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Analysis of the expression of chondroadherin in mouse ocular and non-ocular tissues

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Purpose: Chondroadherin (Chad) is a unique member of the small leucine rich repeat proteoglycan gene family. It is expressed at high levels in certain zones of cartilage and also has been detected in bone, tendon, bone marrow, and chondrosarcoma cells. Recently, we demonstrated that Chad is expressed in human and mouse lens and that its expression is decreased in the absence of mimecan/osteoglycin. This finding prompted us to explore the expression of Chad in other ocular and non-ocular tissues.
Methods: Reverse transcription-polymerase chain reaction amplification (RT-PCR), in situ hybridization (ISH), and immunohistochemistry (IHC) were used to determine the expression of Chad in human and mouse tissues.
Results: RT-PCR analysis showed strong expression of Chad in mouse brain, heart, lung, and embryo. Moderate expression was detected in mouse thymus, spleen, testis, and ovary, and very low expression in kidney and liver. Chad was highly expressed in human cornea, brain, and skeletal muscle, and moderately in human retina and lens. By ISH, Chad mRNA was detected in cornea, lens, and retina of postnatal day 21 mouse eyes. By IHC, immunostaining for Chad was seen in epithelial and endothelial layers of the cornea, as well as in lens, ciliary body, and retina of the adult mouse eye. Strong immunostaining was noted in retinal rod, cone, and plexiform layers. IHC analysis of tissue microarray demonstrated presence of Chad in brain (cerebellum), skeletal and cardiac muscles, lung, gastrointestinal tract, ovary, and cartilage. In most tissues, Chad expression was associated with either peripheral nerves and/or blood vessels. In the stomach and intestines, positive immunostaining was observed in Meissner's plexus and enteroendocrine cells. Intriguingly, positive immunostaining also was observed in Purkinje cells of the cerebellum and pancreatic islets.
Conclusions: The present work establishes the expression and localization of Chad in several new locations including cornea, lens, ciliary body, retina, cerebellum, pancreatic islet, blood vessels, and peripheral nerves. The surprisingly broad expression pattern of Chad suggests potential roles for this protein in cell specific and/or tissues specific function(s). The results reported here are an essential prerequisite for future studies aimed at understanding the biological roles of Chad in health and disease.

The extracellular matrix of many connective tissues contains a class of proteoglycans known as small leucine rich repeat proteoglycans (SLRPs). Experimental evidence demonstrates that these proteins are involved in the regulation of collagen fibril diameter, interfibrilar spacing, and the adhesive properties of different types of collagens [1-4]. The SLRPs have also been recognized as molecules that exhibit direct and indirect cell signaling properties, thereby influencing cellular growth, differentiation and migration [5-7]. Structural analysis of the SLRPs showed that they consist of core proteins that can carry different glycosaminoglycan (GAG) side chains and/or have N-linked sugar modifications. The core protein usually contains 8-11 leucine rich repeats (LRR) flanked by conserved cysteine residues. The LRR is a structural motif that allows the molecule to adopt a horseshoe-like configuration and play a role in protein-protein interactions [8]. The LRR is used in many molecular recognition processes, including cell adhesion, signal transduction, and DNA repair [9,10]. The SLRPs can carry two types of GAG side chains. Chondroitin sulfate/dermatan sulfate GAGs modify the SLRP family members, decorin and biglycan, whereas keratan sulfate GAGs modify the family members, fibromodulin, PRELP, and osteoadherin in non-corneal tissues, and keratocan, lumican, and mimecan in the cornea [11-17]. The GAG chains of SLRPs function in the maintenance of interfibrillar spacing, normal tissue hydration, and the interactions of SLRPs with growth factors and their receptors [6,7,18]. Many of the SLRPs can be isolated from different tissues without GAG chains and these are considered "part time" proteoglycans [2,5].

At the genomic level, the various members of the SLRP gene family map to relatively few chromosomes: Opticin, PRELP, and fibromodulin map to human chromosome 1q3; asporin, osteoadherin, and mimecan map to human chromosome 9q2; epiphycan, keratocan, lumican, and decorin, to human chromosome 12q2 [19-21]. Clustered chromosomal localization suggests that these genes arose from several duplication events and that functional overlaps between related genes should be expected. However, their continued existence also indicates that each one of these genes plays an individual role in the maintenance of normal homeostasis, as illustrated by their distinct tissue specific patterns of expression and regulation [22].

Based on similarities in their amino acid sequence, spacing of the N-terminal cysteines, and gene structure, the SLRP

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family members have been subdivided into 3 classes. Class I members include biglycan, decorin, and asporin; class II includes lumican, keratocan, fibromodulin, PRELP, and osteoadherin; and class III includes mimecan/osteoglycin, epiphican/Pg-Lb, and opticin (reviewed in [1,2,4,5,10]).

Chondroadherin constitutes as a unique (class IV) member of the SLRP gene family [21,23]. Initially isolated from bovine tracheal cartilage as a 36 kDa protein, subsequent determination of its sequence at both protein and cDNA levels revealed that the protein is a member of the SLRP gene family [23,24]. Similarly to other SLRPs, Chad contains 10 leucine rich repeats. In contrast to them, Chad possesses two cysteine loops in the C-terminal domain and lacks an N-terminal extension [23]. The carbohydrate composition of Chad shows the presence of xylose, galactose, mannose, and fucose, an indication that the core protein carries short oligosaccharides resembling the linkage structure of CS/DS GAG chains [23]. The absence of hexosamines indicates that Chad does not carry the repeat disaccharide of GAGs [23]. Chad lacks the Asn-Xaa-Ser/Thr sequenced, potential site for N-glycosylation. A short oligosaccharide, lacking sialic acid and hexosamine, has been located on serine 122 [24]. The human gene has been mapped to human chromosome 17q21 and the mouse gene to a corresponding mouse chromosome (11) [25,26]. The genomic organization of Chad also is different compared to the conventional SLRPs [25,26]. The mouse gene consists of four exons separated by one large and two smaller introns [26]. Chad has been reported to promote attachment of osteoblastic cells to solid state substrates and to bind chondrocytes via their integrin $\alpha 2\beta 1$ receptors [27,28]. It also has been shown to bind to collagens type II and type VI [29,30]. The expression of Chad has been reported to be limited to cartilage, bone, tendon, bone marrow cells, and chondrosarcoma cells [31,32].

