Identification, cDNA cloning, and expression analysis of dermatopontin in the goldfish *Carassius auratus*

Norihiko Komatsu · Nobuhiro Ogawa · Kurin Iimura · Kazuhiro Ura · Yasuaki Takagi

Abstract Fish scales are a potential source of collagen for fabricating scaffolds for cells during tissue engineering because fish collagen has a low risk of zoonosis. Since the assembly of collagen fibrils has a significant impact on the functionality of the scaffold, the ability to replicate the fibril assembly of human tissues is critical. To determine the mechanism of fish collagen fibril assembly, we first identified non-collagenous proteins (NCPs), the potential regulators of fibril assembly in vivo, and then used tandem mass spectrometry to analyze the NCPs contained in the basal plates of goldfish *Carassius auratus* scales, a collagenous plate which is characterized by a plywood-like assembly of collagen fibrils similar to that found in the cornea. We identified a 19-kDa acidic protein as dermatopontin, the NCP which is a possible regulator of fibril assembly in the mammalian cornea. We cloned a goldfish dermatopontin cDNA of 1,074 bp containing an open reading frame encoding 196 amino acids. Reverse transcription-PCR revealed that dermatopontin mRNA was expressed in a wide range of tissues, including scale, skin, fin, eye, and skeletal muscle. In situ hybridization revealed that dermatopontin mRNA was expressed primarily in the basal plate-producing hyposquamal scleroblasts of the scales, suggesting that the dermatopontin is linked to the collagen fibril assembly of the basal plate.

Keywords Biglycan · Collagen assembly · Dermatopontin · Lysyl oxidase · MS/MS analysis · Scale · Teleost

N. Komatsu · N. Ogawa · K. Iimura · K. Ura · Y. Takagi (✉)
Graduate School of Fisheries Sciences, Hokkaido University,
Minato-cho 3-1-1, Hakodate, Hokkaido 041-8611, Japan
e-mail: takagi@fish.hokudai.ac.jp

Present Address:
N. Komatsu
Saku Branch, Nagano Prefectural Fisheries Experimental
Station, Takayagi 282, Saku, Nagano 385-0042, Japan

Present Address:
N. Ogawa
Atmosphere and Ocean Research Institute, The University of
Tokyo, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8564, Japan

Present Address:
K. Iimura
Department of Applied Biological Chemistry, Graduate School
of Agricultural and Life Sciences, The University of Tokyo,
Yayoi 1-1-1, Bunkyo, Tokyo 113-8657, Japan

Introduction

The exploitation of seafood by-products is an important component of improving the sustainability of the seafood industry. Our research is currently focused on the fabrication of a cellular scaffold for tissue engineering applications using fish type I collagen [1], which can be extracted from fish offal (e.g., skin, scales, and fins).

Collagen is the primary component of the extracellular matrix (ECM) in an animal's body and functions not only as a structural support for tissues, but also as an adhesive substrate for cells and a fundamental regulator of cellular growth and differentiation [2–5]. Because of these properties, collagen has been widely used as a scaffold to support cellular growth and differentiation in both research and tissue engineering [6]. To date, the primary source of medical collagen has been domestic animals (pig and cattle). Recently, however, fish collagen has attracted increased attention [7] as it has a low risk of contamination with diseases that are infectious to human beings, such as bovine spongiform encephalopathy. Additionally, the use

of fish collagen raises fewer religious objections than animal collagen.

Collagen-based scaffolds, which have a three-dimensional assembly of collagen fibrils that mirror the structure in human tissues, are generally believed to have improved functionality for tissue engineering applications. The underlying rationale for this notion is that tissue function in humans is dependent on the tissue-specific three-dimensional assembly of collagen fibrils. In the corneal stroma, for example, multiple layers of type I collagen fibrils are stacked, and the fibrils in one layer are aligned in parallel, whereas those in different layers are aligned perpendicular, resulting in a plywood-like structure [8]. This structure provides tensile strength as well as transparency to the tissue. Thus, to fabricate a functional cornea substitute using collagen, researchers must replicate this complicated collagen fibril assembly. Due to the difficulty associated with artificial regulation of collagen fibril assembly using industrial techniques, technology derived from cellular mechanisms (bio-inspired technology) is gaining increased attention [1].

Previous studies have revealed that non-collagenous proteins (NCPs) secreted from ECM-producing cells play an important role in the regulation of collagen fibril assembly in animal tissues. For example, knock-out of dermatopontin, one of the NCPs contained in the corneal stroma of mammals, results in wider spacing between collagen fibrils (i.e., fewer collagen fibrils per unit area of the section) and disrupts fibrillar organization in the cornea [9]. However, the mechanisms controlling fish collagen fibril assembly are poorly understood, and there has been no documentation of NCPs associated with collagen fibril structure to date.

