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Cloning, molecular characterization, and expression analysis of Copine 8

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Abstract

Copines are ubiquitously expressed, phospholipid-binding proteins that have been conserved through evolution. In this paper, we report the cloning and molecular characterization of a new member of the Copine family, Copine 8. This gene has been isolated and characterized using a combination of bioinformatic and experimental approaches. Using an algorithm to cluster ESTs (expressed sequence tags) that are available through the public "GoldenPath" database, Copine 8 was initially identified as a gene predominantly expressed in prostate and testis. Cloning and molecular analysis revealed that this gene is expressed in low-levels in most tissues examined. Two different isoforms of this gene have been isolated. Strongest expression of Copine 8 mRNA is seen in the prostate, heart, and brain. Taken together, this data suggest that Copine 8 may have an important role to play in prostate regulation and development.

Keywords: Copine; Cloning; Functional genomics; Expression analysis; Isoforms; Prostate

Prostate cancer is the second leading cause of cancer-related mortality in men. It is estimated that 1 in 5 men in the United States will develop prostate cancer in their lifetime [1]. To date, there are no curative therapies available for this disease after it has metastasized from the site of origin [2]. Thus, it is of great importance to identify new genes that are expressed in the prostate, and understand their functions. This may lead to better understanding of the etiologies associated with the disease progression and to the development of targeted therapy of prostate cancer. To achieve this goal, we focused on utilizing the EST database to generate clusters of ESTs that are expressed in normal prostate and/or prostate cancer. Using this approach, we previously identified genes whose transcripts are specifically expressed in normal prostate and/or prostate cancer [3–6]. Using a similar approach, we describe here the cloning and characterization of *Copine 8*, a new member of the gene family that produces the calcium-dependent, phospholipid binding proteins called copines [7–9]. This gene expressed two isoforms of the Copine 8 protein. They are expressed at a low level in most tissues analyzed and at a high level in prostate, brain, and heart.

Materials and methods

EST mining and identification of Copine 8. Location of ESTs and mRNAs in, and their alignment to, Human Genome Working Draft were obtained from the "GoldenPath" (http://genome.ucsc.edu/index.html). A total of 4,205,362 EST entries (as of 8/20/2001) and 70,982 mRNA entries (as of 7/24/2001) were extracted from J. Kent's database (http://www.soe.ucsc.edu/~kent/test/gs.6/oo.27/psl). When there was more than one chromosome address to an EST, a single

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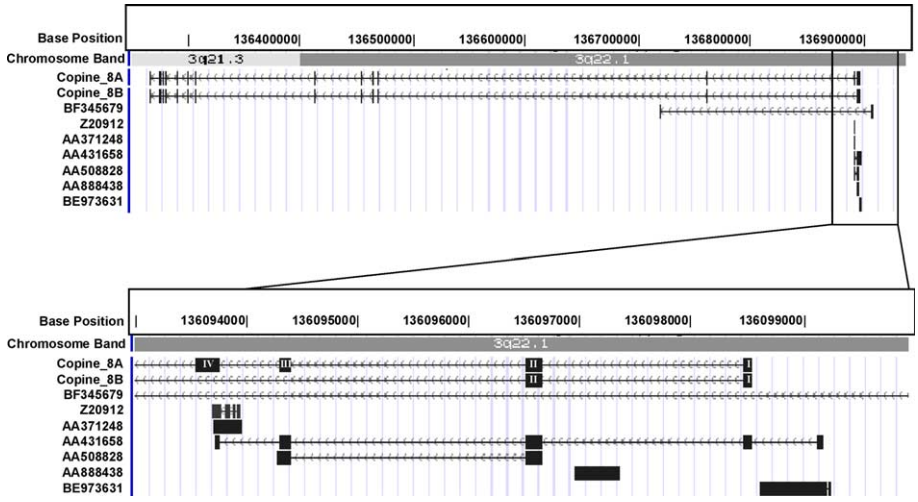

Fig. 1. Alignment of Copine 8 cluster to the human Genome Working Draft ("GoldenPath," December 22, 2001 freeze) using BLAT. Both isoforms of Copine 8 span 3q21.3 and 3q22.1 regions of chromosome band (panel A). Genomic region of the EST clusters magnified in panel B. The first four exons of Copine 8A and the first two exons of Copine 8B are shown in Roman numerals. ESTs BF345679 and Z20912 were not in "GoldenPath" database of August 6, 2001 freeze, when the cluster was first identified.

