Evolution of CpG island promoter function underlies changes in KChIP2 potassium channel subunit gene expression in mammalian heart

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Scaling of cardiac electrophysiology with body mass requires large changes in the ventricular action potential duration and heart rate in mammals. These changes in cellular electrophysiological function are produced by systematic and coordinated changes in the expression of multiple ion channel and transporter genes. Expression of one important potassium current, the transient outward current (\(I_{to}\)), changes significantly during mammalian evolution. Changes in \(I_{to}\) expression are determined, in part, by variation in the expression of an obligatory auxiliary subunit encoded by the KChIP2 gene. The KChIP2 gene is expressed in both cardiac myocytes and neurons and transcription in both cell types is initiated from the same CpG island promoter. Species-dependent variation of KChIP2 expression in heart is mediated by the evolution of the cis-regulatory function of this gene. Surprisingly, the major locus of evolutionary change for KChIP2 gene expression in heart lies within the CpG island core promoter. The results demonstrate that CpG island promoters are not simply permissive for gene expression but can also contribute to tissue-selective expression and, as such, can function as an important locus for the evolution of cis-regulatory function. More generally, evolution of the cis-regulatory function of voltage-gated ion channel genes appears to be an effective and efficient way to modify channel expression levels to optimize electrophysiological function.

cis-regulatory evolution | physiological scaling

Large differences in body mass among mammals require significant changes in heart rate and ventricular action potential duration to scale cardiac physiological function to body mass (1, 2). These changes in cardiac function are produced by systematic changes in myocyte electrophysiological function and in the pattern and level of expression of multiple voltage-gated ion channels and transporters (2). Some of the most prominent changes are seen in the changing role of the transient outward potassium current (\(I_{to}\)). The \(I_{to}\) current is a rapidly activating and inactivating current that is expressed in the ventricular myocytes of most, but not all, mammalian hearts (3). Expression of this current in heart appears to be an innovation of mammals. This current has not been described in the hearts of nonmammalian vertebrates. An essentially identical current (known as \(I_A\)) is, however, expressed in the brains of most vertebrate and many invertebrate species (4). A subset of the genes that encode the neuronal \(I_A\) current in mammals has been coopted for \(I_{to}\) expression in heart (2, 3).

The ventricular action potential in most nonrodent mammals has a “spike and dome” morphology, with a long plateau phase before action potential repolarization. In these species, \(I_{to}\) modifies the early phase of action potential morphology, altering the initial rate of calcium ion entry and the strength of myocyte contraction (5). The \(I_{to}\) channel is composed of an \(\alpha\)-subunit encoded by the Kv4.3 gene (KCND3) and an auxiliary subunit encoded by the KChIP2 gene (KCNIP2) in these species (3). Differences in \(I_{to}\) and KChIP2 expression within the ventricles (6, 7) contribute to regional differences in ventricular contractility and coordination of ventricular contraction (5). The broad phylogenetic distribution of this particular pattern of \(I_{to}\) gene expression (3) suggests that it was established early in mammalian evolution.

\(I_{to}\) and KChIP2 expression vary significantly from the common mammalian pattern in some rodents. In guinea pig, \(I_{to}\) expression (8) and KChIP2 expression have been lost, although the action potential is similar in most other species to other nonrodent mammals. In contrast, mouse and closely related species express \(I_{to}\) at very high levels. In these species the ventricular action potential is very short with a triangular waveform (2, 3, 9), due to the large size and rapid activation of the \(I_{to}\) current. The short action potential duration and altered morphology contribute to the scaling of ventricular action potential duration (2). In mouse and related species, \(I_{to}\) is encoded by both Kv4.3 and a second \(\alpha\)-subunit gene Kv4.2, which is only expressed in the hearts of this subset of rodents (2). Cardiac Kv4.2 gene expression is produced by evolutionarily mediated changes in the cis-regulatory function of the Kv4.2 gene in these species (2). There is a complementary increase in KChIP2 gene expression in these species that also contributes to increased \(I_{to}\) expression.

Despite complex biosynthetic pathways, voltage gated ion channel expression in heart is primarily determined by the level of channel gene transcription (10, 11). The size of the \(I_{to}\) current is directly dependent upon the level of KChIP2 gene expression (7, 12). The large changes in KChIP2 gene expression in mammalian heart and compact gene structure make it a favorable model in which to study the contribution of cis-regulatory evolution to the evolution of physiological function in mammals.