The basal plate of teleost fish scales represents an interesting model to study the cellular mechanisms of the three-dimensional assembly of fish collagen fibrils. The basal plate is formed of type I collagen fibrils assembled in the plywood-like manner characteristic of fibril assembly in the cornea [1]. The objective of our study was to isolate and identify NCPs from the basal plates of the goldfish *Carassius auratus* scales. To this end, we acid-extracted the ECM from the basal plates and conducted time-of-flight tandem mass spectrometry (TOF-MS/MS). We performed cDNA cloning, mRNA expression analysis by real time (RT)-PCR, and in situ hybridization of the goldfish dermatopontin, one of the NCPs identified by MS/MS.

Materials and methods

Fish and rearing

Goldfish (approx. fork length 15 cm) were purchased from a commercial dealer. The fish were maintained in aerated tap water at 25 °C and fed a commercial diet (4C; Nippon Formula Feed Mfg. Co., Ltd., Yokohama, Japan).

Protein extraction from basal plates of fish scales

After deeply anesthetizing the fish in a 0.1 % solution of 2-phenoxyethanol, we removed approximately 700 scales from five fish using forceps. Lateral-line scales were not included. The scales were then washed overnight in a series of cold (4 °C) distilled water baths (with stirring) to remove cellular debris, following which the basal plates were removed using forceps and a scalpel. The wet weight of the pooled basal plates was recorded to two decimal points.

To obtain NCPs, we extracted the basal plate with 10 volumes (v/w) of 0.5 M acetic acid under gentle rotation at 4 °C for 1 day. As acetic acid is commonly used to extract native collagen molecules from tissues, we assumed that the collagen molecules with associated NCPs were extracted by this method. Following extraction, the samples were centrifuged at $2,000 \times g$ for 10 min at 4 °C, and both the supernatant and precipitate were collected. The supernatant was then centrifuged at $21,500 \times g$ for 10 min at 4 °C and stored on ice. The precipitate from the first centrifugation was re-extracted as described above, centrifuged twice, and the final supernatant was mixed with the original supernatant and stored on ice. The protein content of the mixed supernatant was quantified using a Micro BCA^{TM} Protein Assay kit (Pierce, Rockford, IL).

Two-dimensional gel electrophoresis

To characterize the protein profile of the supernatant, we performed two-dimensional gel electrophoresis (2D-E) using the Ettan IPGphor 3 IEF System (GE Healthcare Biosciences, Little Chalfont, UK) for isoelectric electrophoresis (p*I* range 3–10) and a slab electrophoresis chamber (Atto, Tokyo, Japan) for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 15 % separating gel), in accordance with the manufacturers' protocols. A total of 600 μg of proteins were separated and detected by silver staining.

Identification of proteins by MS/MS

For the MS/MS analysis, the protein spots separated by 2-DE were excised and processed according to the manufacturer's protocol (Applied Biosystems, Foster City, CA) with a slight modification: a trypsin enhancer (Promega, Madison, WI) was added to enhance trypsin digestion of the sample. The digested peptides were purified using a ZipTip (Millipore, Billerica, MA) and then analyzed using

the MALDI TOF-TOF model 4700 Proteomics Analyzer (Applied Biosystems). The tryptic peptide mass fingerprinting and MS/MS spectra obtained were used for a Mascot search [10] in the Actinopterygii protein database. Proteins were judged to be identified when the amino acid sequences of at least two peptides were significantly ($P < 0.05$) matched with those in the database.

cDNA cloning of goldfish dermatopontin

Goldfish were anesthetized in a 0.1 % 2-phenoxyethanol solution, and 30 scales were removed from the left flank. The lateral line scales were not used. Total RNA was isolated from the scales using ISOGEN (Nippon gene, Tokyo, Japan) and a FastPure^{TM} RNA kit (Takara, Otsu, Japan). After DNase I (Takara) treatment, first-strand cDNA from 500 µg of total RNA was synthesized using a PrimeScript^{TM} RT reagent kit (Perfect Real Time; Takara). First-strand cDNA was synthesized from 500 µg of total RNA by 5′ and 3′ RACE (rapid amplification of cDNA ends) using a SMART^{TM} RACE cDNA Amplification kit (Clontech, Palo Alto, CA).

The goldfish dermatopontin fragment was obtained by first performing PCR using the primers 5′-ccccgctctgttga tatcatcccaccagc-3′ (forward) and 5′-gccctatagtgagtcgtattag-3′ (reverse), which were designed based on zebrafish *Danio rerio* sequence data (GenBank accession no. NM_001030085). After the partial sequences had been identified, 3′ RACE was performed using the gene-specific primer 5′-aactggaagaattcgcggcc-3′ and adopter primer 5′-aactggaagaattcgcggcc-3′. Following the first 3′ RACE PCR, a nested PCR was performed using the gene-specific primer 5′-tcgcggccgcaggaa-3′ and adopter primer 5′-tcgcg gccgcaggaa-3′. Then, 5′ RACE was performed using the mixed adopter primers 5′-ctaatacgactcactatagggcaagcag tggtat-3′ and 5′-ctaatacgactcactatagggc-3′ and the gene-specific primer 5′-gccctatagtgagtcgtattag-3′.