address on the chromosome was retained in most cases. Entries with long intronic sequences were ignored. A total of 4,011,049 out of 4,276,344 alignments satisfied our conditions and were used for cluster formation. A total of 133,104 clusters were formed based on overlap of aligned sequences. Precise procedure of selecting and clustering ESTs will be described elsewhere. The tissue of origin for clustered ESTs was determined from the library data in the National Center for Biotechnology Information (NCBI) dbEST files (ftp://ftp.ncbi.nih.gov/genbank/gbest*.seq.gz). Similar information for mRNAs was extracted from the NCBI GenBank primate repository (ftp://ftp.ncbi.nih.gov/genbank/gbpri*.seq.gz). The library database was built and libraries were assigned to designated tissue types as described previously [10]. Clusters with 75% or more ESTs from prostate libraries were selected. The Copine 8 cluster was made of five ESTs from five different libraries (four from prostate and one from testis). The corresponding gene is located in chromosome 3q22.1 (Fig. 1).

Primers. The nucleotide sequence of the primers used in this study: T407 > 5′-GAG TCT AGT CCT CAA CTC TAA CCA-3′; T408 > 5′-GCT AAT CTC TGG ATC CTT CGT GTC-3′; P2 > 5′-GCA AAA TTT CAA CAA ACC TG-3′; P11 > 5′-AGA TTT CAC TGC CTC AAA CG-3′; P18 > 5′-ATG TTG CTC ATC TTC TTC ATT CTC-3′. Actin specific primers have been reported elsewhere [11]. All primers were synthesized by Lofstrand Labs.

Dot blot and Northern blot hybridizations. The human multiple tissue RNA dot blot (RNA Masterblot; Clontech) and Northern blot (Multiple Tissue Northern blot; Clontech) hybridizations were carried out as described below. Briefly, the RNA membranes were prehybridized for more than 2 h in hybridization solution (Hybrisol I; Oncor) at 45 °C. PCR with P11 and P2 primers were used to generate ~1.1 kb probe common to both isoforms. The probes were labeled with ^{32}P by random primer extension (Lofstrand Labs) and added to the blots and hybridized for another 16 h. The blots were then washed 2× 15 min each in 2× SSC, 0.1% SDS, at room temperature and then washed 2× 15 min in 0.2× SSC, 0.1% SDS, at 60 °C. Finally, the membranes were exposed on X-ray film for 1–2 days.

PCR analysis. PCR was performed on cDNA from 24 different human tissues using the Rapid-Scan gene expression panel (OriGene Technologies) or the MTC cDNA panel (Clontech) following the manufacturer's instructions. The thermocycling protocol was: initial denaturation at 94 °C for 3 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and elongation at 72 °C for 1 min. The PCR primers used are indicated in the figure legends. The PCR reactions were analyzed on 1.5% agarose gels stained with ethidium bromide.

In vitro translation. The in vitro transcription and translation of the cDNA was carried out using T7 RNA polymerase and rabbit reticulocyte lysate (RRL; Promega) following the manufacturer's instructions. [^{35}S]Met (ICN) was incorporated in the reaction for visualization of translated products. The reaction mixture was heated at 95 °C in reducing sample buffer and then analyzed under reducing conditions on a polyacrylamide gel (4–20% PAGE, Tris/glycine; Bio-Rad) together with prestained protein molecular weight markers (Bio-Rad). The gel was dried and subjected to autoradiography.

Results

Computer analysis of the Copine 8 cluster and cDNA cloning of Copine 8

A new cluster was initially identified by computer analysis as prostate and testis specific (Fig. 1). It was made of five ESTs from five different libraries (four from prostate and one from testis), which mapped to chromosome 3q22.1. However, a recent update of the EST database indicates that there are two additional ESTs from brain in this cluster as well. Primers T407 and T408 were made based upon the EST sequence data. Origene Technologies used these primers to isolate a clone by screening a human prostate library. The primer P2 was constructed based upon the 3′ sequence information from this clone. Finally, the primers P2 and T407 were utilized to amplify the full length cDNA from pooled prostate cDNA (Marathon Ready; Clontech). The amplified products were cloned into the PCR 2.1 Topo vector (Topo TA Kit; Invitrogen). Selected colonies were isolated and sequenced. Two different isoforms of

the gene (isoforms A and B) were identified. The protein from the B form has 18 additional amino acids at its N-terminus compared to the A form (Fig. 2). The sequences were analyzed using the BLAST program (NCBI).