Recently, using cDNA microarray analysis we found that Chad is expressed in the mouse lens and that its expression is suppressed in the absence of mimecan [33]. To extend this initial observation, we performed analyses of Chad mRNA and protein expression using whole eye samples and tissue array immunolabeling. We show here that Chad and its mRNA are expressed in mouse cornea, ciliary body, lens, and retina. In addition to the eye, we found Chad expression associated with peripheral nerves of several non-ocular tissues, as well as with Purkinje cells of the cerebellum. Intriguingly, Chad also is expressed in enteroendocrine cells, pancreatic islet cells, and mouse ovary, suggesting roles for this protein in biological functions of many tissues. Considering the fact that, at present, there are no reports describing the localization of Chad in tissues other than cartilage and bone, our study, by demonstrating the broad expression pattern of this protein, provides important new information which will contribute to understanding the physiological, cell and tissue specific function(s) of Chad.

METHODS

Mice were housed in animal care facilities according to NIH guidelines [34] and IACUC approved protocols. All experiments were performed in compliance with the ARVO statement for use of animals in ophthalmic and vision research. For RNA and protein isolation, mouse eyes were rapidly collected and frozen in liquid nitrogen. For in situ hybridization (ISH), mouse eyes were fixed in 4% paraformaldehyde in phosphate buffered saline overnight. Total RNA from normal mouse tissues was obtained from Ambion Inc. (Austin, TX). Mouse macrophage cell line C2D (MHCII$^{-/-}$ and TLR4^{Lps-n}

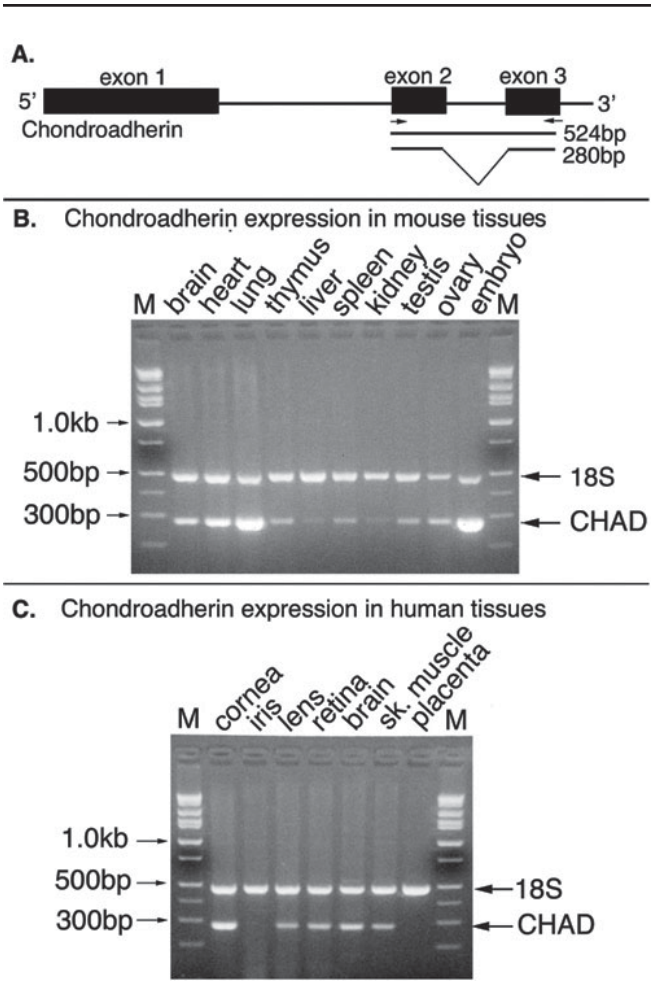

Figure 1. Semi-quantitative RT-PCR performed to assess Chad expression in mouse and human tissues. **A**: Schematic, showing part of the human Chad gene and primers for PCR amplification of Chad cDNA. The primers were designed to flank an intron and to amplify a 280 bp cDNA product. The genomic structure of mouse Chad is similar to that of human; mouse primers also amplify a 280 bp cDNA product. **B**: Chad expression in mouse tissues. The 280 bp PCR product corresponding to Chad cDNA was amplified together with QuantumRNA 18S internal standard in the same (multiplex) PCR reaction. To modulate amplification efficiency of 18S rRNA, 18S primers were mixed with 18S competimers in a 3:7 ratio. PCRs shown are representative of at least two repetitions of each PCR. **C**: Chad expression in human tissues. The 280 bp PCR product corresponding to Chad cDNA was amplified together with QuantumRNA 18S internal standard in the same (multiplex) PCR reaction. To modulate amplification efficiency of 18S rRNA, 18S primers were mixed with 18S competimers in a 3:7 ratio. PCRs shown are representative of at least two repetitions of each PCR. The "M" labels a lane with a Hi-Lo DNA molecular weight marker.

genotype) was a gift from Dr. S. K. Chapes, Kansas State University [35]. These cells express both Chad and mimecan (unpublished). Whole human eyes were provided by the Missouri Lions Eye Bank, Columbia, MO.

Reverse transcription-polymerase chain reaction (RT-PCR): Total RNA was isolated from mouse and human eye tissues using Totally RNA Total RNA Isolation Kit (Ambion). RNA (2 μg) was reverse-transcribed using the anchor primer oligonucleotide $(dT)_{18}$, and Superscript II Reverse Transcriptase (Life Technologies, Inc., Gaithersburg, MD). The single stranded cDNA products (2 μl) were used as templates in PCR amplification reactions as described [33]. The gene specific primers for mouse and human Chad were designed from nucleotide sequences contained in GenBank NM_007689 and BC036360, respectively. The following primers, synthesized by Integrated DNA Technologies Inc. (Coralville, IA), were used; Mchad+830: 5'-GAA GTT CTC AGA TGC TGC CTT CTC GGG-3' and Mchad-1110: 5'-GGT CGG GGA TTT GCA GCT GCG AAG G-3'; Hchad+821: 5'-GAA GTT CTC AGA TGG TGC CTT CCT GG-3' and Hchad-101: 5'-GGT GGG GAA CTT GCA GCT GCG GAA GG-3'. The resulting 280 bp PCR products were resolved by agarose gel electrophoresis, excised from the gel, cloned into pGEM-T vector (Promega Corp., Madison, WI), and sequenced. A Hi-Lo DNA molecular weight marker (Minnesota Molecular, Minneapolis, MN) was used to determine fragment size. Quantum RNA18S Internal Standard (Ambion, catalog number 1716, amplifying a 488 bp fragment) was used as endogenous standard in all PCR reactions. A 3:7 ratio of 18S primers to 18S competimers was used, as previously described [33].