The amplified cDNAs were ligated into a pGEM-T Easy vector (Promega), and XL-1 Blue was transformed. Nucleotide sequences were analyzed with a Big-Dye Terminator v3.1 Cycle Sequencing kit using an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). Multiple amino acid sequence alignments were produced with the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

Expression analyses of goldfish dermatopontin mRNA

The tissue distribution of dermatopontin mRNA expression was examined using reverse transcription-PCR (RT-PCR). Goldfish were deeply anesthetized using a 0.1 % 2-phenoxyethanol solution and then decapitated. Each animal was dissected, and the brain, caudal fin, eye, heart, kidney, liver, scales, skeletal muscle, skin (without scales), swim bladder, and blood cells were collected. Each scale was cut in half with scissors, and the anterior half (lacking epithelial tissue) was used in subsequent analyses. Total RNA was extracted using ISOGEN and a FastPure^{TM} RNA kit (Takara). After DNase I (Takara) treatment, first-strand cDNA was synthesized from 91.5–100 µg of total RNA using a PrimeScript^{TM} RT Reagent kit (Perfect Real Time; Takara). The primers used for PCR [5′-gacttcaggatgagctcagca-3′ (forward) and 5′-accafcaacaagccc attg-3′ (reverse)] were designed based on the cDNA data obtained in this study. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal standard. The primers for *gapdh* [5′-caagccatctctgtgttccag-3′ (forward) and 5′-accagttgaagcagggatga-3′ (reverse)] were designed from sequence data in GenBank (GenBank accession no. AY641443).

In situ hybridization was performed to localize dermatopontin mRNA expression in the scale. A 504-bp fragment (nt 488–991) of dermatopontin cDNA was amplified by PCR using the primers 5′-ccagttacggctacttcataa-3′ (forward) and 5′-gtcttgttactccatctgag-3′ (reverse) and ligated into a pGEM-T Easy vector (Promega). The PCR product in the pGEM-T Easy vector was further amplified with M13 primers and used as a template to synthesize digoxigenin-labeled probes with T7 and SP6 RNA polymerase (Roche, Mannheim, Germany).

The scale-forming cells (scleroblasts) of the ontogenetic scales in goldfish, and particularly the hyposquamal scleroblasts which produce the basal plate, are flat and small [11], leading us to predict that the hybridization signal would be difficult to detect. To improve the likelihood of detection, we used regenerating scales in which the scleroblasts were activated and therefore relatively larger than those in ontogenetic scales [11]. Individual goldfish were anesthetized in a solution of 0.1 % 2-phenoxyethanol and 30 scales removed from the left flank. The lateral line scales were not used. After 7 days, the skin containing the regenerating scales was dissected and fixed in 10 % formalin solution overnight at 4 °C. After fixation, the samples were decalcified in Morse's solution (10 % sodium citrate, 22.5 % formic acid) [12] for 1 day at 4 °C. The samples were then dehydrated using conventional procedures and embedded in TissuePrep (Fisher Scientific, Fair Lawn, NJ); and 6-µm sections were cut frontally. After deparaffinization and rehydration, the sections were treated with 1.0 µg/ml proteinase K in phosphate buffered saline (pH 7.4) for 15 min at 37 °C and then hybridized with the probes at 42 °C overnight. The sections were washed twice with 2× SSC and then twice with 0.1× SSC at 50 °C for 30 min. The signal was visualized with alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche) and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate solution (Roche).
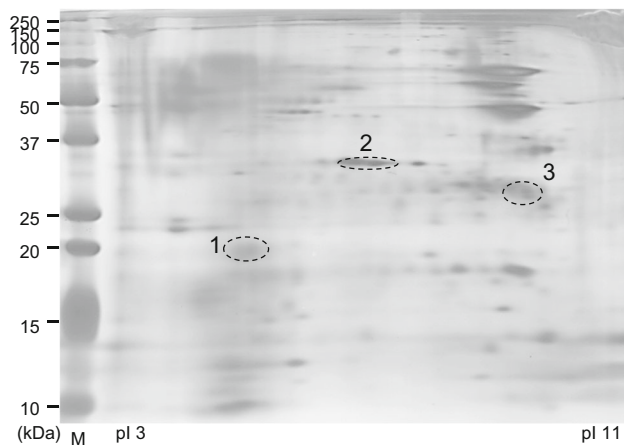
Fig. 1 Two-dimensional gel electrophoresis profile of an acetic acid extract from the basal plates of goldfish *Carassius auratus* scales. The gel was stained with silver. Spots *encircled with a dotted line* were identified by tandem mass spectrometry (MS/MS) analysis, *M* marker, *pI* isoelectric point

Results

Identification of proteins by MS/MS

More than 100 spots with molecular weights of 10–100 kDa were detected by 2D-E (Fig. 1). The acidic spots, with molecular weights of 37–75 and <20 kDa, respectively, were smeared. Clear spots with molecular weights of 35–25 kDa were observed in the neutral region. A number of clear spots with molecular weights of 10–75 kDa were observed in the alkaline region.

Twenty-five spots from the 2-DE gel were extracted for MS/MS analysis, but we succeeded in identifying only three proteins, namely, dermatopontin, lysyl oxidase, and biglycan (spots #1, #2, and #3 in Fig. 1), as described below.