Complete nucleotide sequence analysis (deposited in the GenBank with Accession Nos. AF465770 and AF465771) of the cDNAs reveal that they belong to the Copine family of genes. We name the new gene *Copine 8*. Strongest homology was found between Copine 8 and *N*-Copine [14] at both the nucleotide and protein levels (Fig. 2). There are at least 16 exons in the *Copine 8* gene (Fig. 3). Start codons are present in exons 2 and 5 and stop codons are detected in exons 3, 4, and 16. Copine 8A has a longer cDNA (~2.5 kb) but a shorter open reading frame (ORF) of 1674 bases or 558 amino acids. By con-

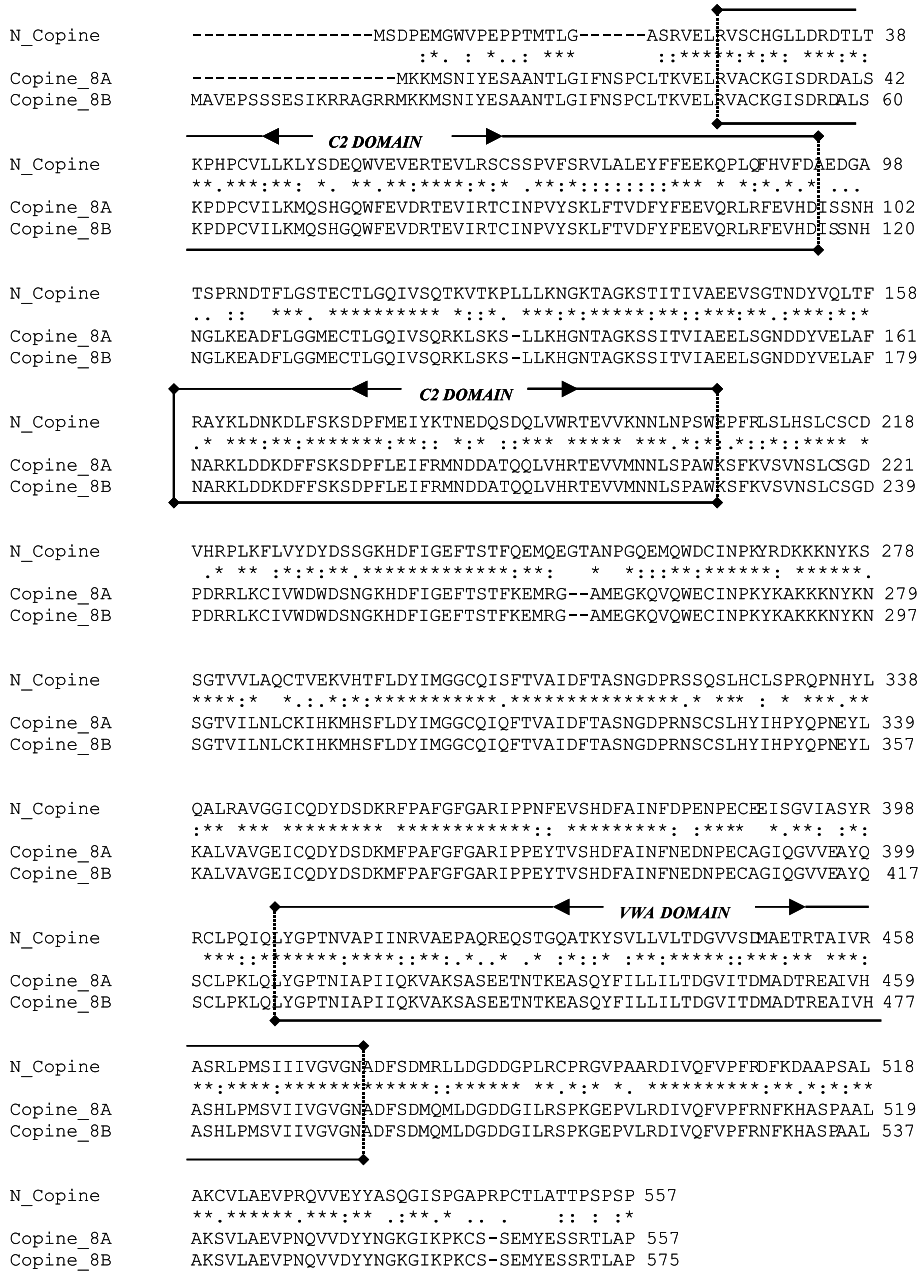

Fig. 2. Multiple alignment of Copine 8 isoforms with *N*-Copine. Amino acid sequences of Copine 8A (557aa) and Copine 8B (575aa) were aligned to *N*-Copine (557aa) using *ClustalW* (*1.81*) protocol of European Bioinformatics Institute (http://www2.ebi.ac.uk/clustalw). Alignment characteristics are as follows: *, identical or conserved residues; :, conservative substitutions; ., semi-conservative substitutions. Using CDART (Conserved Domain Architecture Retrieval Tool; http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi?cmd = rps), two C2 domains (protein kinase C conserved region 2) and one VWA domain (von Willebrand factor type A) were predicted (rectangular areas).

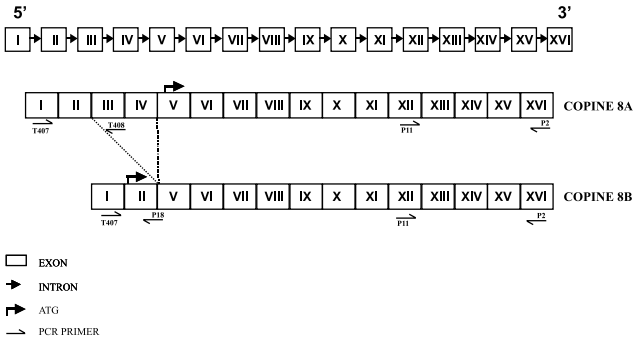

