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論文題目	Study on developmental roles of retinoic acid and its regulation in metamorphosing Japanese flounder larvae（変態期ヒラメ仔魚におけるレチノイン酸とその代謝の発生学的機能に関する研究）
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Doctoral Dissertation Abstract

Study on developmental roles of retinoic acid and its regulation in metamorphosing Japanese flounder larvae

（変態期ヒラメ仔魚におけるレチノイン酸とその代謝の発生学的機能に関する研究）

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Introduction

Japanese flounder (Paralichthys olivaceus), a genus of the Paralichthyidae family, is the most economically cultured flatfish in East Asia. In the early larval stages, Japanese flounder grows bilateral symmetrically as other teleosts. However, during the metamorphosis, flounder forms the asymmetrical body shape, which involving blind-side eye migration and ocular-side pigmentation, making itself an interesting model for asymmetry.

As an important commercial fish, aquaculture industries of Japanese flounder were blossoming in the past three decades. Most cultured flounders are released to the marine environment, aiming at maintaining wild resources, and the others grow to edible size for selling on fish markets. In flounder aquaculture, the frequent occurrence of hyperpigmentation at the blind-side skin always causes a reducing commercial value in markets and low survival rate for release. Thus, many studies were taken by researchers worldwide, focusing on the pigmentation of the Japanese flounder. In these studies, researchers traced the progenitor of pigment cells and established critical genetic markers for different pigment cell types. All of these data provided fruitful knowledge for our researches today.

Vitamin A (VA) is an essential nutrient for flounder development. Retinoic acid (RA), a derivate of VA, functioning as a morphogen that regulates tissue development. The RA is synthesized from retinal by the Aldehyde dehydrogenase 1 family member A1 (Aldh1a) and binds to RA receptors (Rars) to exert function in specific tissues. The redundant RA can be catabolized to 4-hydroxy-RA by cytochrome P450 26 (Cyp26) family in the local tissue. In the previous study, we incubate metamorphosing flounders in all-trans RA for 3 days and observed the hyperpigmentation and fused vertebral column in these flounders after they grew to juveniles. Thus, we suppose the RA plays a critical role in asymmetrical pigmentation and bone formation during metamorphosis. To understand the RA's role in metamorphosing flounder larvae, we examined the expression profile of genes related to RA metabolism (Chapter 1); screened out the developmental-related genes induced by RA (Chapter 2); and investigated the RA's role in regulatory system of asymmetrical pigmentation (Chapter 3).

Chapter 1. Expression profile of RA synthases, catabolic enzymes and receptors in Japanese flounder

In Japanese flounder, transient RA-exposure at metamorphosis causes fusion of centra due to the loss of intervertebral ligament and notochord tissues of the vertebral joints, and

destruction of bilateral asymmetry of body color by ectopic pigmentation of blind-side skin, suggesting that RA plays critical roles in metamorphic development in this teleost (Fig. 1). Elucidation of the expression profile of RA-related genes during metamorphosis, including RA-synthases, catabolic enzymes and receptors, at post-embryonic development is essential for understanding the functions of RA in metamorphosis. At present, however, the expression profile of RA-related genes is largely unclear in the Japanese flounder as in other model fish species.

