which was detected using anti-CTRP5 antibody and anti-V5 tag antibodies (data not shown). Furthermore, western blot analysis of Cos-7 transfected with pCTRP5 under the control of the 579 bp pCTRP5 promoter demonstrated high levels of expression of both the wild-type and mutant pCTRP5 proteins (Figure 5). The above results confirmed the functionality of the putative pig promoter. The pEFα promoter driving expression of pCTRP5 V5 fusion genes were used as controls.

**Determination of the translational start site of pCTRP5:** To investigate the potential translational start site of the pCTRP5 gene, Cos7 cells were transfected with two different expression constructs. These constructs contained either the potential alternative in-frame start sites (pEF CTRP5 wt T and pEF CTRP5 mt T) or truncated versions of the expression vectors (pEF CTRP5 wt ST and pEF CTRP5 mt ST) that lacked the potential alternative start site (Figure 6). The transfected Cos-7 cell lysates were analyzed using western blot analysis with an anti-CTRP5 antibody and an anti-V5 epitope antibody. Detection with the above antibodies revealed that transfected cells expressed pCTRP5 protein with a molecular weight of 31 kDa. Translation from the in-frame alternative start site would have resulted in the expression of a 36 kDa protein. These results suggest that, even though a potential in-frame ATG was present in exon 1, only the second ATG was used for translation of CTRP5 (Figure 6).
Figure 2. Genomic structure of pCTRP5 and protein homology of pig CTRP5 with the human protein. A: The pCTRP5 was amplified from genomic DNA by PCR, cloned, and sequenced. The pCTRP5 consists of two exons; its coding sequence is highlighted in yellow, the Ser163 codon is highlighted in red, a potential in-frame alternative start codon is highlighted in pink, the transcriptional start site (TSS) is highlighted in blue, and various transcription factor binding sites are highlighted in green.

B: The protein homology between the pCTRP5 and hCTRP5 protein is represented. The pCTRP5 protein sequence is shown in black and the human in blue. The underlined residues indicate the five amino acid differences between the pig and human protein. The Ser shown in red indicates residue 163.
Determination of the pCTRP5 transcriptional start site: 5′ RACE was performed to determine the transcriptional start site of the endogenous pCTRP5 gene. RNA purified from the pig retinal-pigmented epithelium was used as the starting template for the RACE adaptor ligation and reverse transcription reaction. Sequencing of a PCR product from the 5′ RACE reaction determined that the transcriptional start site was located at −65 bp from the initiation codon ATG (Figure 2A). The detected transcriptional start site (TSS) was found to be downstream of a potential in-frame initiation codon (77 bp downstream to the alternate initiation codon), indicating that it was not used for the generation of the 5′ RACE product.

Expression of pCTRP5 in various eye tissues and localization in the eye: We examined pCTRP5 gene expression in different pig eye tissues using quantitative reverse transcription polymerase chain reaction (qRT–PCR) on cDNA produced from total mRNA isolated from the retina, optic nerve, lens, RPE, choroid, and ciliary body of a 222-day-old pig. The highest level of pCTRP5 expression was detected in the RPE, although pCTRP5 messages were expressed in the ciliary body, optic nerve, and choroid. Minimal expression levels of the pCTRP5 transcript were detected in the pig retina and lens (Figure 7A).

Western blot analysis was performed using the protein lysates of the above tissues. The pCTRP5 protein was primarily detected within the RPE (Figure 7B) as ~31 kDa protein (Figure 7B) but not in other eye tissues, indicating that the RPE was the predominant tissue expressing the CTRP5 protein. To localize the presence of CTRP5 protein in the pig eye, IHC was performed with an anti-CTRP5 antibody. The pCTRP5 protein was localized predominantly to the basal RPE layers of the eye (Figure 7C). Staining of CTRP5 was

---

**Figure 3.** Expression constructs designed for studying expression of pCTRP5 and pCTRP5 promoter activity. Six constructs were made to investigate pCTRP5 expression and putative pCTRP5 promoter activity. The initial vector was pEF CTRP5 wt. All other vectors were based on modifications to this expression vector. Figure abbreviations are as follows: wt=wild type Ser163 allele, mt=Arg163 mutation, T=V5 epitope tagged, S=pCTRP5 short. The short pCTRP5 genomic fragment is missing 461 bp of the upstream sequence, including the possible promoter and alternative in-frame start site. Constructs made with EF1α promoter were used as experimental controls for promoter expression studies.