In situ hybridization: The pGEM-T vector containing a 280 bp mouse Chad cDNA fragment was used as template for synthesis of cRNA probes, according to the manufacturer's recommendation (Ambion) and labeled with ^{35}S-UTP (>1000 Ci/mmol, Amersham Biosciences Corp., Piscataway, NJ). For the ISH experiments, custom service from Phylogeny Inc. (Columbus, OH) was used as previously described [30].

Antibodies: A polyclonal antibody against mouse Chad was raised by immunizing a rabbit with a peptide from the C-terminal region of the Chad protein (SSP AKF KGQ RIR DTD ALR SC) coupled to Keyhole Limpet Hemocyanin. For antibody generation, a custom service provided by Abgent, Inc. (San Diego, CA) was used. The purified antibody was shown to react with mouse Chad by immunoblot and IHC search

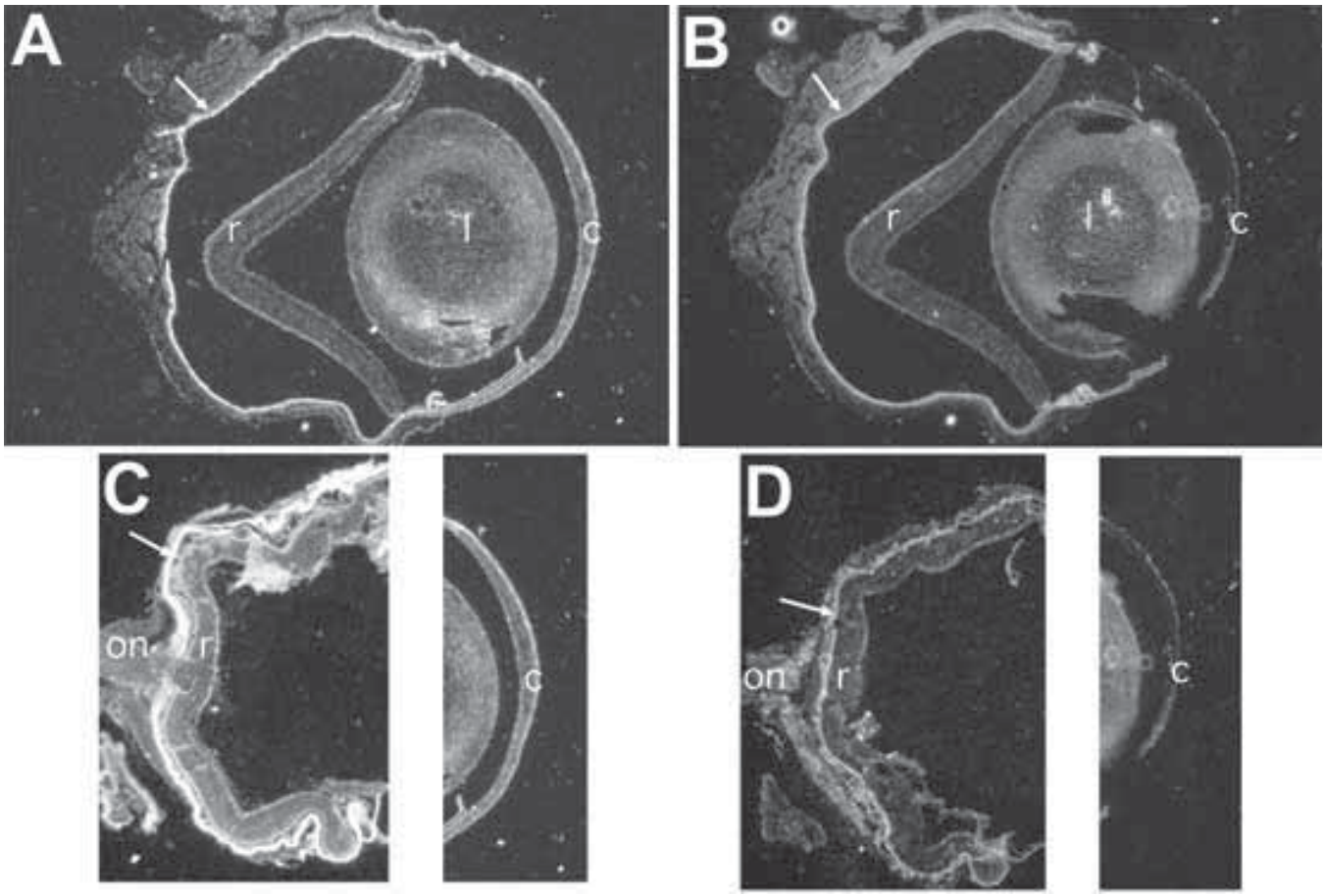

Figure 2. In situ hybridization analysis of Chad mRNA in postnatal day 21 mouse eyes. Chad expression was detected in cornea (c), lens (l), and retina (r) by ISH. In each slide, the pigmented layer of the retina, which is refractile in darkfield illumination, is indicated by a white arrow. **A**,**C**: Postnatal day 21 mouse eye (x2.5) treated with antisense Chad probe. **B**,**D**: Postnatal day 21 mouse eye (x2.5) treated with sense Chad probe.

analyses. The secondary antibody, goat anti-rabbit IgG conjugated to horseradish peroxidase (catalog number A-0545), was obtained from Sigma-Aldrich Co. (St. Louis, MO).