In a Mascot search [10] for spot #1, the amino acid sequences of two peptides, (R)QGFNFQCPHGEVLVAVR(S) and (K)YFEAVLDREWQFYCCR(Y), matched that of zebrafish dermatopontin (GenBank accession no. NM_001030085). In a Mascot search for spot #2, the amino acid sequences of two peptides, (K)NQGTADFLPSRPR(Y) and (R)VKNQGTADFLPSRPR(Y), matched that of Atlantic salmon *Salmo salar* lysyl oxidase (GenBank accession no. DQ167812). Similarly, from spot #3, the amino acid sequences of three peptides, (R)HIEHGALSYLTNLR(E), (K)VFYNGISLFDNPIR(Y), and (R)YWEVQPSTFR(C), matched that of zebrafish biglycan (GenBank accession no. NM_001002227).

cDNA cloning and mRNA expression analyses of goldfish dermatopontin

Dermatopontin is closely linked to collagen fibrillogenesis as well as the organization of collagen fibrils in mammals [9, 13, 14]. It should also be noted that at the time this study was initiated, knowledge of fish dermatopontin was limited to a single zebrafish dermatopontin gene on the database, which was predicted to yield a protein. Therefore, we targeted dermatopontin for further analysis and conducted cDNA cloning studies of this protein.

The cloned goldfish dermatopontin cDNA had 1,074 bp (DDBJ, accession no. AB576186) and contained an open reading frame encoding 196 amino acids. The alignments of an amino acid sequence predicted from goldfish dermatopontin cDNA (obtained in this study) with the sequences of zebrafish, *Xenopus laevis*, chicken *Gallus gallus domesticus*, and human *Homo sapiens* (obtained from databases) are shown in Fig. 2. Data for zebrafish dermatopontin from cloned cDNA, which was uploaded from our laboratory during this study [15], are shown in Fig. 2. The two amino acid sequences which were found to match with zebrafish dermatopontin by MS/MS analysis were found in the goldfish sequence identified in our study (Fig. 2). However, one valine in the zebrafish sequence (QGFNFQCPHGEVLVA**V**R) was substituted with isoleucine (QGFNFQCPHGEVLVA**I**R) in goldfish. The predicted molecular weight of goldfish dermatopontin, excluding signal peptides (21 amino acids inferred by SignalP 3.0 Server; http://www.cbs.dtu.dk/services/SignalP-3.0/), was 21.0 kDa.

The amino acid sequence of goldfish dermatopontin had 88 % homology with that of zebrafish, 62 % with *Xenopus*, 57 % with chicken, and 56 % with human. All ten cysteine residues observed in mammalian dermatopontin were conserved in goldfish dermatopontin.

Mammalian dermatopontins have three characteristic motifs [16, 17]. The first is a sequence of six amino acids, DRE/QWXF/Y (where X is any amino acid). Similar sequences at appropriate sites were all conserved in goldfish dermatopontin (Fig. 2). The second motif is the integrin-binding RGAT sequence and the third is a consensus sequence NYD that is observed in many amine oxidases; however, these motifs were not conserved in either goldfish or zebrafish dermatopontin (Fig. 2).

RT-PCR analysis revealed that dermatopontin was expressed in all the tissues examined in this study (Fig. 3).