The results presented in this paper demonstrate that the KChIP2 gene contains a CpG island promoter that is not simply permissive for gene transcription but also contributes significantly to the tissue specificity of gene expression and is a primary locus for the evolution of KChIP2 gene expression patterns in mammalian heart.

Results

Expression of the KChIP2 Gene in Cardiac Ventricle. KChIP2 mRNA expression in the left ventricular wall of hearts from six different species was determined (Fig. 1A). KChIP2 mRNA was expressed at moderate levels in nonrodent mammals. Expression was high in mouse and rat, consistent with the high level of \(I_{to}\) expression in these species (2, 3). There was an almost complete absence of KChIP2 expression in the guinea pig ventricle, concordant with the lack of \(I_{to}\) expression in this species (8).

KChIP2 gene regulatory function was initially analyzed in three species: mouse, representative of small rodents that have a large \(I_{to}\) guinea pig, a rodent that lacks significant KChIP2 expression in heart (2, 3).

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and $I_0$ expression; and human, representative of the majority of mammals that have a relatively small but functionally important $I_0$.

**KChIP2 Transcription Start Site Mapping.** The transcription start site (TSS) for the KChIP2 gene was mapped in mouse and human heart and brain and guinea pig brain (Fig. 1A). Mapping of the TSS in guinea pig heart was not attempted due to the very low level of transcripts in this tissue. In the mouse, transcription was initiated primarily at a single primary transcription start site. The primary TSS in guinea pig heart was not attempted due to the very low level of transcripts in this tissue. In the mouse, transcription was initiated primarily at a single primary transcription start site. The primary TSS in human heart was marked with an “H.” (D) Analysis of CpG frequency around the KChIP2 transcription start site of the mouse, human, and guinea pig genes. The standard threshold for CpG island prediction (observedCpG/expectedCpG > 0.65) is marked with a dashed red line. The lower CpG frequency in the mouse KChIP2 gene and the loss of apparently nonfunctional CpG islands is reflective of genome-wide trends in this species. The transcription start site was aligned for all three species and is marked by an arrow. The exons are marked by gray bars. At the most commonly used TSS in each species, the [-1,+1] dinucleotide was C:A, a pyrimidine:purine pair, which conforms to the consensus at this location (13). In the human the second most commonly used site, equivalent to the primary site in mouse and guinea pig, was also a pyrimidine:purine pair, although in this case a C:G pair. The plots use a 200-bp window to calculate obsCpG/expectedCpG.

**Comparison of KChIP2 Proximal Promoter Function and mRNA Expression.** The KChIP2 gene is located within a gene-rich region of chromosomes 19 and 10 in mouse and human, respectively. The gene is closely bounded in the 5′ direction by another transcription unit (C10orf76), resulting in a relatively compact upstream regulatory region. Equivalent, ~2.5 kb, 5′-intergenic regions containing the core promoter were selected from mouse, guinea pig, and human genomic sequences and then tested using an in vitro transcription assay in cultured rat myocytes. There was a good correspondence between the function of this region and the relative level of mRNA expression in cardiac ventricles from the corresponding species (Fig. 2 A and B), suggesting that this region was likely to make a primary contribution to the species differences in KChIP2 transcriptional function in heart.

The presence of 5′-UTR sequence in the constructs raised the possibility that some of the differences in reporter gene expression between the different species could be due to differences in the translational efficiency of the transcribed mRNAs. To test this possibility, transcription from the reporter genes was measured directly using real-time PCR (Fig. 2C). The species differences in transcriptional activity were maintained when assessed directly from mRNA levels.
obtaining a large number of ferret myocytes for transfection. Error bars are

relative to Euarchontoglires (the superclade containing mouse, human, and
guinea pig). The fact that, with the exception of cellular electrophysiology, myo-
cyte morphology and function are generally very well conserved (2) makes the
culture system. This observation supports our general hypothesis that

cis-regulatory function is the primary mechanism respon-
sible for changes in ion channel expression in heart and that
transcription factor function and expression remain relatively
invariant in mammalian heart (2).

**KChIP2 Proximal Promoter Function.** An alignment of the proximal
promoter region from mouse, human, and guinea pig shows that
regions upstream of the CpG island are relatively poorly
conserved (Fig. 3A). The conserved regions at the 5′ end of this
sequence are associated with the upstream genes’ transcription
unit.

To isolate the regions responsible for the species differences in
promoter function, a series of 5′ deletions were performed (Fig.
3B). Increasingly large deletions had relatively little effect on
both the absolute level of activity (Fig. 3C) and the relative level
of activity (Fig. 3D) of promoter function for the three species.