Immunoblot analysis: C2D cells were harvested and suspended in a cell lysis buffer containing protease inhibitor cocktail (Complete Mini; Roche Diagnostics, Mannheim, Germany). The frozen mouse eye tissue was ground under liquid nitrogen to make a fine powder which was also suspended in cell lysis buffer. The insoluble material was removed by centrifugation. An aliquote of the resulting supernatant was diluted 1:1 in sample buffer and loaded onto a polyacrylamide gel. After electrophoresis, the proteins were transferred to PDVF membrane. The blot was incubated with antibody RB5522 specific for mouse Chad, rinsed, exposed to a horseradish peroxidase conjugated secondary antibody, rinsed again, and incubated in SuperSignal West Pico Chemiluminescent reagent (Pierce Biotechnology, Inc., Rockford, Il; catalog number, 34077). The blot was then exposed to blue lite autorad film (ISC BioExpress, Kaysville, UT).

Immunohistochemisry: Custom IHC on slides containing normal adult mouse tissues was performed using the service provided by SuperBioChips Laboratories (Seoul, Korea), as previously described [33]. Briefly, sections of mouse tissues were immunostained with rabbit anti-Chad polyclonal antibody raised against synthetic peptide corresponding to amino acids 305-325 of mouse Chad (see above, Antibodies). Biotin conjugated secondary antibody was reacted with avidin-biotin-peroxidase complex solution (Vector Laboratories Inc., Burlingame, CA), with immunoreactivity developed in 3,3'-diaminobenzidine (DAB).

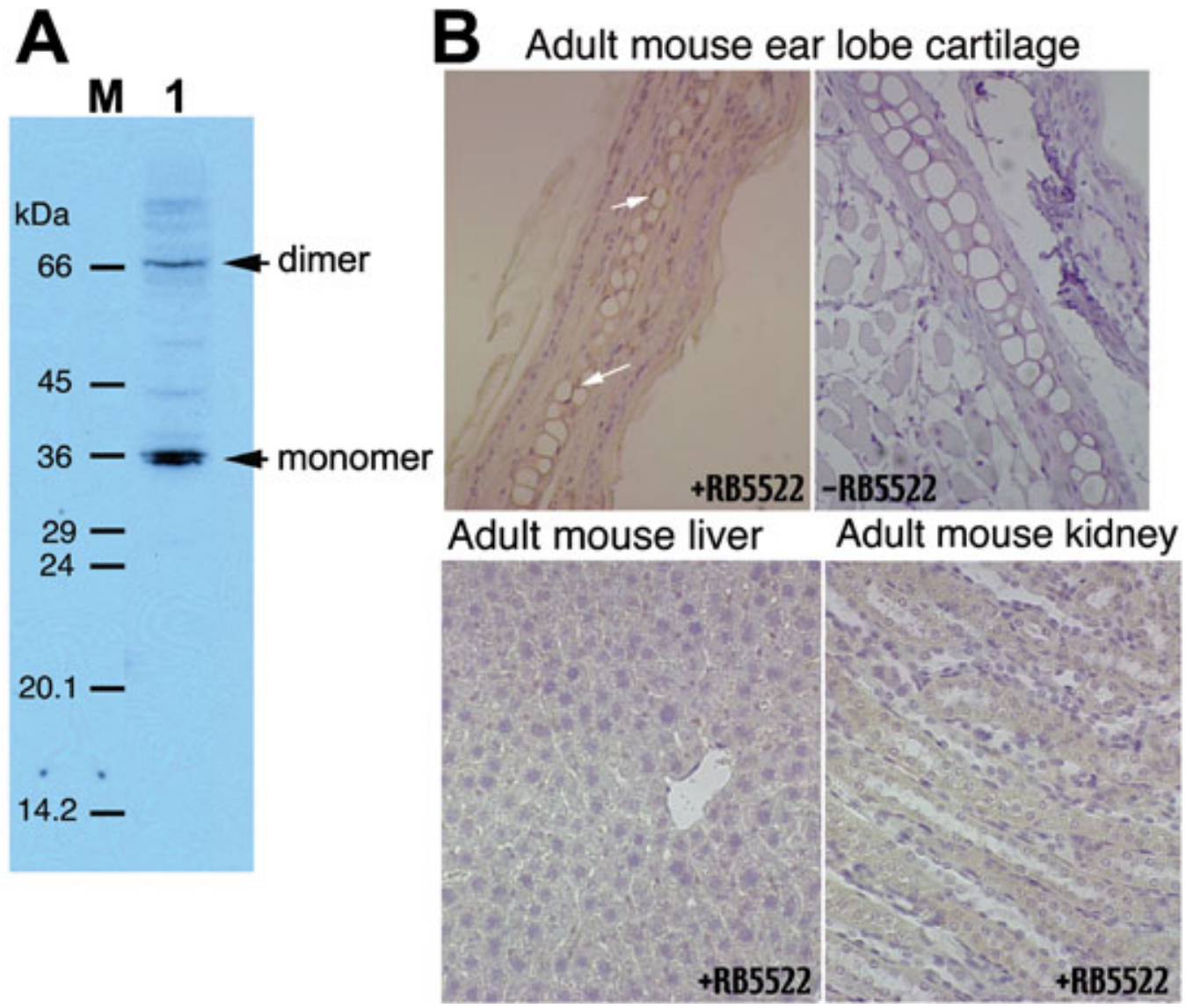

Figure 3. Immunoblot and immunohistochemistry analyses confirming specificity of RB5522 antibody to mouse Chad. **A**: The antibody RB5522 selectively recognizes 36 kDa monomeric and 67 kDa dimeric forms of Chad in C2D protein lysates. **B**: IHC shows the presence of Chad in mouse ear lobe cartilage and its absence in liver and kidney. Positive immunostaining appears brown and is highlighted by arrows.

