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学 位 論 文 題 目

Functional analysis of dermatopontin (Dpt) in zebrafish *Danio rerio*
(ゼブラフィッシュ *Danio rerio* におけるデルマトポンチン (Dpt) の機能解析)

In regenerative medicine, tissue transplantations are severely limited by the problem of donor shortage and immune rejection by patients. Therefore, synthetic scaffold having an excellent ability to support tissue regeneration, biocompatibility and safety is called for biomedical applications. Collagen, the major component of extracellular matrix (ECM), exhibits biodegradability, weak antigenicity and superior biocompatibility compared with other natural polymers, and is requisite for cellular functions of each tissue. Therefore, collagen is considered to be the most suitable and commonly used xenogeneic ECM component intended for therapeutic applications. The other important reason for the usage of collagen in biomedical application is that collagen can form fibers with extra strength and stability through its self-aggregation and cross-linking, which is essential for the tissue structure maintenance. Thus, proper understandings of collagen fibrillogenesis both *in vivo* and *in vitro* are important to effectively apply the collagen for biomaterial scaffolds.

Recent studies of non-collagenous proteins such as proteoglycans (decorin, lumican etc.) and glycoproteins (DPT etc.) in the ECM have revealed their critical effects on collagen fibril formation. DPT is unique since it accelerates collagen fibrillogenesis *in vitro*, and stabilizes the collagen fibrils interacting with lysyl oxidase *in vivo*. The DPT-null mice show a 24%-decrease in stromal thickness in the cornea, as well as significant disruption of fibril spacing within the posterior lamellae of the corneal stroma with 40% lower collagen content. All these data suggest that DPT strongly relates to collagen fibrillogenesis *in vivo*, and thus, DPT-collagen hybrid material may have characteristic activity as biomaterial scaffolds intending to promote successful tissue repair. In contrast to other already identified non-collagenous proteins in the ECM, however, the functional characterization of DPT has not been fully elucidated, and this inhibits the application of DPT as biomaterial scaffolds.

The zebrafish, *Danio rerio*, is an excellent model of gene function analysis alternative to

mammalian models such as mice and rats. However, little is currently known about DPT in teleosts. In the present study, I carried out molecular biological characterization, spatiotemporal localization, and morpholino-mediated knockdown analysis of Dpt in zebrafish, and then tried to find out Dpt functions and the role during collagen fibrillogenesis.

Firstly, I cloned the full-length of zebrafish *dpt* cDNA and analyzed its gene structure. The results showed that zebrafish *dpt* maintained a conservation and syntenic relationship with its mammalian counterparts. A comparison of the cDNA sequence with the genomic sequence revealed that zebrafish Dpt includes 4 exons and 3 introns, and the localization and organization are the same as in human and mouse. Zebrafish *dpt* also contains the three 6-residue repeat region, D-R-[E/Q]-W-[N/Q/K]-[F/Y], a potential glycosaminoglycan binding site, which were localized in the first, second and fourth exons as in human and mouse. In addition, a cell adhesion promoted peptide G-Q-V-V-V-A-V-R in bovine was also found as G-E-V-L-V-A-V-R in zebrafish Dpt sequence. However, the integrin binding site R-G-A-T sequence in mammalian DPT has been altered to R-G-A-Q in zebrafish, which has likely resulted in loss of this activity. At last, a potential sequence N-Y-D involved in biogenesis of topaquinone may not be maintained in zebrafish, for the reason that N is substituted for S and D is substituted for G in zebrafish. All these results revealed that zebrafish *dpt* has some specific modifications but is co-orthologous with that of mammalian *Dpt*, which gives the potential possibility using zebrafish to study DPT function *in vivo*.

Secondly, I described the spatiotemporal expression pattern of zebrafish *dpt* and Dpt using RT-PCR, Q-PCR, ISH and IHC. The results determined by RT-PCR and Q-PCR showed that *dpt* transcripts were expressed at zebrafish all developmental stages from as early as 0.5 hpf to 42 dpf with some changes in expression levels: they increased from 0.5 hpf to 2 hpf, then decreased from 3 hpf to 12 hpf, and remained relatively the same levels thereafter. In adult tissues, strong *dpt* expressions were found in the skin and muscle, relatively strong expressions in the scale, and weak expressions in the gut, heart, brain, eye, kidney and fin. ISH and IHC revealed a ubiquitous expression of zebrafish Dpt transcripts and protein in connective, muscle, nervous, and epithelial tissue cells. These distribution patterns of zebrafish *dpt* mRNA and Dpt protein were similar to that in mammals. Thus, my results clearly suggest that zebrafish Dpt may play similar roles as in mammals. On the other hand, I also found zebrafish *dpt* mRNA expressed in brain and four layers of the retina: the ganglion cell layer, inner nuclear layer, outer plexiform layer, and outer nuclear

layer. Such expression patterns were not reported in mammals. Thus, Dpt may have some undiscovered roles in zebrafish. Positive *dpt* transcript signals were also observed in myosepta, dermis and cartilage, which contain collagen.

Thirdly, I carried out morpholino-mediated gene function analysis of zebrafish Dpt. The morphological analysis of 3-dpf larvae revealed that both Dpt-MO^{start}- and Dpt-MO^{splice}-morphants showed similar shortened body axis phenotype which suggested a possible defect in convergent extension. This hypothesis was supported by observations of morphants in the somitogenesis stage (12-hpf), which also showed the aberrant head-to-tail body axis. The histological examinations of Dpt-morphants also showed that the muscle fibers of the morphants were irregularly formed, the meninges, a collagenous membrane that surrounds spinal cord, was not visible, and the cells in the eye were irregularly arranged. Subsequently, alcian blue staining revealed a compressed and flattened cartilage structure in craniofacial region of 5-dpf Dpt-morphant. Moreover, the SEM analysis found a perturbation in the surface contour of the epidermis cells with decreased density of microridges. Based on the results, I concluded that Dpt serves an important role during collagen fibrillogenesis in zebrafish. In addition, I propose five potential novel aspects of function that zebrafish Dpt may involve: convergent extension, craniofacial cartilage development, muscle fiber formation, arrangement of cells in the eye, and epidermis cell structure maintenance.

In conclusion, I characterized the zebrafish Dpt and elucidated its expression pattern during embryogenesis and in adults in zebrafish. I also proposed some new functions of Dpt which are unique in zebrafish compared to mammals, and provided useful basic information for further characterization of Dpt function. The present study also proves the involvement of Dpt in collagen fibril formation during zebrafish embryogenesis, and thus adds further support of the possibility to apply DPT-collagen hybrid material for biomaterial scaffolds intending to promote successful tissue repair.