Notably, even the shortest sequence, which encompasses the
CpG island containing only the core promoter and 5′-UTR,
retained most of the difference in expression between mouse,
human, and guinea pig genes (Fig. 3C and D), suggesting that
the primary differences between the species are located within
the CpG island.

**Substitution of the CpG Island Between Mouse and Guinea Pig.** To
further confirm that the CpG island region plays the predominant
role in determining the species differences in promoter function,
this region was swapped between the mouse and guinea pig
proximal regulatory regions (Fig. 4A). Substituting the guinea pig
sequence into the mouse background produced a more than
fourfold reduction in expression relative to the mouse sequence
(Fig. 4B). The reverse experiment produced a more than eight-
fold increase in expression relative to the guinea pig sequence.

The upstream 5′ regulatory region clearly modifies the activity
of the CpG island to some extent, as seen by comparison of
mouse to the guinea pig–mouse hybrid and guinea pig to the
mouse–guinea pig hybrid (Fig. 4A and B). Nonetheless, it is
clear that the CpG island is the major determinant of the species
differences in KChIP2 promoter function.

**Localization of Sequence Changes Within the CpG Island Responsible
for Changes in Functional Activity.** A series of swaps between the
mouse and guinea pig CpG island were performed to localize the
sequences responsible for the differences in transcriptional ac-
tivity. This region was divided into four domains (Fig. 4C):
regions “a” and “b” correspond to roughly equal halves of the
core promoter, with “b” containing the transcription start site.
Regions “c” corresponds to a poorly conserved region of the 5′-
UTR that is largely deleted in guinea pig and “d” corresponds to
a well-conserved region of the 5′-UTR that contains a conserved
inverted repeat. Transcription assays showed that region “d” did
not contribute to the functional differences (Fig. 4C and D). The
other three regions contributed approximately equally. Consid-
erable effort was expended to localize specific nucleotide changes
responsible for these differences. Individual sequence changes
had very modest effects on function, suggesting that the concerted
effect of multiple changes is required to produce these differences
in regulatory activity, as has been found in other systems (16).

**Phylogenetic Comparison of KChIP2 CpG Island Promoter Function.**
Expression of KChIP2 varies most markedly in rodents. The
function of the CpG island promoter from eight rodents and
three nonrodents was compared (Fig. 5A). High KChIP2 pro-
moter function appears to be phylogenetically restricted to
mouse and closely related species. Most rodents have
intermediate function, similar to the level seen in nonrodents. Very
low KChIP2 promoter function was restricted to guinea pig
and chipmunk (Tamias striatus).

**KChIP2 Proximal Promoter Function in Neurons.** Mouse, guinea pig,
and human KChIP2 proximal promoter function was tested in
cultured rat cortical neurons. Mouse and human promoter
function was indistinguishable in neurons (Fig. 5B). Guinea pig

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**Fig. 2.** (A) Comparison of mouse, guinea pig, and human KChIP2 proximal
promoter region activity using an in vitro transcription assay in neonatal rat
cardiomyocytes. (B) Expression of KChIP2 mRNA in the left ventricle of
mouse, guinea pig, and human heart. The data are replotted from Fig. 1A
to aid comparison. (C) Comparison of firefly luciferase mRNA abundance
when driven from mouse, guinea pig, and human KChIP2 proximal pro-
moter region constructs. (D) Comparison of mouse, guinea pig, and human
KChIP2 proximal promoter activity in cultured embryonic ferret car-
diomyocytes. Values for the negative control construct were relatively high
when compared with the rat cultures primarily because of the difficul-
ty in obtaining a large number of ferret myocytes for transfection. Error bars are
SEM (n = 3–6).

The transcription activity assays were performed using cul-
tured rat myocytes. The use of this culture system assumes that
the evolution of KChIP2 cis-regulatory function is fast relative to
any potential evolutionarily mediated changes in transcription factor network function. This assumption is consistent with the
fact that, with the exception of cellular electrophysiology, myo-
cyte morphology and function are generally very well conserved
in mammals (2). The observation that mouse, guinea pig, and
human KChIP2 promoter activity matches relative mRNA
abundance rather than the degree of phylogenetic relatedness
to the cell culture system also supports this assumption (Fig. 2
A and B).