This chapter elucidates the expression profile of RA-synthases (*aldh1a1*, *aldh1a2*, and *aldh1a3*), catabolic enzymes (*cyp26a1*, *cyp26b1* and *cyp26c1*) and receptors (*raraa*, *rarab*, *rarba*, *rarbb*, *rarga* and *rargb*) during metamorphosis to better elucidate RA signaling and functions in the development of flounders (Fig. 2). Among the above twelve RA-related genes, clear mRNA expression signals were detected by in situ hybridization (ISH) for *aldh1a1*, *aldh1a2*, *aldh1a3*, *cyp26a1*, *cyp26b1*, *cyp26c1, raraa*, *rarab*, *rarba*, *rarbb*, *rarga* and *rargb* in embryos (Table 1), suggesting the perfect specificity of RNA probes prepared here. Of note, during embryogenesis, *aldh1a*s, *cyp26*s and *rar*s were detected together in tissues including tectum, dorsal somite, eye, pectoral fin bud and pharyngeal arch, indicating the critical role of RA signaling in these tissues' development. In metamorphosing flounders, RA metabolism was particularly active in the central nervous system. The cells that expressed *aldh1a*s along the margin of the tectum are likely radial glia cells and ependymal cells (Fig. 3). RA supplied by these cells can be transported via cerebrospinal fluid (CSF). With the *cyp26*s that were expressed near ventricles and central canal, RA may be responsible for brain growth and maintenance (Fig. 3). Throughout metamorphic development, *aldh1a2* is strongly expressed in the intestine and liver, which may function as the main sources of RA during metamorphic development (Fig. 4). *aldh1a3* is expressed in the larval pituitary, giving interesting implications for endocrine function (Fig. 3). *cyp26a1* is detected in the pyloric region in the intestine. As the pyloric caecum is formed during metamorphosis, RA may function in the outgrowth of the pyloric caecum (Fig.4; Table 2). No ISH signal was detected for *aldh1a*s or *cyp26*s from the skin and vertebral column susceptible to the teratogenic effects by RA (Table 2). Since flounder skin and vertebra transcriptome included these genes, the ISH result could be related to both low expression level of gene and sensitivity of ISH. Additionally, genes encoding RARs were detected in the vertebra and both sides of skins by RT-PCR at G-stage, suggesting the RA supplied from other places can be taken by these tissues (Table 3). Obtained data suggest the comprehensive function of RA signaling during embryogenesis and metamorphosis, *rar*s

expressed in both sides of skins may be the key point why the hyperpigmentation can be induced on the blind-side skin.

Chapter 2. Comparative transcriptomic analysis between DMSO and RA-treated metamorphosing flounders

Retinoic acid (RA) regulates the expression of various genes critical for vertebrate development. To characterize the molecular mechanism(s) underlying the malformation induced by RA (Fig. 1), we used RNA-seq to identify genes that were up- and down-regulated by RA.

Comparison of expression profiles between RA-treated and control animals revealed that among 21,071 annotated genes, 251 genes were significantly up-regulated and 188 genes were significantly down-regulated by RA (Fig.5). By Gene Ontology (GO) analysis, the differential expressed genes are most enriched in retinoid metabolic process, extracellular matrix organization and complement and coagulation cascades (Fig. 6). We found that negative feedback works in response to RA to decrease the RA level by up-regulating genes encoding RA degradation enzymes (*cyp26a1*, *cyp26b1, cyp26c1*) and enzymes that convert vitamin A (VA) to retinyl ester (*lrata*) and retinal to VA (*dhrs3a* and *dhrs3b*), and also by down-regulating genes encoding retinal synthase (*bco1* and *bco1l*) (Fig. 7). RNA-seq data also indicated that RA down-regulates *col2a1a*, and up-regulates *pth3r*, *mmp9* and *mmp13b*, the products of which presumably are related to the vertebral anomaly (Table 4). As for pigmentation, RA did not affect the expression of *sox10a* and *sox10b*, but did up-regulate *gch2* twice, suggesting that RA stimulates differentiation of chromatophore precursors to *gch2*-positive chromatoblasts (Table 5). Thus, our RNA-seq analysis provided data that clarify the molecular basis of the RA-induced abnormalities and the functions of endogenous RA in flounder metamorphosis.

