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

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Abstract

Fish collagen is a potential source of a scaffold for cells during tissue engineering, because it has low risk of zoonosis. Since the collagen fibril assembly has a significant impact on the functionality of the scaffold, the ability to replicate the fibril assembly of human tissues is critical. As a first step in determining the mechanism of fish collagen fibril assembly, we identified non-collagenous proteins (NCPs), the potential regulators of fibril assembly *in vivo*. We applied tandem mass spectrometry to analyze NCPs contained in the basal plate of goldfish *Carassius auratus* scales, a collagenous plate having a cornea-like, plywood-like assembly of collagen fibrils. We identified a 19-kDa acidic protein as dermatopontin, the NCP that is a possible regulator of fibril assembly in the mammalian cornea. We cloned a goldfish dermatopontin cDNA of 1074 bp containing an open reading frame encoding 196 amino acids. RT-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 in the scale, suggesting that the dermatopontin is linked to the construction of fibril assembly of the basal-plate collagen.

Key words: biglycan, collagen assembly, dermatopontin, lysyl oxidase, MS/MS analysis, scale, teleost

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 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 having a three-dimensional assembly of collagen fibrils replicating the structure in human tissues are generally believed to have improved functionality for tissue engineering applications. This is because the 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 gives 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. Because of the difficulty associated with artificial regulation of collagen fibril assembly using industrial techniques, technology derived from cellular mechanisms (bioinspired technology) is gaining increased attention [1].

Previous studies have revealed that non-collagenous proteins (NCPs) secreted from ECM-producing cells have 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 larger collagen-fibril spacing (fewer collagen fibrils per unit area of the section) and disrupted 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 three-dimensional assembly of fish collagen fibrils. The basal plate is formed of type I collagen fibrils having a cornea-like plywood-like assembly [1]. Our objective was to isolate and identify NCPs from the scale basal plates of the goldfish *Carassius auratus*. We acid-extracted the ECM of the scale basal plate and conducted time-of-flight tandem mass spectrometry (MS/MS). Additionally, we performed cDNA cloning and mRNA expression analysis by RT-PCR and *in situ* hybridization of goldfish dermatopontin, one of the NCPs identified during MS/MS analysis.

Materials and methods

Fish and rearing

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

Protein extraction from scale basal plates

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

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. Acetic acid is commonly used to extract native collagen molecules from tissues. Therefore, we assumed that the collagen molecules with associated NCPs were extracted by this method. Following extraction, the samples were centrifuged at 2,000x*g* for 10 min at 4°C, and both the supernatant and precipitate were collected. The supernatant was further centrifuged at 21,500x*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™ Protein Assay Kit (PIERCE, Rockford, IL, USA).

Two-dimensional gel electrophoresis

To characterize the protein profile of the supernatant, we performed two-dimensional gel electrophoresis (2-DE) using the Ettan IPGphor 3 IEF System (GE Healthcare Biosciences, Buckinghamshire, UK) for isoelectric electrophoresis (pI range 3–10) and a slab electrophoresis chamber (Atto, Tokyo, Japan) for SDS-PAGE (15% separating gel). A total of 600 μg of proteins were separated according to the manufacturers' protocols. The separated proteins were detected by silver staining.

Identification of proteins by tandem mass spectrometry

To perform mass spectrometry, the protein spots separated by 2-DE were excised and processed according to the manufacturer's protocol (Applied Biosystems, Tokyo, Japan), with a slight modification: a trypsin enhancer (Promega, Madison, WI, USA) was added to enhance trypsin digestion of the sample. The digested peptides were purified using a ZipTip (Millipore, Billerica, MA, USA) and applied to MS/MS (MALDI-TOF-TOF, Applied Biosystems 4700). The tryptic peptide mass fingerprinting and subsequently obtained MS/MS spectra 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™ RNA kit (Takara, Otsu, Japan). After DNase I (Takara) treatment, first-strand cDNA from 500 μg of total RNA was synthesized using a PrimeScript™ RT reagent kit (Perfect Real Time: Takara). First-strand cDNA was synthesized from 500 μg of total RNA by 5′ and 3′ RACE using a SMART™ RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA).