The frontal sections of goldfish skin that contained scales regenerated in 7 days are illustrated in Fig. 4. Three types of scale-forming scleroblasts, i.e., flat episquamal, square hyposquamal, and oval- to droplet-shaped marginal scleroblasts, were attached onto the scale matrix (Fig. 4a). In situ hybridization revealed that dermatopontin mRNA was primarily expressed in the hyposquamal scleroblasts (Fig. 4b). No positive reaction was detected when the sense probe was used (data not shown).

```
goldfish.nuc    1:-----MSSAHVLQALCLLMVFSTASAQHGYMYETGEEWVNTWRQGFNFQCPHGEVLVAIR 55
zebrafish.nuc   1:-----------------MLFSMTTAQQGYMHESGEDWVNAWRQGFNFQCPHGEVLVAVR 42
xenopus.nuc     1:-MMLLLALMLHLVAVTWAQY-DYYYSQHDYSP--DDQWVNLFRQGFNFQCPHGQVVVAIR 56
chicken.nuc     1:MDLVLLWVFLPLVSVAWGQYGDYYYGPYNYGD--NDEWANVYRXGFNFQCPHGQVIVAIR 58
human.nuc       1:MDLSLLWVLLPLVTMAWGQYGDYGYPYQQYHDYSDDGWVNLNRQGFSYQCPQGQVIVAVR 60
                                            ..    .. . ..  . .*      . *.*  *.*.**..***.*.* **.*

goldfish.nuc   56:SYFSEKEGSDRLWNFECQKTPYDWGEPNECWWDDINRAGLEWSSVCTNNGLVAGVQSKYF 115
zebrafish.nuc  43:SYFSEKEGSDRLWSFECQRTPYGWGEPSECWWDDINRAGMEWSSVCSNNGLVAGVQSKYF 102
xenopus.nuc    57:STYSKKEGSDRQWNYGCMPTPHELGEPTECWWEEINRAGMEWYQTCSNNGLVAGFQSQYF 116
chicken.nuc    59:SVFSKKEGSDRLWNYACMPAAQSLGEPTECWWEEINRAGTEWYQTCSNNGLVAGFQSQYF 118
human.nuc      61:SIFSKKEGSDRQWNYACMPTPQSLGEPTECWWEEINRAGMEWYQTCSNNGLVAGFQSRYF 120
                  * .*.****** **   *.        *.. .***.*.*.*  .****** ** ** **

goldfish.nuc  116:EAVLDREWQFYCCRYARRCPYSCWKTNDVPEYHKEEGEMVVPSYGYFIRGAQTTFSGVLR 175
zebrafish.nuc 103:EAVLDREWQFYCCRYAQRCPYSCWKTYDVPEYYREEGEMVVPSYGYFIRGAQTTFSGVLR 162
xenopus.nuc   117:EAVLDREWQFYCCRYSKRCSYSCWLTTEYPGHYGEESDMVLYSYGYYIRGATTTFSGVER 176
chicken.nuc   119:PSVLDREWQFYCCRYSRRCPYSCWLTTEYPGHYGEDMDMMMYTYDYYIRGATTTFSAVDR 178
human.nuc     121:ESVLDREWQFYCCRYSKRCPYSCWLTTEYPGHYGEEMDMISYNYDYYIRGATTTFSAVER 180
                  **********.***.*....*....*. .* ..*.*.****.****.* *

goldfish.nuc  176:DRQWKYIICRMTDFDCQFENF 196
zebrafish.nuc 163:DRQWKYIICRMTDFDCQFQNY 183
xenopus.nuc   177:DRQWKYIICRMTDFNCEFENF 197
chicken.nuc   179:DRQWKFIVCRMTEYDCPFQNV 199
human.nuc     181:DRQWKFIMCRMTEYDCEFANV 201
                  *****.*.****...* * *
```

Fig. 2 Alignments of amino acid sequences of dermatopontin of goldfish, zebrafish, *Xenopus*, chicken, and human. *Black box* sequences matched with zebrafish dermatopontin by MS/MS analysis, *open box* sequences of six amino acids (D-R-E/Q-W-X-F/Y, where X is any amino acid), *dotted box* cysteine residues, *underline* integrin-binding R-G-A-T sequences, *dotted underline* N-Y-D sequences, *asterisk* conserved amino acids, *dot symbol* amino acid similarity
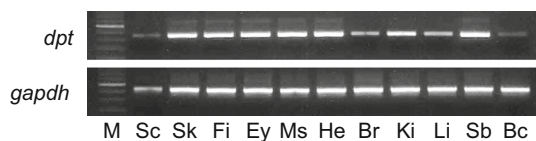

Fig. 3 Tissue distribution of goldfish dermatopontin (*dpt*) mRNA expression based on reverse transcription-PCR. *gapdh* Glyceraldehyde 3-phosphate dehydrogenase, *M* marker, *Sc* scale, *Sk* skin, *Fi* caudal fin, *Ey* eye, *Ms* skeletal muscle, *He* heart, *Br* brain, *Ki* kidney, *Li* liver, *Sb* swim bladder, *Bc* blood cells

Discussion

This is the first study to apply MS/MS analysis to identify NCPs contained in teleost fish scales. Although the identification efficiency was low, a protein having an apparent molecular weight of 19 kDa was identified as dermatopontin. Dermatopontin, formerly known as tyrosine-rich acidic matrix protein (TRAMP) [18], was first purified from bovine skin [19]. It is an acidic protein (isoelectric point: approx. 4.2) composed of 183 amino acids with an apparent molecular weight, as determined by SDS-PAGE, of 22 kDa. Its N-terminal region is rich in tyrosine residues, many of which are sulfated [18, 20]. The apparent molecular weight and isoelectric point (approx. 5.0) of the goldfish dermatopontin identified in our study largely corresponded with those of bovine dermatopontin. We also cloned and sequenced a dermatopontin homolog from the cDNA of goldfish scales and found that it had 175 amino

acids in the mature form (predicted molecular weight 21 kDa). Thus, the apparent molecular weight of goldfish dermatopontin as determined by SDS-PAGE in our study (19 kDa) was smaller than the predicted weight of the mature form, and at this time we are unable to explain this difference. Purification and amino acid sequence analysis of the protein will further clarify the mature form of dermatopontin in goldfish tissues. Analysis of the deduced amino acid sequence also revealed that all ten cysteine residues conserved among mammalian dermatopontins were also conserved in goldfish dermatopontin, suggesting that the latter shares a similar 3D conformation to mammalian dermatopontins. Mammalian dermatopontins have five loop structures composed of five intramolecular disulfide bonds [16].