Chapter 3. RA and its role in the asymmetrical pigmentation in flounder

Japanese flounder has its unique asymmetric body color, with a pigmented ocular-side and a white blind-side. This body color asymmetry develops by restricted differentiation of chromatophores on the ocular-side during metamorphosis. In this study, we found that in flounder, Sox10-positive chromatophore progenitors, which firstly appeared in the spinal cord migrated to both sides of skins in metamorphosing larvae, then become Sox10-negative when the metamorphosis happened (Fig. 8). Transient exposure of metamorphosing larvae to retinoic acid (RA) induced progenitors on the blind-side to differentiate into *gch2*-positive

chromatoblasts with no changes in the numbers of larval-type melanophores (Fig. 9). On the other hand, exposure to an RA receptor antagonist, BMS493, suppressed the differentiation of *gch2*-positive chromatoblasts on the ocular-side (Fig. 10). Thus, we demonstrated that RA signaling is essential for flounder chromatophore progenitors to differentiate into chromatoblasts. At the time of chromatoblast differentiation on the ocular-side, *cyp26b1*, which inactivates RA, was upregulated on the blind-side skin compared to the ocular-side (Fig. 11). Therefore, we surmise that ocular-side specific pigmentation is regulated by the inhibition of RA-signaling by *cyp26b1* on the blind-side (Fig. 12).

Conclusion

Chapter 1. RA synthases, catabolic enzymes and receptors were detected in both embryogenic and metamorphosing stages. Obtained data suggest that RA-signaling is particularly active in the central nervous system and gut development, including the differentiation of pyloric caecum. Throughout metamorphic development, *aldh1a2* is strongly expressed in the intestine and liver, which may function as the main sources of RA during metamorphosis. The detection of *rar*s at both sides of skins at G-stage suggests the critical role of RA signaling in pigmentation.

Chapter 2. Comparative transcriptomic analysis reveals that perturbations of RA signaling induced the negative feedback of RA metabolism and activation of the extracellular matrix organization. The expression of *mmp*s and downregulated *col* family genes may cause the loss of intervertebral ligaments between centra. *gch2* was up-regulated 2-folds, indicates the ectopic pigmentation on the blind-side of the skin during RA treatment could be related to the increase of *gch2* positive chromatoblast at blind-side skin.

Chapter 3. Chemical exposure experiment reveals a critical role of RA in asymmetrical pigmentation of flounder during metamorphosis. RA functions as the initiator of the *gch2,* which is a marker for chromatoblasts. Depletion of RA signaling at mid-metamorphosis suppressed the *gch2* expression. Since the *cyp26b1*, the main RA catabolic enzyme was up-regulated on the blind-side skin during mid-metamorphosis, we assume the up-regulated *cyp26b1* on the blind-skin leads to the decrease of RA level and then the asymmetrical pigmentation.

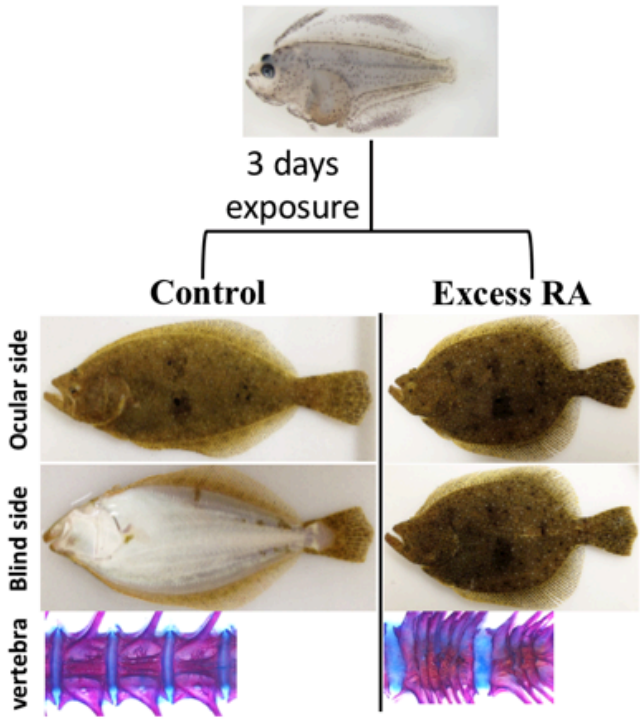

Figure 1. Three days exposure in RA during metamorphosis caused hyperpigmentation on blind-side of skin and fused centra in 122 dpf flounder.