To obtain the goldfish dermatopontin fragment, PCR was first performed using the primers 5′-ccccgctctgttgatatcatcccaccagc-3′ (forward) and 5′-gccctatagtgagtcgtattag-3′ (reverse), which were designed

based on zebrafish *Danio rerio* sequence data (GenBank accession no. NM_001030085). After determining the partial sequences, 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′-tcgcggccgcaggaa-3′. Then, 5′ RACE was performed using the mixed adopter primers 5′-ctaatacgactcactatagggcaagcagtggtat-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, Madison, WI, USA), 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, Tokyo, Japan). 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 RT-PCR. Goldfish were deeply anesthetized using a 0.1% 2-phenoxyethanol solution, and then decapitated. Each animal was dissected and 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™ RNA kit. After DNase I (Takara) treatment, first strand cDNA from 91.5–100 μg of total RNA was synthesized using a PrimeScript™ RT reagent Kit (Perfect Real Time). The primers for PCR, 5′-gacttcaggatgagctcagca-3′ (forward) and 5′-accafcaacaagcccattg-3′ (reverse), were designed based on the cDNA data obtained in this study. 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 conducted 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 M 13 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 basal-plate producing hyposquamal scleroblasts, are flat and small [11]. Because of this, we predicted 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 were therefore larger compared with those in ontogenetic scales [11]. Individuals were anesthetized in a solution of 0.1% 2-phenoxyethanol and 30 scales were 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 for overnight at 4°C. After fixation, the samples were decalcified in Morse's solution (10% sodium citrate and 22.5% formic acid) [12] for 1 day at 4°C. The samples were then dehydrated conventionally, embedded in TissuePrep (Fisher Scientific, Fair Lawn, NJ, USA), and sections (6 μm) 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 were 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 nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate solution (Roche).

Results

Identification of proteins by tandem mass spectrometry

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

We excised 25 spots from the 2-DE gel for MS/MS analysis, but succeeded to identify only three proteins: 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 organization of collagen fibrils in mammals [9, 13, 14]. Additionally, at the time we initiated this study, 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.

The cloned goldfish dermatopontin cDNA had 1074 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) is given in Figure 2. Data for zebrafish dermatopontin from cloned cDNA, which was uploaded from our laboratory during this study [15], was used in Fig. 2. The two amino acid sequences matched with zebrafish dermatopontin by MS/MS analysis were found in the obtained goldfish sequence (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 10 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 and the third motifs are the integrin-binding RGAT sequence and 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 7-day regenerating scales are illustrated in Figure 4. Three-types of scale-forming scleroblasts, flat episquamal, square hyposquamal, and oval- to droplet-shaped marginal scleroblasts, were attached on 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).

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: ca. 4.2) composed of 183 amino acids with an apparent molecular weight on SDS-PAGE of 22 kDa. Its N-terminal region is rich in tyrosine residues, and many of them are sulphated [18, 20]. The apparent molecular weight and isoelectric point (ca 5.0) of the goldfish dermatopontin identified in our study largely agreed with those of bovine dermatopontin. Additionally, we 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 on the SDS-PAGE (19 kDa) was smaller, but its reason is presently unknown. Purification and amino acid sequence analysis of the protein will further clarify matured form of dermatopontin in goldfish tissues. The deduced amino acid sequence also revealed that all 10 cysteine residues conserved among mammalian dermatopontins were also conserved in goldfish dermatopontin, suggesting that it shares a similar three-dimensional 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 organization of collagen fibrils in mammals. Dermatopontin purified from porcine skin accelerated type I collagen fibrillogenesis *in vitro*, and the synthesized fibrils were relatively thin and had a narrower range of diameters in the presence of dermatopontin [13]. Dermatopontin-null mice exhibited decreased thickness of the dermis, a lower skin collagen content, and had larger diameter collagen fibrils with irregular contours [14]. The range of collagen fibril diameters in the

dermis of dermatopontin-null mice was also greater. Moreover, Cooper et al. [9] reported that the corneal stroma of dermatopontin-null mice had larger collagen-fibril spacing and disrupted fibrillar organization. Because the expression of goldfish dermatopontin mRNA in the regenerating scales was primarily detected in the hyposquamal scleroblasts responsible for the production of the basal plate, the data obtained in mammals to date strongly suggest that dermatopontin regulates collagen fibrillogenesis and 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 in a wide range of tissues [15, 16, 20, 21], suggesting that it has multiple functions. Two functions of dermatopontin, other than regulating collagen fibrillogenesis, have been proposed [16]. These include promotion of cell adhesion and fine tuning of activity of TGF-β, a cytokine playing a central role in wound healing of 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 recognition sequence of α3β1 integrin in dermatopontin was not clarified, the binding site of the proteoglycan receptor was identified as an eight amino acid sequence, GQVVVAVR [23]. A similar sequence was also observed in goldfish and zebrafish dermatopontins, but substitutions of some amino acids were observed [G**E**V**L**VA**I**R (goldfish residues 48–55) and G**E**V**L**VAVR (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 goldfish scale basal plates may also increase the strength of the plates by promoting cross-linking of collagen fibrils. Biglycan is a member of the Class I small leucine-rich proteoglycans (SLRPs) [26]. In tendon tissue, biglycan-null mice exhibit a decrease in the number of fibrils with a larger diameter, and an increase in those with a smaller diameter [27]. However, unusually large fibrils also occur 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, and disruption in fibril packing and lamellar organization [28]. However, single knockout mice lacking either decorin or biglycan do not exhibit significant changes in corneal collagen fibrils [28]. A compensatory up-regulation 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 fine-tunes the function of decorin [28]. Although decorin was not detected in this study, it is possible that biglycan with decorin has an important role in the regulation of collagen fibrillogenesis in the scale basal plate of goldfish.