We directly tested the effect of cell species using cultured
ferret embryonic ventricular myocytes. Ferret is an out-group
relative to Euarchontoglires (the superclade containing mouse,
guinea pig, and human) and both KChIP2 mRNA (Fig. 1A) and
I_{Na} (15) are expressed in ferret heart at similar levels to that seen
in human. The differences in mouse, guinea pig, and human
KChIP2 transcriptional activity are retained in the ferret cultures
(Fig. 2D) and are independent of both the species (ferret versus
rat) and developmental stage (embryonic versus neonatal) of the
culture system. This observation supports our general hypothesis
that cis-regulatory function is the primary mechanism respon-
sible for differences in ion channel expression in heart.
promoter activity in neurons was reduced to ∼37% of the mouse and human levels. This level was significantly higher than that seen in myocytes (Fig. 2A) but remains lower than mouse and human. There are no established species differences in $I_A$ function in mammalian neurons and it is unlikely that KChIP2 expression in equivalent neurons varies significantly between these species.

The reduction in guinea pig KChIP2 promoter activity in neurons suggests that changes in a neuron-specific enhancer element may be required to compensate for the weaker promoter function in neurons. Such an element was not identified in functional tests using the large first intron.

Discussion

The results reported here support the hypothesis that the large differences in the level of KChIP2 expression in mammalian heart are primarily due to evolutionarily mediated changes in the cis-regulatory function of the gene. This is demonstrated by the close match between KChIP2 mRNA expression and promoter function for different species and by the independence of the
relative promoter function from the phylogenetic origin of the cultured cells in which functional promoter tests were performed.

Expression of the KChIP2 gene is primarily restricted to electrically excitable cells and this gene is expressed in both cardiac myocytes and a subset of neurons (17–19). Transcription in both brain and heart tissues is initiated from the same CpG island promoter. Surprisingly, a major locus of evolutionary change for KChIP2 gene expression in heart lies within this CpG island core promoter. The species differences in KChIP2 mRNA expression in heart were matched by similar differences in the functional activity of this relatively small CpG island promoter from the same species.

Prior studies on the evolution of cis-regulatory function have generally found that evolutionarily mediated changes in gene regulatory function were localized to tissue-specific enhancer/repressor elements separate from the core promoter (16, 20, 21). Evolution of these tissue-restrictive elements is considered an effective way to modify regulatory function in a tissue-specific way, thereby limiting the pleiotropic effects of changes in gene regulatory function (22). Evolution of KChIP2 regulatory function deviates from this pattern in that a major locus of change is the CpG island promoter itself.

The changes in regulatory function of the KChIP2 core promoter in cardiac tissue are, to some degree, tissue specific. For example, there are relatively large differences in mouse and human KChIP2 core promoter function in heart with essentially no difference in neuronal function. There are, however, limits to the tissue specificity of these changes. In the more extreme case of guinea pig, where KChIP2 expression in cardiac myocytes is largely eliminated, there was a significant reduction in neuronal function, although this was not as extreme as the reduction seen in myocytes. It is possible that compensatory changes may occur in other regulatory regions of the guinea pig gene, although these were not identified. The results suggest that tissue-selective changes in CpG island core promoter function are possible, although there are limitations in isolating the effect of these changes.

Approximately half of the tissue-restricted genes in mammals have CpG island promoters (23) and the KChIP2 gene is an apparently typical example of this class of genes. Results for the KChIP2 gene suggest that, at least for some of these tissue-specific genes, the CpG island promoter is not merely permissive for gene expression but can be a significant contributor to tissue-selective expression. As such, for this class of promoters, the core promoter may itself be an important locus for evolutionarily mediated changes in tissue-selective regulatory function.

There is significant variation in the physiological function of the I_{Kr} current in different rodent hearts. In mouse and closely related species, the I_{Kr} current acquires a new function, becoming the major current underlying action potential repolarization (2, 9). The large I_{Kr} current produces a ventricular action potential of very short duration that matches the very fast heart rates in these species (2). Enhanced KChIP2 gene regulatory function makes an essential contribution to the required changes in cardiac electrophysiological function in these species. For guinea pig (Cavia), and possibly Tamias, the ventricular action potential retains the classic spike and dome morphology seen in most mammals, albeit without the initial rapid phase 1 repolarization contributed by the I_{Kr} current. The degenerative loss of I_{Kr} current expression in guinea pig may reflect reduced importance of I_{Kr} for coordinating contractile function in these relatively small hearts or the reduced requirement for the space repolarization capacity provided by the I_{Kr} in hearts that already have relatively large repolarizing I_{Kr} and I_{Kc} currents.