Fig. 3. Alternative splicing of Copine 8A and Copine 8B. The Copine 8 gene contains at least 16 exons (exons and introns are represented by boxes and lines, respectively). Exons II and V contain starting codons and exons III, IV, and XVI possess stop codons. The A form transcript has all 16 exons. In B form, the exons III and IV are excised out. The A form protein uses the start codon in exon V whereas the B form uses the start codon in exon II. Different primers used in this study are shown as arrowheads with corresponding IDs.

trast, Copine 8B has a shorter cDNA (~2.2 kb) but an ORF of 1728 bases or 576 amino acids. This is due to alternate splicing of the gene as indicated in Fig. 3.

Expression analysis of the Copine 8 gene

To determine experimentally the tissue expression of Copine 8, we initially performed PCR analysis on a commercially available cDNA panel (Rapid-Scan) containing cDNAs from 24 different human tissues using primers from a region common to both isoforms. As shown in Fig. 4A, significant expression of Copine 8 is observed in brain, heart, kidney, prostate, and fetal brain. The gene is also weakly expressed in most tissue types examined. Dot blot analysis using a probe amplified from a region common to both isoforms confirmed this observation (Fig. 4B). Semi-quantitative PCR analyses of the two isoforms using isoform-specific primers demonstrate that both forms are strongly expressed in prostate and weakly expressed in testis, brain, and heart (Fig. 4C).

Northern blot analysis of Copine 8

Northern blot analysis was performed to determine the transcript size of Copine 8. Premade blots containing