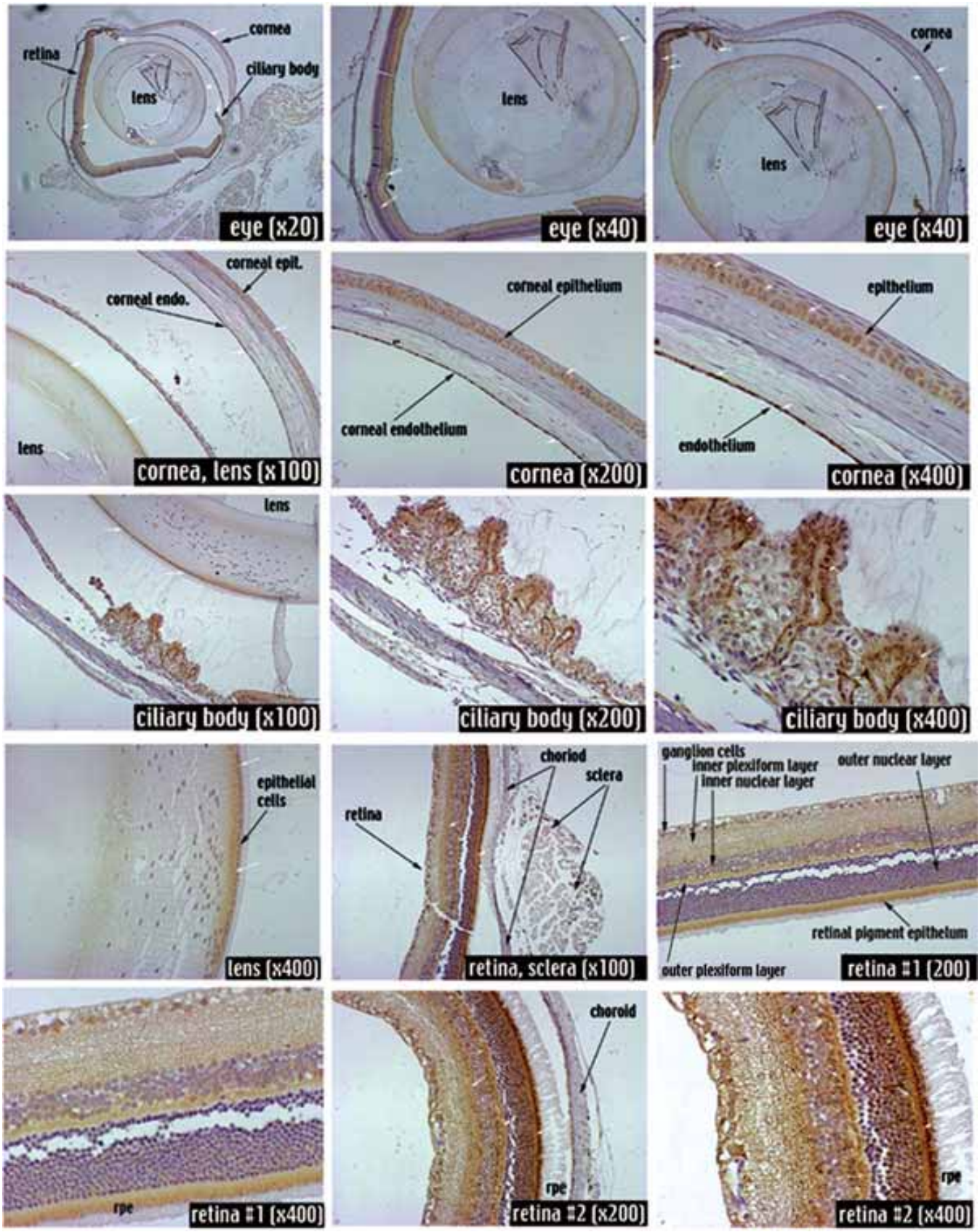

Figure 4. Immunohistochemistry analysis of Chad in mouse ocular tissues. Chad expression was detected in epithelial and endothelial layers of the cornea, the lens, the ciliary body, retinal rod and cone cells, and the plexiform layers. Positive immunostaining appears brown and is highlighted by arrows.

RESULTS

Expression of chondroadherin mRNA in ocular and non-ocular tissues: We began our study with examination of the expression pattern of Chad in human and mouse tissues using RT-PCR. Primers were designed to flank an intron and to detect a 280 bp mRNA amplicon (Figure 1, top). PCR reaction fidelity was verified by sequencing. As shown in Figure 1, Chad expression was detected at different levels in all of the 10 mouse tissues and most of the human tissues used in this study. Very high levels of mRNA were observed in mouse lung and embryo (Figure 1B), and in human cornea (Figure 1C). Chad was moderately expressed in mouse brain, heart, thymus, testis, and ovary (Figure 1B) and in human brain and skeletal muscle (Figure 1C). In human eye, in addition to the cornea, Chad mRNA was also detected in the lens and retina (Figure 1C). Chad expression was very low in mouse liver and kidney (Figure 1B), and was not detected in human iris and placenta (Figure 1C).

The expression of Chad in mouse eye was examined by ISH. Chad expression was detected in cornea, lens, and retina of postnatal day 21 mouse eyes. Digital images of sections that show these results are presented in Figure 2. The signal in corneal epithelium and endothelium, as well as in neural retina was significantly more abundant than the signal in the lens.

Expression of chondroadherin protein in mouse ocular and non-ocular tissues: Our purified polyclonal antibody RB5522 to mouse Chad recognized two strong bands of about 36 kDa and a band of about 67 kDa on western blot analysis