Aiming to obtain collagen-associated NCPs from the scale basal plate, the present study employed a 0.5 M acetic acid solution as an extraction solution, because the acidic solution is usually used to extract collagen molecules. However, more broader spectrum of proteins in the basal-plate matrix seem to be contained in the extract, because many spots were obtained by 2DE analysis. On the other hand, the present extraction method may also be an incomplete one, because a large part of basal plate matrix was discarded as a precipitates of the extraction in this study. Protein denaturation agents such as urea, guanidine-HCl or CHAPS may be contained in the solution to extract proteins from the precipitates. In fact, Jiang et al. [29] showed a need of sequential extraction of dog bone for complete proteome analysis. 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. They 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 the present study.

Zebrafish is the most frequently used model teleost in the recent biomedical research [30], but we used goldfish scales to identify NCPs of the basal plate in the present study. This is because goldfish is much bigger in body and scale size than zebrafish. The bigger scale size makes to collect basal plate samples much easier. The bigger body size may also beneficial in future. It may be possible to purify both collagen and dermatopontin from goldfish, and conduct *in vitro* fibrillogenesis experiments to clarify function of goldfish dermatopontin. In porcine, dermatopontin was reported to accelerate collagen fibrillogenesis, and the produced fibrils in the presence of dermatopontin became thinner than its absence [13]. Another beneficial point to use goldfish is that goldfish and zebrafish are included in the same family Cyprinidae; thus, zebrafish genome data can be applied, at least in part, when MS/MS analysis is performed. In fact, the peptides from two identified proteins matched that

of zebrafish data in the present MS/MS analysis. The PCR primers designed based on zebrafish dermatopontin also worked well. Therefore, we believe that gold fish is a suitable species for the purpose of the present study.

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Figure captions

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. *Dotted circle* spots identified by MS/MS analysis, *M* marker, *pI* isoelectric point

Fig. 2 Alignments of amino acid sequences of dermatopontin of goldfish, zebrafish, *Xenopus*, chiken 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 mRNA expression based on RT-PCR. *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