**Figure 4.** Western blot of lysates of cells transfected with pig CTRP5 and human CTRP5 constructs. Western blot analysis was performed with anti-V5 antibody on lysates from Cos-7 cells transfected with pEF CTRP5 wt T (lane 2), pEF CTRP5 mt T (lane 3), pCMV CTRP5 wt T (lane 4), pCMV CTRP5 mt T (lane 5), and nontransfected Cos7 cells (lane 1). Lanes 4 and 5 were transfected with hCTRP5 expression vectors as a positive control. Detection with anti-V5 antibody produced a single band with a molecular weight around 31 kDa for both the pCTRP5 (lanes 2 and 3) and hCTRP5 (lanes 4 and 5) V5-tagged protein.
also observed on the apical and lateral regions throughout the retina (Figure 7C). From the above analysis it appears that, although the pCTRP5 transcript was detected in various eye tissues, it was predominantly expressed and localized to the RPE layer in pig eye.

The localization pattern of pCTRP5 in the ciliary epithelium (CE) of pigs was demonstrated by IHC using the anti-CTRP5 antibody. Immunofluorescence microscopy showed that pCTRP5 was localized to predominantly to the apical and few basal membrane surfaces of the pigmented and nonpigmented cells, and was abundantly present in the CE layers (arrows in Figure 7D). Among the ciliary epithelial layers, pCTRP5 was found to be significantly localized in the outer layer between both layers, unlike the mouse CTRP5 that was reported to be localized in the apposed apical membrane surfaces of the CE layers [6]. The variation observed in the localization pattern of CTRP5 between mouse, human, and pig retinal sections could be due to the difference in the specificity of anti-human/-mouse CTRP5 polyclonal antibodies against the pig protein. Similar to the pattern observed in the mouse RPE cells, pCTRP5 also showed a

### Table 1. List of transcription factors binding sites identified in the 579 bp upstream promoter region of the pig CTRP5 gene.

| Binding site     | Identification* | Location from start of translation (bp) |
|------------------|-----------------|----------------------------------------|
| Alpha INF        | S01153          | (−378 to −373)                         |
| AP-2             | S01936          | (−470 to −463)                         |
| GCF              | S01964          | (−54 to −48) (−169 to −163) (−271 to −265) (−385 to −379) (−387 to −381) |
| H4TF-1           | S01969          | (−256 to −248) (−262 to −254) (−304 to −293) |
| H4TF-2           | S00742          | (−398 to −394)                         |
| JVC repeat sequences | S01193       | (−254 to −247) (−260 to −253) (−299 to −292) (−319 to −312) (−324 to −317) (−500 to −493) |
| LBP-1            | S02121, S00487 | (−503 to −499) (−521 to −517)          |
| LF-A1            | S00250          | (−534 to −529)                         |
| NF-kB            | S01498          | (−208 to −200)                         |
| SDR              | S01561          | (−335 to −326)                         |
| Sp1              | S00781, S00978, S00857, S00979 | (−321 to −313)                                |
| T-Ag             | S00973, S00974 S02135, S01375 | (−192 to −188) (−277 to −273) (−320 to −316) (−374 to −370) (−443 to −439) (−458 to −454) |
| TCF-1            | S02023          | (−379 to −375)                         |
| UCE.2            | S00437          | (−192 to −188) (−277 to −273) (−320 to −316) (−374 to −370) (−443 to −439) (−458 to −454) |
| v-Myb            | S01896          | (−523 to −518)                         |

Selected transcription factor binding sites were identified using Prosan V1.7 and TSSG searches. *Motif identifiers from the Ghosh database.
DISCUSSION