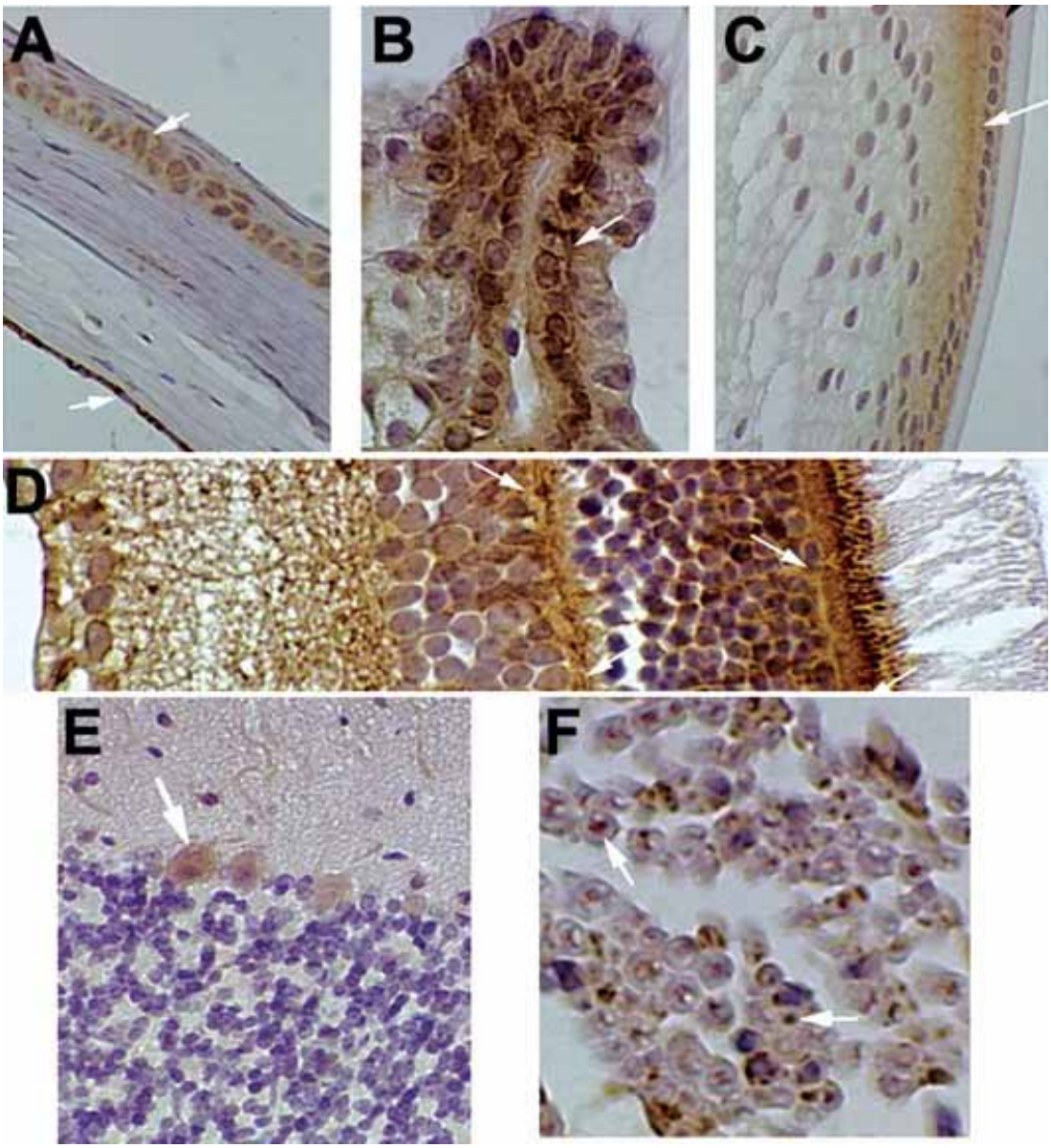

Figure 5. Higher magnification IHC micrographs of selected mouse tissues. Some mouse tissues from Figure 4 and Figure 6 were examined at higher magnification. Positive immunostaining appears brown and is highlighted by arrows. **A** Cornea. **B**: Ciliary body. **C**: Lens. **D**: Retina. **E**: Cerebellum. **F**: Peripheral nerve.

(Figure 3A). These results are consistent with previously published data showing that Chad exists in several isoforms resulting from a cleavage near the C-terminus and two sites of removal of the signal peptide [23,24,28]. The two major isoforms, differing in molecular mass by about 1 kDa, have been detected on SDS-PAGE gels as duplicate bands around 36 kDa. In addition, a band abound 67 kDa, corresponding to dimeric protein, has also been detected on SDS-PAGE gels [23,28,29]. From our western blot analysis (Figure 3A) we concluded that the antibody RB5522 recognizes bands migrating at the same level as the known isoforms of Chad. The specificity of our antibody was further confirmed by IHC