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. *Bar* 50 μm

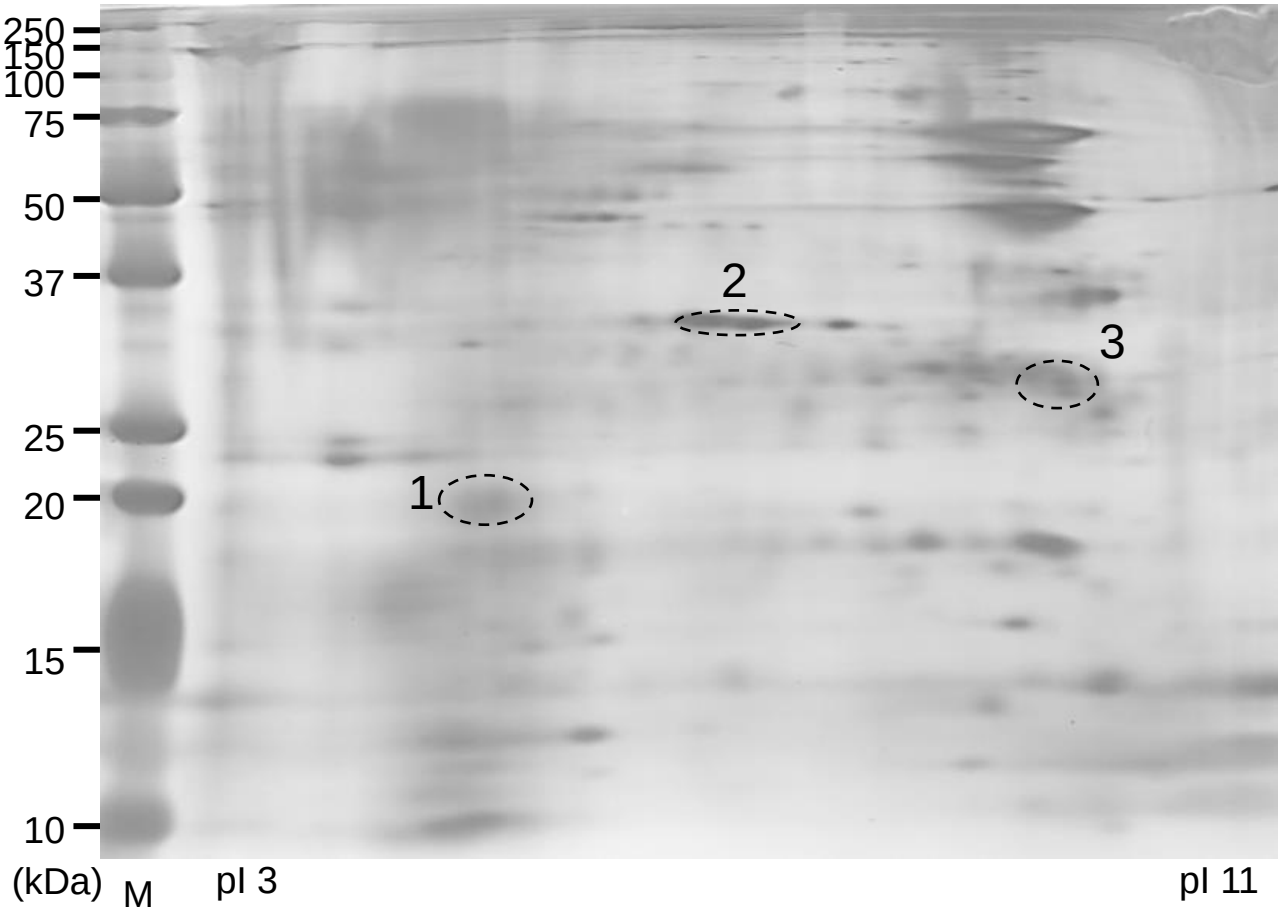

Fig.1
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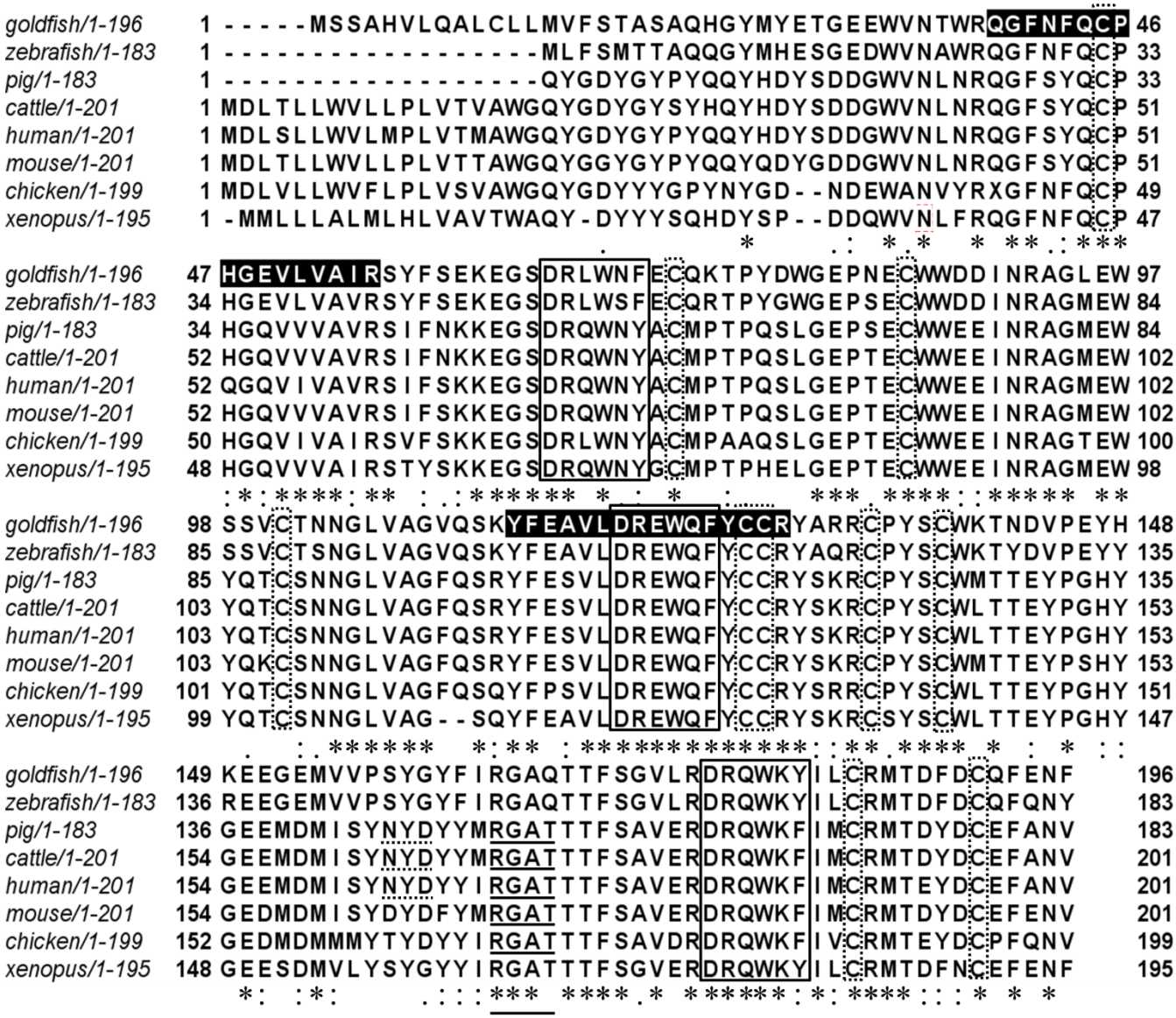