Expression of most voltage-gated ion channels in heart is transcriptionally regulated (10, 11). A general property of excitable cells is that they are computational systems, in the sense that the output of the system is determined by the sum of the variables that are expressed in the cell (24). As a consequence, relatively modest quantitative changes in voltage-gated ion channel expression can have large effects on cellular electrophysiological function. The evolution of KChIP2 cis-regulatory function within the rodent clade is an example of how ion channel gene cis-regulatory function can evolve over relatively short phylogenetic distances in a quantitative fashion to significantly alter electrophysiological function. Evolution of cis-regulatory function is a flexible and efficient way to modify cellular electrophysiological function and would seem to be the most reliable way to match channel expression levels to optimal electrophysiological function. It has been suggested that relatively complex cellular homeostatic mechanisms could be used to match voltage-gated ion channel expression levels to the required cellular electrophysiological function (25). A simpler alternative is that the cis-regulatory function of ion channel genes can evolve over time to achieve this matching, in much the same way that the evolution of cis-regulatory function establishes a wide range of phenotypic properties in most other cell types (26, 27). This is not to say that there is not some environment-specific tuning of voltage-gated ion channel gene expression levels within individual cells, but it seems most likely that cis-regulatory evolution is the primary mechanism used to achieve appropriate channel expression levels in electrically excitable cells (24).

Materials and Methods
All animal procedures have been approved by the institutional animal care and use committee of Stony Brook University.

Analysis of KChIP2 and Luciferase mRNA Expression. Analysis of mRNA expression in left ventricle wall was performed using real-time PCR as described previously (2, 28). Animals were killed and the hearts were quickly removed before further dissection. Human RNA samples were obtained from two independent commercial suppliers (Ambion and BioChain). For luciferase mRNA quantitation, rat neonatal myocytes were isolated, transfected, and cultured for 48 h, as described below. After culture, cells were lysed by adding RLT buffer and passing the lysate over a Qiashredder column (Qiagen). Total RNA was prepared from tissues and cells using Qiagen RNeasy columns with DNase treatment. RNA samples were quantitated, rediluted to give nominally equal concentrations, and quantitated a second time using spectrophotometric analysis.
cDNAs were prepared from a starting RNA amount of 5 μg per sample. In vitro reverse transcription was performed using SuperScript III reverse transcriptase (Invitrogen), as described previously (29). Real-time PCR was performed using the SYBR Green Quantitect PCR kit (Qiagen) with a 7300 Real-time PCR system (Applied Biosystems). Experimental samples were analyzed in triplicate. Threshold crossing points were converted to expression values automatically (30). Real-time PCR products were analyzed by gel electrophoresis and sequenced to confirm specificity of the amplification. Gene expression across RNA samples was normalized using 18S and 28S rRNAs as internal controls.

Multiple (typically three) primer pairs were used to analyze expression to detect primer-dependent artifacts (2). The results from each primer pair were averaged. Primer sequences used for real-time PCR are listed in Table S1.

BAC Library Screening. BAC clones encompassing the KChIP2 proximal promoter regions of guinea pig and hamster were identified in BAC libraries (BACPAC Resources Center) using a nonradioactive probe labeled with Digoxigenin-11-dUTP (DIG-11-dUTP alkali-labile; Roche). Probe sequences were based on cDNA sequences or conserved regions from multiple species. A 40-bp double-stranded DNA probe was synthesized using two 24-mer oligonucleotides with overlapping 3′ ends (5′ GGAGCCCCGAGACGACTCGAGA-GTC 3′ and 5′ TAAGGGAGGTGTCGACGACGGTC 5′), using Klenow fragment DNA polymerase and a mixture of dNTPs and DIG-dUTP. Hybridization was carried out using buffers optimized for DIG labeled probe (DIG Easy Hyb; Roche). Signals were developed using immunological detection method with anti-DIG-AP, Fab fragments (Roche) and CDP-Star (Roche). Positive BAC clones identified from the screening were then obtained from the BACPAC Resources Center. The BAC clones were analyzed by fingerprinting, end sequencing, and finally sequencing of the specific region of interest.

Isolation, subcloning, and sequencing of genomic DNA regions were performed using standard techniques (as described in SI Materials and Methods).

TSS Mapping by RNA Ligase-Mediated RACE PCR. The transcription start site of the KChIP2 gene was mapped in mouse, guinea pig, and human with the RNA Ligase-Mediated RACE PCR technique (31) using the Ambion First Stand Capture Kit (Applied Biosystems). Guinea pig brain and mouse brain and heart RNA were prepared as described previously (2). Human brain and heart RNA were obtained from commercial suppliers (BioChain and Ambion, respectively).