The Ser163Arg mutation in hCTRP5 is known to cause the autosomal dominant pathology associated with early-onset LAZs and late-onset retinal degeneration [3,9]. Developing suitable animal models that mimic the human phenotype is critical to understanding the disease pathology caused by gene mutations. The phenotype of these patients resembles that of patients with AMD and RD. There are multiple mouse models showing pathological features mimicking those of AMD [15]. In spite of having many phenotypic similarities with AMD patients, the mouse eye differs significantly from the human eye, due to the mouse eye’s smaller size and the absence of a cone-rich region, the macula. A pig model can serve as an alternative best model to study AMD due to the presence of the cone-rich region, called the area centralis, and its bigger eye size compared to that of the mouse. To generate a pig model for L-ORD in future, we cloned and characterized the genomic pCTRP5 gene. This is the first study describing pCTRP5 and its expression and localization pattern in the pig eye. This study also confirmed the presence of an independent promoter for pCTRP5 that is similar to but smaller than the human promoter [11].

To characterize pCTRP5, we cloned the gene from genomic DNA and sequenced it. The first release (April 2009) of the high-coverage Sus scrofa assembly for chromosomes 1 to 18 and X of the pig genome is available through Ensembl browser [23]. We compared the pCTRP5 gene sequence we generated with the public domain sequence and found a 100% match between the two sequences. Initially, the hCTRP5 transcript was found to be present in the 3′ untranslated region of the full length hMFRP transcript [6]. We have since identified an autonomous hCTRP5 promoter [11]. The genomic organization between the coding exons in pCTRP5 (exon 1 and exon 2) and hCTRP5 (exon 1 and exon 2) is very similar. They both have the same-size exons of 215 bp and 517 bp for, respectively, the first and second coding exons. The gene sequence homology of both the DNA and protein was compared with other ortholog sequences. The DNA and protein alignments of the pCTRP5 gene showed, respectively, a 92% DNA homology and a 98% amino acid homology with hCTRP5. ClustalW sequence analysis of the 579 bp sequence upstream to the CTRP5 gene in pigs and humans revealed an alignment score of 74%, indicating that they are conserved.

The CTRP5 message is reported to be expressed as two isoforms in humans: a dicistronic transcript of CTRP5 with the MFRP gene in its 5′ region, and a monocistronic transcript without MFRP [24]. The CUB domains present in human MFRP interact with the C1q domain in CTRP5, suggesting
Figure 7. Expression and localization of CTRP5 in the pig eye. A: Expression of pCTRP5 was studied by qRT–PCR using total mRNA extracted from different tissues of a 222-day-old pig eye. The pCTRP5 expression is presented as bars using an arbitrary scale on the y axis. Values are presented as mean (±SEM) of three independent observations after normalization with the control gene (HGPRT). B: Western blot analysis of CTRP5 protein extracted from a 222 days pig. Retina (lane 1), optic nerve (lane 2), lens (lane 3), RPE (lane 4), ciliary body (lane 5), Choroid (lane 6). Detection with an anti-CTRP5 antibody shows significant expression of pCTRP5 protein in the RPE with an expected size molecular weight of approximately 31 kDa. C: Localization of CTRP5 in retinal sections as evaluated by IHC analysis of retinal sections with human monoclonal anti-CTRP5 antibody and Alexa Fluor 488 staining (green, arrows), nuclei (*) stained with DAPI (blue). D: Localization of CTRP5 in the ciliary body as shown by IHC with human monoclonal anti-CTRP5 antibody and Alexa Fluor 555 (red, arrows), nuclei stained with DAPI (blue).
that these two may be functionally related, similar to other dicistronic genes [13,25,26]. The MFRP gene in pigs is reported to be around 1,707 bp, encoding a putative protein of 508 amino acids, as found in the Ensembl browser [23]. Like human MFRP, pig MFRP also has 13 exons. Domain analysis of the pMFRP gene showed that it consists of two CUB domains (CUB1: 132 to 238 bp; CUB2: 289 to 403 bp), Ldla domain (244–287 bp) and a Frizzled domain (Fz: 454 to −563 bp). Unlike the human gene, only the monocistronic transcripts are reported for the pig MFRP and CTRP5 genes in the Ensembl browser, suggesting that the bicistronic MFRP-CTRP5 transcripts may be absent in pigs.