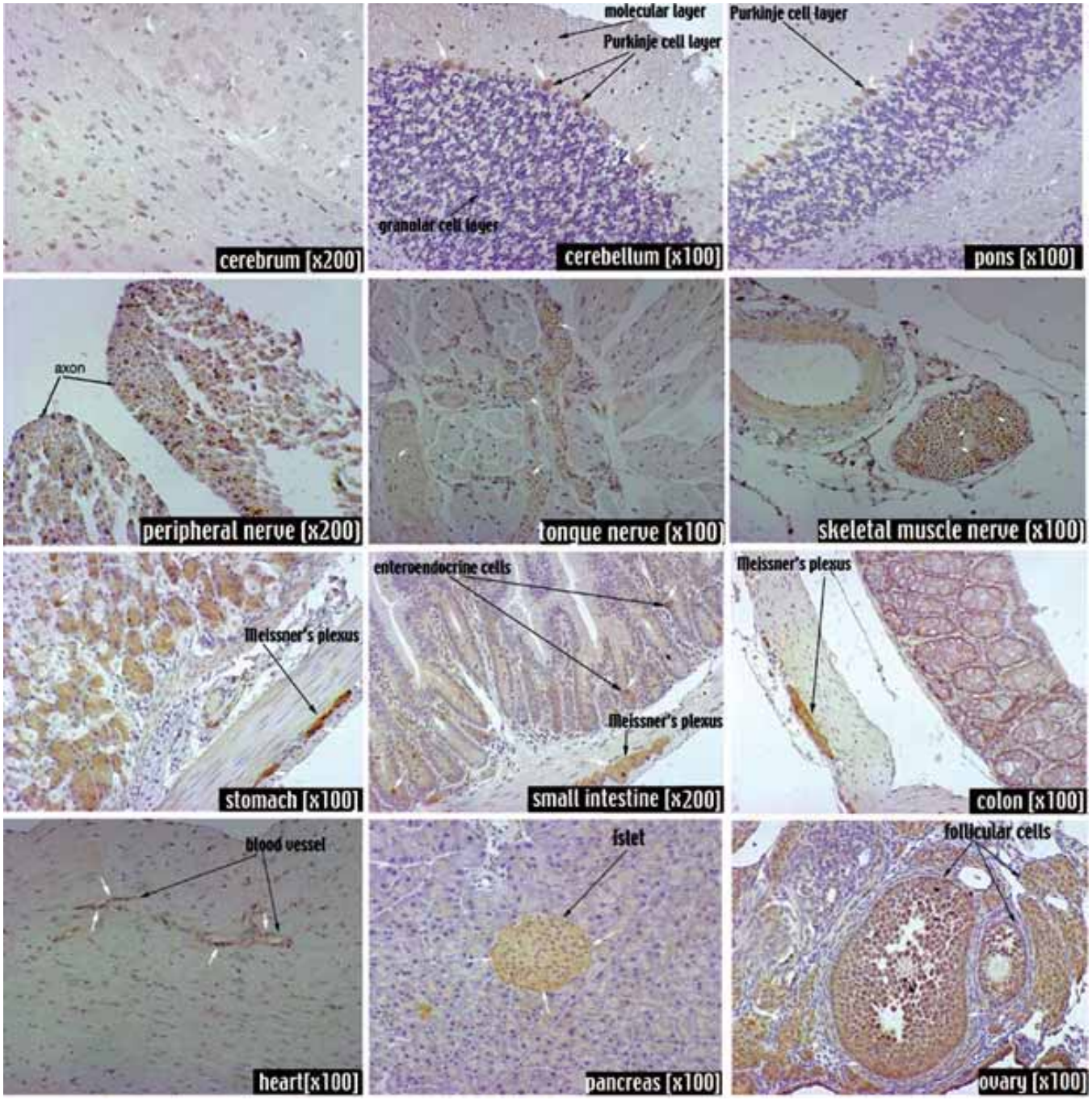

Figure 6. Immunohistochemical localization of Chad in selected tissues represented on the ZE1 tissues microarray slide. Immunoreactivity for Chad was found in Purkinje cells of the cerebellum and several peripheral nerves, including peripheral nerves in the orbit, the tongue, and skeletal muscle. In the heart, immunoreactivity for Chad was associated with blood vessels. In the gastrointestinal tract, Chad expression was localized to enteroendocrine cells of the stomach and intestine. Strong expression was also observed in Meissner's nerve plexus of the stomach, intestine, and colon. Intense immunoreactivity for Chad was also found in the cells of the pancreatic islet and follicular cells in ovary. Positive immunostaining appears brown and is highlighted by arrows.

analysis. As shown in Figure 3B, adult mouse ear lobe cartilage showed specific immunolabeling for Chad, whereas none appeared in liver and kidney. The absence of immunolabeling in liver and kidney is consistent with our RT-PCR results that show barely detectable Chad mRNA in these tissues (Figure 1).

To determine the expression of Chad in mouse ocular tissues, IHC analyses on mouse eyes were performed. In the cornea, immunolabeling of Chad revealed deposits of this protein in corneal epithelial and endothelial layers, whereas only traces of labeling were detected in the stroma, in association with individual keratocytes (Figure 4 and Figure 5). Immunolabeling also was present in the lens, ciliary body, and retina. In the retina, Chad immunolabeling was prominent in rod and cone layers, as well as in outer and inner plexiform layers. There was no Chad expression in sclera (Figure 4). The results from expression analyses of mouse eye are summarized in Table 1.

Table 1. Expression of chondroadherin in mouse ocular tissues

Tissue	Expression
Cornea	
epithelium	++
stroma	±
endothelium	+++
Iris	-
Ciliary body	+++
Lens	
epithelial cells	+
fiber cells	±
Retina	
pigment epithelium	-
rod and cone layer	+++
outer nuclear layer	-
outer plexiform layer	+++
inner nuclear layer	-
inner plexiform layer	+++
ganglion cell layer	-
Sclera	-

Summary of expression analyses for chondroadherin in mouse ocular tissues based upon RT-PCR, in situ hybridization, and immunohistochemistry. The minus sign indicates no expression by immunochistochemistry; the ± sign indicates low expression by immunohistochemistry; the single plus sign indicates moderate expression by immunochistochemistry; and the triple plus sign indicates high expression by immunohistochemsitry. The cornea, lens, and retina were all positive by PCR and by in situ hybridization, while the retina was also positive by the following entries from the Gene Expression Omnibus (GEO) profiles (Entry: GDS592|GPL1073, Experiment: Large scale analysis of the mouse transcriptiome; Entry: GDS182|GPL81, Experiment: Large scale analysis of the mouse transcriptome).

IHC analysis performed on tissue microarray was used to examine the expression of Chad in 24 mouse tissues. Representative micrographs from this study are shown in Figure 6 and at higher magnification in Figure 5. Immunoreactivity for Chad was found in Purkinje cells of the cerebellum and several peripheral nerves, including peripheral nerves in the orbit, the tongue, and skeletal muscle. In the heart, immunoreactivity for Chad was associated with blood vessels. In the gastrointestinal tract, Chad expression was localized to enteroendocrine cells of the stomach and intestine. Strong expression was also observed in Meissner's nerve plexus of the stomach, intestine, and colon (Figure 6). Intense immunoreactivity for Chad was also found in the cells of the pancreatic islet and follicular cells in ovary. Skin, kidney, and bladder were negative for Chad (not shown). Liver was also negative for Chad (Figure 3B).

The surprisingly broad expression pattern of Chad was further confirmed by searching the Gene Expression Omnibus (GEO) databases at the NCBI. The Gene Expression Omnibus is a high throughput gene expression/molecular abundance data repository. Data is gathered from NCBI's Gene Expression Omnibus gene expression and hybridization array repository. Four entries, showing that CHAD is expressed at different levels in as many as 79 physiologically normal human tissues and 61 physiologically normal mouse tissues, were retrieved from the GEO database. These entries are the following: GEO Profiles Entries GDS592|GPL1073, GDS181|GPL91, GDS596|GPL96, and GDS182|GPL81. Interestingly, these profiles show that CHAD is present in several other regions of the brain and spinal cord, as well as in whole blood samples. Retina and pancreatic islets are among the tissues included in these entries and reported to express Chad.