Dermatopontin is closely linked to collagen fibrillogenesis and the organization of collagen fibrils in mammals. In one study, dermatopontin purified from porcine skin was found to accelerate type I collagen fibrillogenesis in vitro, with the synthesized fibrils being thinner and having a narrower range of diameters in the presence of dermatopontin than in its absence [13]. In another study, dermatopontin-null mice exhibited a decreased thickness of the dermis, lower skin collagen content, and irregularly contoured collagen fibrils with a larger diameter than wild-type mice [14]. The authors also observed that the range of collagen fibril diameters was greater in the dermis of dermatopontin-null mice than in the wild-type mice. Cooper et al. [9] also reported that the corneal stroma of
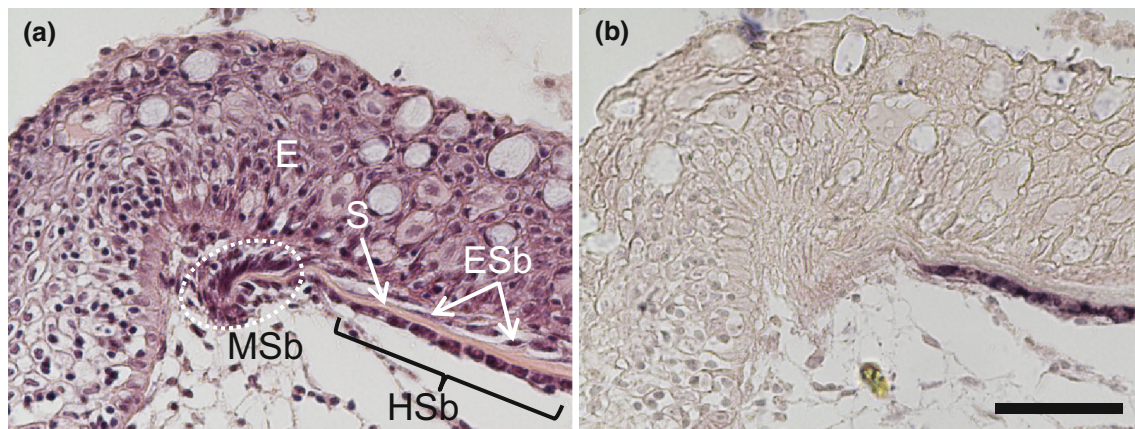
Fig. 4 In situ hybridization analysis of goldfish dermatopontin mRNA expression in a 7-day regenerated scale. **a** Hematoxylin and eosin-stained section, **b** antisense-probe reacted in situ hybridization section. *E* Epithelium, *ESb* episquamal scleroblasts, *HSb* hyposquamal scleroblasts, *MSb* marginal scleroblasts, *S* scale. *Scale bar* 50 μm

dermatopontin-null mice had larger spacing between collagen fibrils and disrupted fibrillar organization. Because the expression of goldfish dermatopontin mRNA in the regenerating scales was primarily detected in the hyposquamal scleroblasts, which are responsible for the production of the basal plate, the data obtained in mammals to date strongly suggest that dermatopontin regulates collagen fibrillogenesis and the formation of the cornea-like plywood-like collagen-fibril assembly of the basal plate. However, there remains a need for further analysis on the function of dermatopontin during collagen assembly in the basal plate.

Our results also demonstrate that goldfish dermatopontin mRNA expression is not specific to scales. We detected a positive signal in a wide range of tissues, including the skin, fin, eye, skeletal muscle, heart, brain, kidney, liver, swim bladder, and blood cells. Dermatopontin in mammals and zebrafish is also distributed throughout a wide range of tissues [15, 16, 20, 21], suggesting that it has multiple functions. Okamoto and Fujiwara [16] suggested that dermatopontin has two additional functions, other than regulating collagen fibrillogenesis. These include promotion of cell adhesion and fine tuning of activity of transforming growth factor beta, a cytokine playing a central role in the healing of wounds in the skin [22]. Recent research suggests that dermatopontin promotes epidermal keratinocyte adhesion via α3β1 integrin and a proteoglycan receptor (likely to be syndecan) [23]. Although the authors of that study did not elucidate the recognition sequence of α3β1 integrin in dermatopontin, they were able to identify the binding site of the proteoglycan receptor as an eight amino acid sequence, GQVVAVR [23]. A similar sequence has also been observed in goldfish and zebrafish dermatopontins, but substitutions of some amino acids were observed [GEVLVAIR (goldfish residues 48–55) and GEVLVAVR

(zebrafish residues 35–42); Fig. 2]. Moreover, the predicted integrin-binding site sequence (RGAT) conserved in mammalian dermatopontins was not conserved in goldfish and zebrafish (Fig. 2). Thus, the cell-adhesion activity of dermatopontin should be studied carefully in fish.