Analysis of the pCTRP5 DNA sequence noted a potential in-frame alternate start site upstream from the presumed coding sequence. Western blot expression analysis indicated that this potential in-frame start was not translated (Figure 6). The 5′ RACE analysis of expressed pCTRP5 mRNA from the RPE indicated that the TSS was located −65 bp to +77 bp from the potential in-frame alternate start site (Figure 2A). The TSS (−65 bp) of pCTRP5 was similar in distance from the initiation codon, as we previously found with the independent hCTRP5 promoter (TSS −62 bp) [11]. Finally, the size of the pCTRP5 protein found in the pig eye was consistent with the predicted size (Figure 6B). These observations indicate that the cloned genomic pCTRP is expressed properly in vitro.

Apart from characterizing pCTRP5 and pCTRP5, sequence analysis revealed the presence of an autonomous promoter for pCTRP5. We recently described an autonomous promoter function for hCTRP5; the results of the present finding are consistent with that report [11]. The sequences to the ATG start site in both human and pig CTRP5 genes are highly conserved, with 73% homology. The cloned promoter region was capable of expression in both Cos-7 (Figure 5) cells and ARPE-19 cells (data not shown), indicating the functionality of the predicted promoter region. Further characterization of this promoter is necessary to determine the core promoter region.

CpG islands (CGIs) are clusters of CpG dinucleotides in GC-rich regions. They are also defined as regions with at least 200 bp, with a GC percentage that is greater than 50% and with an observed/expected CpG ratio that is greater than 60% [27]. In humans and mice, approximately 60% of all promoters colocalize with CpG islands [28]. Similar to hCTRP5, the CpG island search software predicted the presence of a CpG island in the 5′ region of the pCTRP5 sequence. A single CpG island of 578 bp with a GC of less than 70.6% and an observed to expected CpG ratio of 0.66 was identified in pCTRP5, which may be involved in the initiation of DNA replication or transcription. The presence of a prominent CpG island and a conserved upstream region to the pCTRP5 gene confirms the presence of a regulatory region/promoter upstream to pCTRP5.

We have shown that the structure of the pCTRP5 gene is similar to that found in humans [6,9]. The expression and localization of the pCTRP5 protein was also found to be consistent with the hCTRP5 expression in different cell lines, such as Cos-7 and ARPE-19 cells, indicating that these genes are highly similar not only in their structure but also in their function. These findings indicate that a pig model of LAZs and L-ORD is likely to mimic the human disease. The pig rod-to-cone ratio is around 8:1 for the total retina [17,29], compared to roughly 36:1 in the mouse retina [30]. The pig will thus be an excellent model for the human disease because it has a cone-rich retina and a macula-like region, the area centralis [16]. The pig has been used extensively as a model of retinitis pigmentosa [31] and is likely to serve as a useful model of LAZs and L-ORD.

ACKNOWLEDGMENTS
Supported by NIH grants R21 EY018414 (R.M.P.) and R01 EY013198 (R.A.), Foundation Fighting Blindness (R.A.), Research to Prevent Blindness Inc., USA (R.A.).