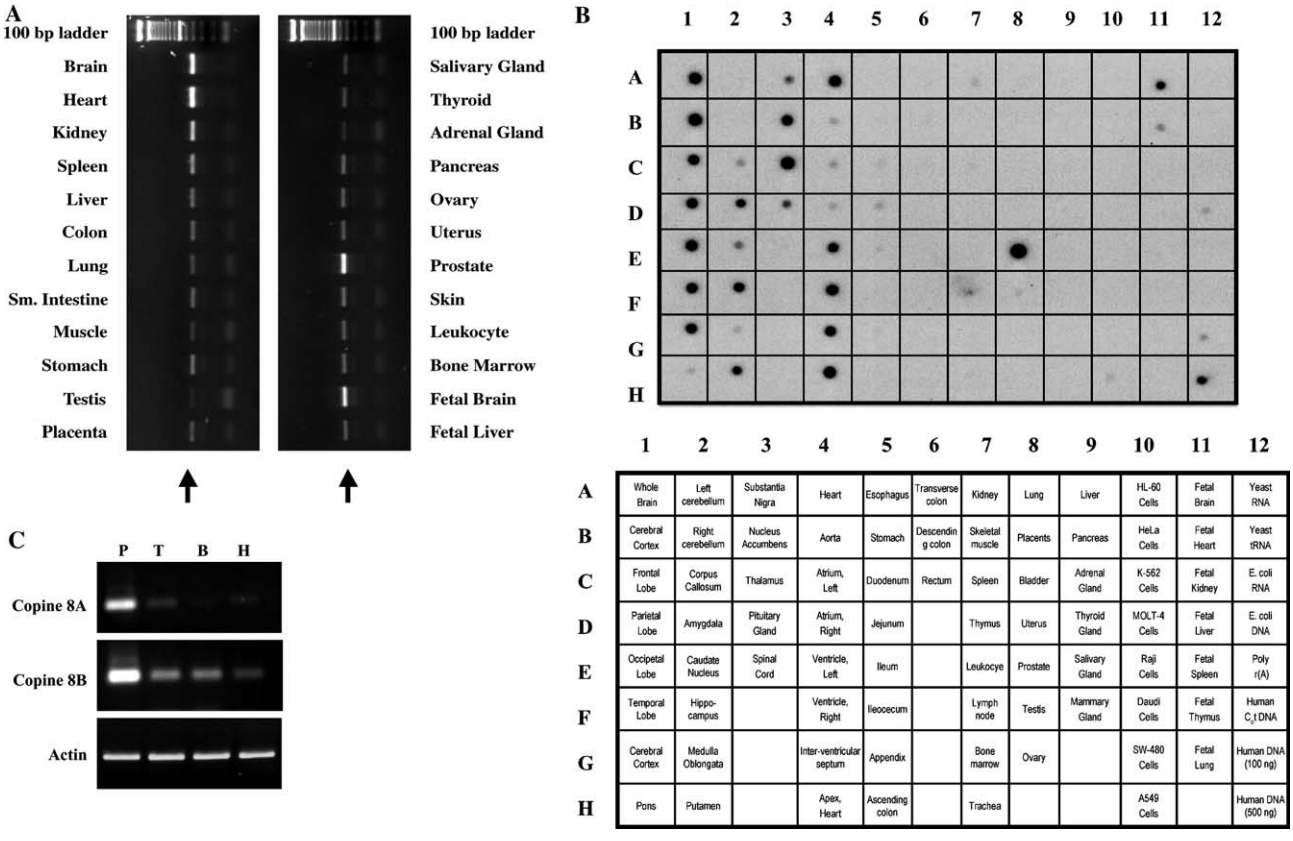

Fig. 4. Tissue distribution of Copine 8 expression. (A) PCR analysis of Copine 8 expression on cDNA from 24 different human tissues. The primers used were P2 and P11. The expected size of the PCR product is indicated by an arrow. (B) RNA hybridization of a multiple tissue dot blot containing mRNA from 50 normal human tissues or cell types using a cDNA probe from the 3′ end of the Copine 8 transcript. This area is common to both isoforms. A template showing the location of each tissue/cell-type is indicated in the bottom panel. (C) Analysis of relative expression of the Copine 8 isoforms in selected tissues. PCR was performed using isoform specific primers to determine the relative expression of each isoform in prostate (P), testis (T), brain (B), and heart (H) cDNA. Primers T407 and T408 were used to amplify Copine 8A (top panel), primers T407 and P18 were used to amplify Copine 8B (middle panel). Actin-specific primers were used as a control (bottom panel). PCR was performed for 35 cycles to visualize Copine 8-specific products and 25 cycles to visualize actin-specific product.