Our MS/MS analysis also identified lysyl oxidase and biglycan. Lysyl oxidase is an enzyme secreted into the ECM which oxidizes lysine residues in collagen and initiates formation of covalent cross-linkages that stabilize collagen fibrils [24, 25]. Thus, lysyl oxidase activity is closely related to the strength of the fibrils and, hence, of the tissue. Lysyl oxidase in the basal plates of goldfish scales may also increase the strength of the plates by promoting cross-linking of the collagen fibrils. Biglycan is a member of the Class I small leucine-rich proteoglycans (SLRPs) [26]. Corsi et al. [27] observed that in the tendon tissue of biglycan-null mice, the number of fibrils with a larger diameter decreased and the number of those with a smaller diameter increased. However, they also reported that unusually large fibrils also occurred in these mice. In cornea tissue, biglycan has a supportive role for decorin, another Class I SLRP that regulates collagen fibrillogenesis in this tissue. Double knock-out mice lacking both decorin and biglycan exhibit abnormally thick collagen fibrils with an irregular contour, as well as disruption in fibril packing and lamellar organization, but single knockout mice lacking either decorin or biglycan do not exhibit significant changes in corneal collagen fibrils [28]. In this study, a compensatory upregulation of biglycan gene expression was observed in decorin-deficient mice, but not the reverse, suggesting that decorin plays a primary role in regulating fibril assembly in the cornea and that biglycan finely tunes the function of decorin [28]. Although decorin was not detected in our study, it is possible that biglycan together with decorin plays an important role in the regulation of

collagen fibrillogenesis in the basal plates of goldfish scales.

With the aim to obtain collagen-associated NCPs from the basal plates of the scales, we used a 0.5 M acetic acid solution as an extraction solution because this acidic solution is the most commonly used method to extract collagen molecules. However, a broader spectrum of proteins in the basal plates appeared in the extract, as evidenced by the many spots obtained by 2D-E analysis. On the other hand, the extraction method may be incomplete as a large part of the basal plate matrix was discarded as precipitates of the extraction in our study. The solution containing protein denaturation agents, such as urea, guanidine-HCl or CHAPS, may be more efficient. For example, Jiang et al. [29] showed the need for sequential extraction of dog bone for complete proteome analysis. In their study, they sequentially used a 1.2 M HCl solution, a 100 mM Tris buffer (pH 7.4) solution with 6 M guanidine-HCl, the same buffer solution containing 6 M guanidine-HCl and 0.5 M tetrasodium EDTA, and a 6 M HCl solution for extraction. These authors reported that only 0.77 mg protein out of 3.77 mg of total proteins obtained by the sequential extraction was extracted by the first acid extraction. However, the acid extract contained most of the bone-specific matrix proteins, suggesting the acid extraction is the efficient method for the purpose of our study.

Zebrafish is the most frequently used model teleost in recent biomedical research [30], but we used goldfish scales to identify NCPs of the basal plate because the goldfish has a much larger body size and scale size than zebrafish. The bigger scale size facilitates the collection of basal plate samples, and the larger body size may also be beneficial in future research. It may be possible to purify both collagen and dermatopontin from goldfish and to conduct in vitro collagen-fibrillogenesis experiments to clarify the function of goldfish dermatopontin. In the porcine model, dermatopontin was reported to accelerate collagen fibrillogenesis, but the fibrils produced in its presence were thinner than those produced in its absence [13]. Another benefit to using goldfish is that goldfish and zebrafish are both in the family Cyprinidae; zebrafish genome data can therefore be used, at least in part, in the MS/MS analysis of goldfish data. In fact, the peptides from two identified proteins matched that of zebrafish data in our MS/MS analysis. The PCR primers designed based on zebrafish dermatopontin also worked well. Therefore, we believe that the goldfish is a suitable species for the purpose of this study.