REFERENCES
1. Kuntz CA, Jacobson SG, Cideciyan AV, Li ZY, Stone EM, Possin D, Milam AH. Sub-retinal pigment epithelial deposits in a dominant late-onset retinal degeneration. Invest Ophthalmol Vis Sci 1996; 37:1772-82. [PMID: 8759344]
2. Milam AH, Curcio CA, Cideciiyan AV, Saxena S, John SK, Kruth HS, Malek G, Heckenlively JR, Weleber RG, Jacobson SG. Dominant late-onset retinal degeneration with regional variation of sub-retinal pigment epithelium deposits, retinal function, and photoreceptor degeneration. Ophthalmology 2000; 107:2256-66. [PMID: 11097607]
3. Ayyagari R, Mandal MN, Karoukis AJ, Chen L, McLaren NC, Lichter M, Wong DT, Hitchcock PF, Caruso RC, Moroi SE, Maumenee IH, Sieving PA. Late-Onset Macular Degeneration and Long Anterior Lens Zonules Result from a CTRP5 Gene Mutation. Invest Ophthalmol Vis Sci 2005; 46:3363-71. [PMID: 16123441]
4. Ayyagari R, Griesinger IB, Bingham E, Lark KK, Moroi SE, Sieving PA. Autosomal dominant hemorrhagic macular dystrophy not associated with the TIMP3 gene. Arch Ophthalmol 2000; 118:85-92. [PMID: 10636420]
5. Subrayan V, Morris B, Armbrecht AM, Wright AF, Dhillon B. Long anterior lens zonules in late-onset retinal degeneration (L-ORD). Am J Ophthalmol 2005; 140:1127-9. [PMID: 16376663]
6. Mandal MN, Vasireddy V, Reddy GB, Wang X, Moroi SE, Pattnaik BR, Hughes BA, Heckenlively JR, Hitchcock PF, Jablonski MM, Ayyagari R. CTRP5 is a membrane-associated and secretory protein in the RPE and ciliary body and the S163R mutation of CTRP5 impairs its secretion. Invest Ophthalmol Vis Sci 2006; 47:5505-13. [PMID: 17122142]
7. Park SY, Choi JH, Ryu HS, Pak YK, Park KS, Lee HK, Lee W. C1q tumor necrosis factor alpha-related protein isofrom 5 is increased in mitochondrial DNA-depleted myocytes and activates AMP-activated protein kinase. J Biol Chem 2009; 284:27780-9. [PMID: 19651784]
8. Wong GW, Krawczyk SA, Kitidis-Mitrokostas C, Revett T, Gimeno R, Lodish HF. Molecular, biochemical and functional characterizations of C1q/TNF family members: adipose-tissue-selective expression patterns, regulation by PPAR-gamma agonist, cysteine-mediated oligomerizations, combinatorial associations and metabolic functions. Biochem J 2008; 416:161-77. [PMID: 18783346]

9. Hayward C, Shu X, Cideciyan AV, Lennon A, Barran P, Zareparsi S, Sawyer L, Hendry G, Dhillon B, Milam AH, Luthert PJ, Swaroop A, Hastie ND, Jacobson SG, Wright AF. Mutation in a short-chain collagen gene, CTRP5, results in extracellular deposit formation in late-onset retinal degeneration: a genetic model for age-related macular degeneration. Hum Mol Genet 2003; 12:2657-67. [PMID: 12944416]

10. Kamaya S, Hawes NL, Chang B, Heckenlively JR, Naggett JK, Nishina PM. Mfrp, a gene encoding a frizzled related protein, is mutated in the mouse retinal degeneration 6. Hum Mol Genet 2002; 11:1879-86. [PMID: 12140190]

11. Chavali VR, Sommer JR, Petters RM, Ayyagari R. Identification of a promoter for the human C1Q-tumor necrosis factor-related protein-5 gene associated with late-onset retinal degeneration. Invest Ophthalmol Vis Sci 2010; 51:5499-507. [PMID: 20554618]

12. Shu X, Clark S, Dodds A, Slingsby F, Day A, Sim R, Wright A. Biochemical Evidence of Pathogenetic Overlap Between Late Onset Retinal Macular Degeneration and Age-Related Macular Degeneration. ARVO Annual Meeting; 2007 May 6–10; Fort Lauderdale (FL).