Supporting Information

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SI Materials and Methods

Isolation, Subcloning, and Sequencing of Genomic DNA Regions. For human, mouse, rat, guinea pig, dog, and ferret, initial selection of genomic fragments was based on sequences from genome databases (Homo sapiens Build 36 for human, Mus musculus Build 36 for mouse, Rattus norvegicus RGSC v3.4 for rat, Cavia porcellus Broad/cavPor3 for guinea pig, Canis lupus familiaris Broad/canFam2 for dog, and Mustela putorius furo MusPutFur1.0 for ferret). Human, mouse, guinea pig, and dog BAC clones containing the KChIP2 proximal promoter region were obtained from the BACPAC Resources Center. For rat and ferret, genomic DNA was used as a template to amplify the region of interest. The genome sequences of the remainder mammalian species were not available. For hamster, the specific KChIP2 DNA fragment was isolated from the BAC clone by a combination of restriction mapping and Southern blotting and then subcloned into pBluescript SK. For Petromyscus (rock mouse), Anomalurus beecrofti (Beecroft’s flying squirrel), Dipodomys heermanni (Heermann’s kangaroo rat), and Tamias striatus (eastern chipmunk), the specific KChIP2 DNA fragments were isolated from genomic DNA by PCR amplification using a pair of primers designed at highly conserved regions around the KChIP2 proximal promoter region and then subcloned into pBluescript SK. The KChIP2 sequences of these species were obtained by sequencing these intermediate subclones and were confirmed by sequencing multiple independent isolates.

Comparisons of the KChIP2 proximal promoter sequences were performed using the Vista (1) and ClustalW (2, 3) alignment programs. Conserved regions were used as landmarks to select orthologous sequences, although these were not exactly identical in length. All sequences terminated immediately before the initiator methionine in the first exon of the KChIP2 gene. The lengths of DNA fragments for the KChIP2 proximal promoter region were: 2,256 bp (mouse), 2,596 bp (guinea pig), and 2,760 bp (human). The lengths of DNA fragments for the KChIP2 CpG island region were: 453 bp (Mus), 457 bp (Rattus), 454 bp (Cricetus), 443 bp (Petromyscus), 463 bp (Anomalurus), 436 bp (Dipodomys), 395 bp (Cavia), 462 bp (Tamias), 457 bp (Homo), 440 bp (Canis), and 439 bp (Mustela).

Genomic regions were obtained using high-fidelity PCR amplification with either Expand Long Template PCR system (Roche) or Platinum Pfx DNA polymerase (Invitrogen), subcloned into the pGL2 luciferase reporter vector (Promega) and confirmed by sequencing.

Table S1. Real-time PCR primers

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<th>Gene</th>
<th>Pair no.</th>
<th>Sequence (5’ to 3’)</th>
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### Table S2. Reverse transcription gene-specific primers

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### Table S3. First round nested PCR primers

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</thead>
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<tr>
<td>Mouse</td>
<td>5′ AGGATAGGGCCGCTCACACA 3′</td>
<td>5′ TCAGGCTTTTTACTGGGC 3′</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>5′ GGACAAGGCGCTGGCTAACG 3′</td>
<td>5′ TCAGGCTTTTTAGTGCGGC 3′</td>
</tr>
<tr>
<td>Human</td>
<td>5′ GCGCTCGAGATCTGTGCTGGGG 3′</td>
<td>5′ TGGCGTCAAGAAAATCTTGCGGCCCCTG 3′</td>
</tr>
</tbody>
</table>

### Table S4. Second round nested PCR primers

<table>
<thead>
<tr>
<th>Species</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>5′ GCGGAATCAGCGAGCTGTGGGGCT 3′</td>
<td>5′ GCGGAATTCCGAGTAGGAGCCGTTCAAATCTC 3′</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>5′ GCGGAATTCGAGGAGCGAGGGGCTGCT 3′</td>
<td>5′ GCGGAATTCCGAGTAGGAGCCGTTCAAATCTC 3′</td>
</tr>
<tr>
<td>Human</td>
<td>5′ GCGCTCGAGACTGTGCTGTGGGGCT 3′</td>
<td>5′ GCGCTCGAGAAAATCTTGCGGCCCCTG 3′</td>
</tr>
</tbody>
</table>