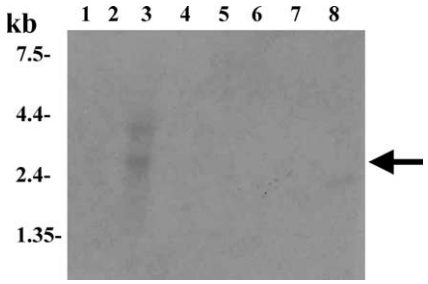

Fig. 5. Northern blot analysis of Copine 8 expression. A cDNA probe constructed from the 3′ end of the Copine 8 transcript common to both isoforms was hybridized to a premade mRNA blot. The lanes are: (1) spleen; (2) thymus; (3) prostate; (4) testis; (5) uterus; (6) small intestine; (7) colon; and (8) peripheral blood leukocyte. The expected position of the Copine 8 transcript is indicated by an arrow.

equal amounts of mRNA from different human tissues were hybridized with a probe from a common region of the two isoforms. A strong band corresponding to a transcript size of ~2.5 kb is detected in prostate (Fig. 5). The size of the transcript is in agreement with the cloned cDNA and other members of the Copine family. A higher molecular weight band is also detected on the blot. The identity of this band is not clear but may be due to the presence of unprocessed RNA in the sample. The absence of signals in other tissues is probably due to the low-level of expression of Copine 8 in these tissues.

In vitro coupled transcription and translation of Copine 8 isoforms

To determine the size of the proteins encoded by the two isoforms of Copine 8, the two cDNAs were transcribed and translated using the T7 promoter-driven rabbit reticulocyte lysate system. As shown in Fig. 6, both isoforms produce distinct products with a molecular weight of ~60 kDa. Although equal amounts of plasmid were used in each reaction, the higher intensity of Copine 8B product may be due to a stronger Kozak initiation sequence utilized by this isoform. No products

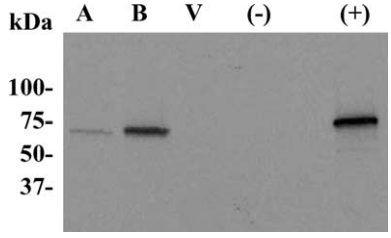

Fig. 6. In vitro translation analysis of Copine 8. Copine 8A and Copine 8B were transcribed with T7-RNA polymerase and translated with rabbit reticulocyte lysate in the presence of [^{35}S]methionine. The translated products were analyzed by SDS–PAGE gel followed by autoradiography with fluorographic enhancement. Lanes are designated as follows: A, Copine 8A; B, Copine 8B; V, vector alone; (−), no DNA; and (+), positive control (luciferase-expressing vector). Distinct products are obtained with Copine 8 isoforms and positive control.

are observed in lanes expressing vector alone or with no DNA. Luciferase cDNA was used as a positive control in this experiment.

Discussion

Here we report the cloning and characterization of Copine 8, a new member of the Copine family. Copine 8 is strongly expressed in the prostate, brain, and heart tissues but is also detected in most other tissues examined at low levels. Copines are highly conserved across species and it is possible that the family members may play a pivotal role in development and mitogenesis [12,13]. Copine 8 has the strongest homology to *N*-Copine with ~60% homology at the protein level. *N*-Copine has been reported to be associated with OS-9, a protein that is frequently over-expressed in osteosarcoma [13]. *N*-Copine has also been implicated in synaptic plasticity and development [12,14]. While Copines are calcium-dependent phospholipid-binding proteins with intrinsic kinase activity [7–9], their physiological function in mammals is not entirely clear. In *Arabidopsis*, Copines have been implicated in plant growth homeostasis [15] and increased drug resistance [16].

Copine 8 was initially selected using an algorithm that identifies genes with tissue-specific patterns of expression in the dbEST. However, experiments showed that the expression of this gene is fairly ubiquitous. In a later version (December 22, 2001) of the "GoldenPath" database we find two new ESTs added to the cluster, both of which are from the brain. There are over 4.5 million human ESTs at present. This number is rapidly increasing as more ESTs are reported to the database. However, certain organs are better represented in this database than others. This may in turn reduce the possibility of a low-abundance transcript being reported from a less-studied organ. Therefore, a combination of bioinformatic and experimental approaches should be undertaken to identify genes that are over-expressed or specific to certain tissues.

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