13. Mandal MN, Vaisireddy V, Jablonski MM, Wang X, Heckenlively JR, Hughes BA, Reddy GB, Ayyagari R. Spatial and temporal expression of MFRP and its interaction with CTRP5. Invest Ophthalmol Vis Sci 2006; 47:5514-21. [PMID: 17122143]

14. Crespí J, Buil JA, Bassaganyas F, Vela-Segarra JI, Díaz-Cascajosa J, Ayala-Ramírez R, Zenteno JC. A novel mutation confirms MFRP as the gene causing the syndrome of nanophthalmos-retinitis pigmentosa-foveoschisis-optic disk drusen. Am J Ophthalmol 2008; 146:323-8. [PMID: 18554571]

15. Elizabeth Rakoczy P, Yu MJ, Nusinovitz S, Chang B, Heckenlively JR. Mouse models of age-related macular degeneration. Exp Eye Res 2006; 82:741-52. [PMID: 16325179]

16. Chandler MJ, Smith PJ, Samuelson DA, MacKay EO. Photoreceptor density of the domestic pig retina. Vet Ophthalmol 1999; 2:179-84. [PMID: 11397262]

17. Gerke CG Jr, Hao Y, Wong F. Topography of rods and cones in the retina of the domestic pig. Hong Kong Med J 1995; 1:302-8.

18. Hendrickson A, Hicks D. Distribution and density of medium- and short-wavelength selective cones in the domestic pig retina. Exp Eye Res 2002; 74:435-44. [PMID: 12076087]

19. Knudsen S. Promoter2.0: for the recognition of PolII promoter sequences. Bioinformatics 1999; 15:356-61. [PMID: 10366655]

20. Takai D, Jones PA. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. Proc Natl Acad Sci USA 2002; 99:3740-5. [PMID: 11891299]

21. Vaisireddy V, Vijayasarthathy C, Huang J, Wang XF, Jablonski MM, Petty HR, Sieving PA, Ayyagari R. Stargardt-like macular dystrophy protein ELOVL4 exerts a dominant negative effect by recruiting wild-type protein into aggresomes. Mol Vis 2005; 11:665-76. [PMID: 16163264]

22. Shu X, Tulloch B, Lennon A, Vlachantoni D, Zhou X, Hayward C, Wright AF. Disease mechanisms in late-onset retinal macular degeneration associated with mutation in C1QTNF5. Hum Mol Genet 2006; 15:1680-9. [PMID: 16600989]

23. Humphray SJ, Scott CE, Clark R, Marron B, Bender C, Camm N, Davis J, Jenks A, Noon A, Patel M, Sehra H, Yang F, Rogatcheva MB, Milan D, Chardon P, Rohrer G, Nonneman D, de Jong P, Meyers SN, Archibald A, Beever JE, Schook LB, Rogers J. A high utility integrated map of the pig genome. Genome Biol 2007; 8:R139. [PMID: 17625002]

24. Kim MJ, Lee W, Park EJ, Park SY. Role of hepatocyte nuclear factor-4alpha in transcriptional regulation of C1qTNF-related protein 5 in the liver. FEBS Lett 2010; 584:3080-4. [PMID: 20621834]

25. Sundin OH, Dharmaraj S, Bhutto IA, Hasegawa T, McLeod DS, Merget CA, Silval ED, Maumenee IH, Lutty GA. Developmental basis of nanophthalmos: MFRP Is required for both prenatal ocular growth and postnatal emmetropization. Ophthalmic Genet 2008; 29:1-9. [PMID: 18363166]

26. Won J, Smith RS, Peachey NS, Wu J, Hicks WL, Naggett JK, Nishina PM. Membrane frizzled-related protein is necessary for the normal development and maintenance of photoreceptor outer segments. Vis Neurosci 2008; 25:563-74. [PMID: 18764959]

27. Gardiner-Garden M, Frommer M. CpG islands in vertebrate genomes. J Mol Biol 1987; 196:261-82. [PMID: 3656447]

28. Antequera F. Structure, function and evolution of CpG island promoters. Cell Mol Life Sci 2003; 60:1647-58. [PMID: 14504655]

29. Beauchemin ML. The fine structure of the pig's retina. Albrecht Von Graefes Arch Klin Exp Ophthalmol 1974; 190:27-45. [PMID: 4547923]

30. Jeon CJ, Strettoi E, Masland RH. The major cell populations of the retina. Exp Eye Res 2002; 74:435-44. [PMID: 12076087]

Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 12 January 2012. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.