References

1. Takagi Y, Ura K (2007) Teleost fish scales: a unique biological model for the fabrication of materials for corneal strama regeneration. J Nanosci Nanotech 7:757–762
2. Schnaper HW, Kleinman HK (1993) Regulation of cell function by extracellular matrix. Pediatr Nephrol 7:96–104
3. Streuli C (1999) Extracellular matrix remodelling and cellular differentiation. Curr Opin Cell Biol 11:634–640
4. Kresse H, Schönherr E (2001) Proteoglycans of the extracellular matrix and growth control. J Cell Physiol 189:266–274
5. Marastoni S, Ligresti G, Lorenzon E, Colombati A, Mongiat M (2008) Extracellular matrix: a matter of life and death. Connect Tissue Res 49:203–206
6. Parenteau-Bareil R, Gauvin R, Berthod F (2010) Collagen-based biomaterials for tissue engineering applications. Materials 3:1863–1887
7. Yamada S, Yamamoto K, Ikeda T, Yanagiguchi K, Hayashi Y (2014) Potency of fish collagen as a scaffold for regenerative medicine. BioMed Res Int. doi:10.1155/2014/302932
8. Parfitt GJ, Pinali C, Young RD, Quantock AJ, Knupp C (2010) Three-dimensional reconstruction of collagen-proteglycan interactions in the mouse corneal stroma by electron tomography. J Struct Biol 170:392–397
9. Cooper LJ, Bentley AJ, Nieduszynski IA, Talabani S, Thomson A, Utani A, Shinkai H, Fullwood NJ, Beown GM (2006) The role of dermatopontin in the stromal organization of the cornea. Invest Ophthalmol Visual Sci 47:3303–3310
10. Perkins DN, Pappin DJ, Creasy DM, Cottrell JS (1999) Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 20:3551–3567
11. Ohira Y, Shimizu M, Ura K, Takagi Y (2007) Scale regeneration and calcification in the goldfish Carassius auratus: quantitative and morphological process. Fish Sci 73:46–54
12. Morse A (1945) Formic acid-sodium citrate decalcification and butyl alcohol dehydration of teeth and bones for sectioning in paraffin. J Dental Res 24:143–153
13. MacBeath JRE, Shackleton DR, Hulmes DJS (1993) Tyrosine-rich acidic matrix protein (TRAMP) accelerates collagen fibril formation in vitro. J Biol Chem 268:19826–19832
14. Takeda U, Utani A, Wu J, Adachi E, Koseki H, Taniguchi M, Matsumoto T, Ohashi T, Sato M, Shinkai H (2002) Targeted disruption of dermatopontin causes abnormal collagen fibrillogenesis. J Invest Dermatol 119:678–683
15. Tan Y, Iimura K, Sato T, Ura K, Takagi Y (2013) Spatiotemporal expression of the dermatopontin gene in zebrafish Danio rerio. Gene 516:277–284
16. Okamoto O, Fujiwara S (2006) Dermatopontin, a novel player in the biology of the extracellular matrix. Connect Tissue Res 47:177–189
17. Takeuchi T (2010) Structual comparison of dermatopontin amino acid sequences. Biologia 65:874–879
18. Cronshaw AD, Macbeath JRE, Shackleton DR, Collins JF, Fothergill-Gilmore LA, Hulmes DJS (1993) TRAMP (Tyrosine Rich Acidic Matrix Protein), a protein that co-purifies with lysil oxidase from porcine skin: identification of TRAMP as the dermatan sulphate proteoglycan-associated 22 K extracellular matrix protein. Matrix 13:255–256
19. Neame PJ, Choi HU, Rosenberg LC (1989) The isolation and primary structure of a 22-kDa extracellular matrix protein from bovine skin. J Biol Chem 264:5474–5479
20. Forbes EG, Cronshaw AD, MacBeath JRE, Hulmes DJS (1994) Tyrosine-rich acidic matrix protein (TRAMP) is a tyrosine-sulphated and widely distributed protein of the extracellular matrix. FEBS Lett 351:433–436

21. Superti-Furga A, Rocchi M, Schäfer BW, Gitzelmann R (1993) Complementary DNA sequence and chromosomal mapping of a human proteoglycan-binding cell-adhesion protein (dermatopontin). Genomics 17:463–467

22. Beanes SR, Danng C, Soo C, Ting K (2003) Skin repair and scar formation: the central role for TGF-β. Exp Rev Mol Med 5:1–11

23. Okamoto O, Hozumi K, Katagiri F, Takahashi N, Sumiyoshi H, Matsuo N, Yoshioka H, Nomizu M, Fujiwara S (2010) Dermatopontin promotes epidermal keratinocyte adhesion via α3β1 integrin and a proteoglycan receptor. Biochemistry 49:145–155

24. Kagan MH, Li W (2003) Lysyl oxidase: properties, specificity, and biological roles inside and outside of the cell. J Cell Biochem 88:660–672

25. Trackman CP (2005) Diverse biological functions of extracellular collagen processing enzymes. J Cell Biochem 96:927–937

26. Schaefer L, Iozzo R (2008) Biological functions of the small leucine-rich proteoglycans: from genetics to signal transduction. J Biol Chem 283:21305–21309

27. Corsi A, Xu T, Chen X-D, Boyde A, Liang J, Mankani M, Sommer B, Iozzo RV, Eichstetter I, Gheron Robey P, Bianco P, Young MF (2002) Phenotypic effects of biglycan deficiency are linked to collagen fibril abnormalities, are synergized by decorin deficiency, and mimic Ehlers–Danlos-like changes in bone and other connective tissues. J Bone Miner Res 17:1180–1189

28. Zhang G, Chen S, Goldoni S, Calder B, Simpson H, Owens R, McQuillan D, Young M, Iozzo R, Birk D (2009) Genetic evidence for the coordinated regulation of collagen fibrillogenesis in the cornea by decorin and biglycan. J Biol Chem 284:8888–8897

29. Jiang X, Ye M, JIang X, Liu G, Feng S, Cui L, Zou H (2007) Method development of efficient protein extraction in bone tissue for proteome analysis. J Proteome Res 6:2287–2294

30. Brittijn SA, Brittijn A, Duivesteijn SJ, Belmamoune M, Bertens LFM, Bitter W, De Bruijin J, Champagne DL, Cuppen E, Flik G, Vandenbroucke-Grauls CM, Janssen RAJ, De Jjong IML, De Kloet ER, Kros A, Meijer AH, Mets JR, Van Der Sar AM, Schaafl MJM, Schlute-Merker S, Spainkll HP, Tak PP, Vereek FJ, Vervoordeldonk MJ, Vonk FJ, Witte F, Yuan H, Richardson M (2009) Zebrafish development and regeneration: new tools for biomedical research. Int J Dev Biol 53:835–850