Taken together, the results presented here establish the expression and localization of Chad in several new locations, including mouse and human eye, peripheral nerves in skeletal muscles and orbit, blood vessels in heart, endocrine cells in pancreas, and gastrointestinal tract.

DISCUSSION

In this study we have used RT-PCR, ISH, and IHC to analyze the expression of Chad in mouse and human ocular and non-ocular tissues. In addition to confirming previous reports showing the presence of Chad in certain zones of cartilage and lens [23,24,31-33] this present work provides novel information about the broad expression of Chad in many tissues, including multiple ocular tissues. To our knowledge this is the first report demonstrating the expression and localization of Chad mRNA and protein in the cornea, retina, Purkinje cells of the cerebellum, peripheral nerves, blood vessels of various tissues, pancreatic islets, and ovary. Noteworthy, the knowledge gained from our analysis, combined with the data on Chad interactions in cartilage, will provide important information for predicting potential roles of this protein within each tissue. Two aspects of these roles should be taken into consideration. First, different cell types including chondrocytes, fibroblasts, and osteoblasts have been shown to attach to Chad

immobilized on culture dishes [36]. The interaction of chondrocytes with Chad is known to occur via integrin α2β1 receptors, a finding that suggests a role for Chad in mediating signals between the cells and the extracellular matrix [28]. A similar role for Chad is possible in other tissues. This notion is supported by the fact that integrins of the β1 class are expressed on a variety of different cell types, including neurons and endothelial cells [37]. Second, Chad also has been shown to interact with collagens type II and VI [29,30]. The collagen type II itself also interacts with cells (chondrocytes) partly via the same receptor as Chad, but these interactions give rise to different cellular responses [29]. Thus, by interacting with both collagens and cells, Chad may be an important molecule for communication between cells and their surrounding matrix, not only in cartilage but also in other tissues. In addition, a potential role of Chad in regulation of collagen fibrillogenesis in different connective tissues also should be expected since all single or double SLRP-null mice generated so far display abnormal collagen fibrillogenesis [4].

Our data suggest that Chad may be important for developing and maintaining eye tissues. It is abundant in corneal epithelium (Figure 4 and Figure 5) where type II collagen also is present during development [38,39]. Chad expression in corneal endothelium, a layer with critical role in the maintenance of corneal hydration, suggests a role in the maintenance of corneal transparency. The absence of Chad from corneal stroma, but its association with individual keratocytes, may indicate that in the cornea the function of Chad is rather related to its cell binding and cell regulatory activities than to function related to regulation of collagen fibrillogenesis. In support of this notion are reports on the carbohydrate composition of Chad, showing that it carries only a short oligosacchride [23,24], whereas the major proteoglycans of the corneal stroma usually carry long side chains of keratan sulfate and/or chondroitin/dermatan sulfate, needed for maintenance of interfibrillar spacing and normal tissue hydration [16-18]. The presence of Chad in the ciliary body may indicate a potential role for this protein in regulating the secretion of aqueous humor. Chad in the lens suggests participation in maintaining optical refraction of the lens or in filtration of substances entering or leaving lens cells. Abundant expression of Chad noted in the retina, including rod and cone layer, outer, and inner plexiform layers may reflect involvement of this protein in normal retinal physiology. Although the role of Chad in the retina remains to be determined in future studies, it may be speculated that its role is similar to the roles of other SLRPs expressed in this tissue. Thus, increased decorin expression has been shown in the inner retinal layers in transient retinal ischemia models and a role in the repair process in injured neural tissues has been suggested for this protein [40,41]. Several retinal diseases have been associated with either mutations in a SLRP family member or mutations in the gene encoding type II collagen, the type of collagen to which Chad is known to bind. Thus, nictalopin, a membrane bound member of the SLRP gene family that is expressed in different tissues, including retina, brain, muscle and kidney, has been shown mutated in patients with X-linked congenital stationary night blindness [42,43]. Mutations in the type II collagen gene have been associated with degenerative vitreo-retinal changes in mice and Stickler syndrome in man [44-46].

An intriguing result from our studies is that Chad is expressed in Purkinje cells of the cerebellum, peripheral nerves of different tissues, and Meissner's plexus in the gastrointestinal tract (Figure 6). Purkinje cells occupy the middle layer of the cerebellar cortex and carry all efferent signals from the cerebellum. These cells have control over motor activities and the function of other parts of the brain [47]. Significant down regulation of the dopamine receptor and transporter has been found in the brain of Purkinje Cell Degeneration mutant mice [48]. Meissner's plexus consists of neuronal ganglia located between the inner circular muscle layer and the submucosa of the stomach and intestines [49]. This neural network it thought to inhibit muscular contractions within the stomach, and to excite peristalsis in the intestines. The endocrine cells in the gastrointestinal tract (also positive for Chad, Figure 6) are in contact with nerve fibers of the Meissner's plexus and form the neuroendocrine complex of the gastrointestinal tract. Changes in this complex have been found in different gastrointestinal diseases [50]. Considering the knowledge above about the function of cells and tissues where Chad is present it will be important to determine what roles Chad may play in their physiology.

A very interesting result from our studies is that Chad is expressed strongly in pancreatic islets (Figure 6). Given the important role of the extracellular matrices in pancreatic endocrine cell function, particularly their utility for generating a bioartificial endocrine pancreas that can be transplanted as a treatment for diabetes [51-53], our results, by showing the presence of Chad in the normal pancreatic islet, provide significant new information. Presently, it is unclear what cell type produces Chad in this tissue. Pancreatic islets of Langerhans contain three major cell types; alpha cells producing the hormone glucagons, beta cells producing insulin, and delta cells producing the hormone somatostatin. Beta cells occupy the central portion of the islet and are surrounded by a ring of alpha and delta cells. The immunolabeling of Chad appears positive for the entire islet (Figure 6). However, the cell type producing Chad, the role of this protein in pancreatic islet, and its potential involvement in regulating glucose metabolism remain to be determined in future studies. We should note that lumican, another member of the SLRPs family, also has been detected in pancreatic islets [54].

In conclusion, this report provides a basis for future studies aimed at addressing the role of Chad in health and disease, including ocular diseases, diabetes mellitus, gastrointestinal, and neuromuscular diseases.

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