Fig. 2
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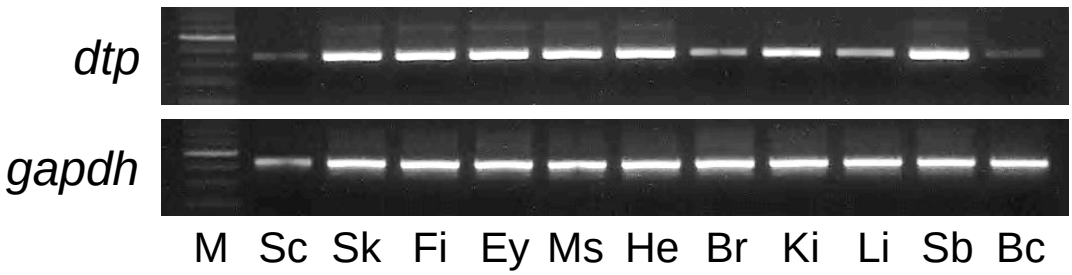

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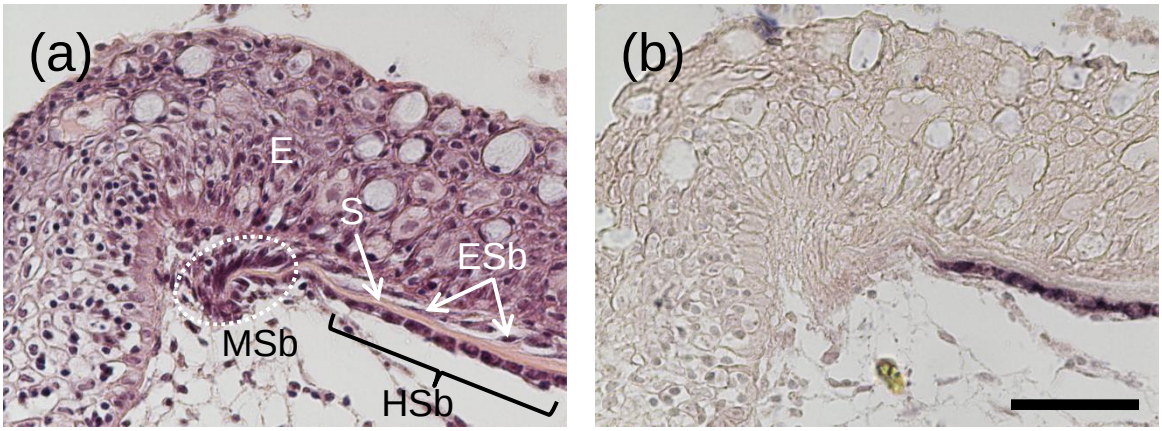

Fig. 4
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