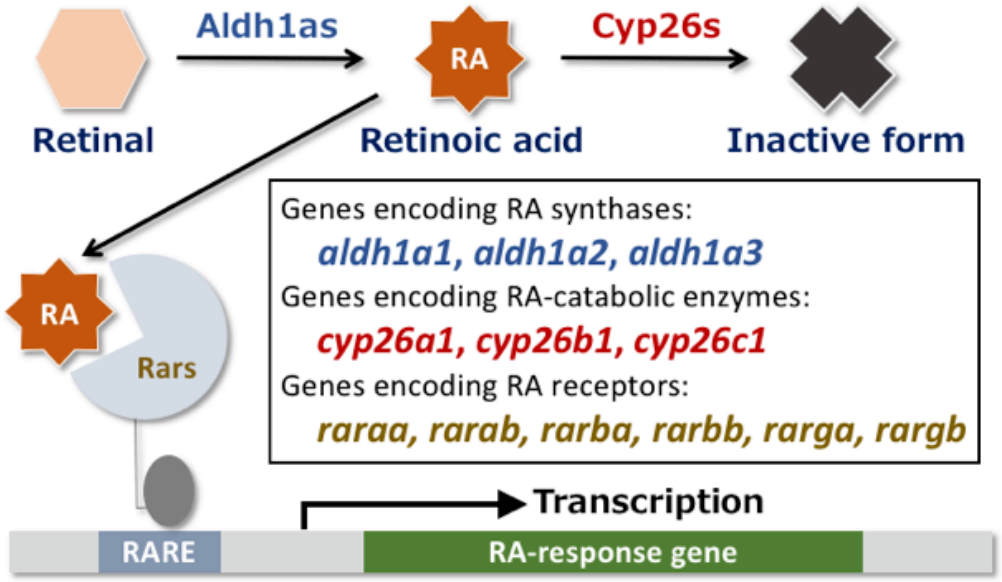

Figure 2. Scheme of RA metabolism pathway. Twelve genes on the list encode important enzymes for RA metabolism.

Table 1. Conclusion of whole mount in situ hybridization results of all genes encoding RA synthases, catabolic enzymes and receptors at hatching stage

	Telencephalon	Tectum	Hindbrain	Otic vesicle	Spinal cord	Gut	Dorsal somite	Dorsal side of eye	Ventral side of eye	Pectoral fin bud	Pharyngeal arch	Proctodeum	Tail bud
aldh1a1				+				+					
aldh1a2		+				+	+	+	+	+	+		
aldh1a3				+					+				
cyp26a1					+				+	+	+	+	
cyp26b1	+	+	+						+	+			
cyp26c1	+			+				+			+		
raraa			+		+					+			
rarab								+	+	+	+		
rarba		+					+						
rarbb					+								
rarga			+					+	+	+	+		+
rargb								+	+	+	+		+

Table 2. Conclusion of section in situ hybridization results of all genes encoding RA synthases, catabolic enzymes during metamorphosing stages

	Tectum	Cerebellum	myelencephalon	Spinal cord	Neurohypophysis	Eye	Intestine	Pyloric caecum	Liver	Kidney	Heart	Skin	Vertebra
aldh1a1	+					+							
aldh1a2	+	+		+		+	+		+	+	+		
aldh1a3					+								
cyp26a1				+				+					
cyp26b1	+	+	+										
cyp26c1													

Table 3. Conclusion of RT-PCR results of all genes encoding RA receptors at G-stage

	Eye	Brain	Gill	Heart	Intestine	Liver	Left skin	Right skin	Spinal cord	Vertebra	muscle
raraa	+	+	+	+	+	+	+	+	+	+	+
rarab	+	+	+	+	+	+	+	+	+	+	+
rarba		+									
rarbb	+	+	+	+	+	+	+	+	+		+
rarga	+	+	+	+	+	+	+	+	+	+	+
rargb	+	+	+	+	+	+	+	+	+	+	+

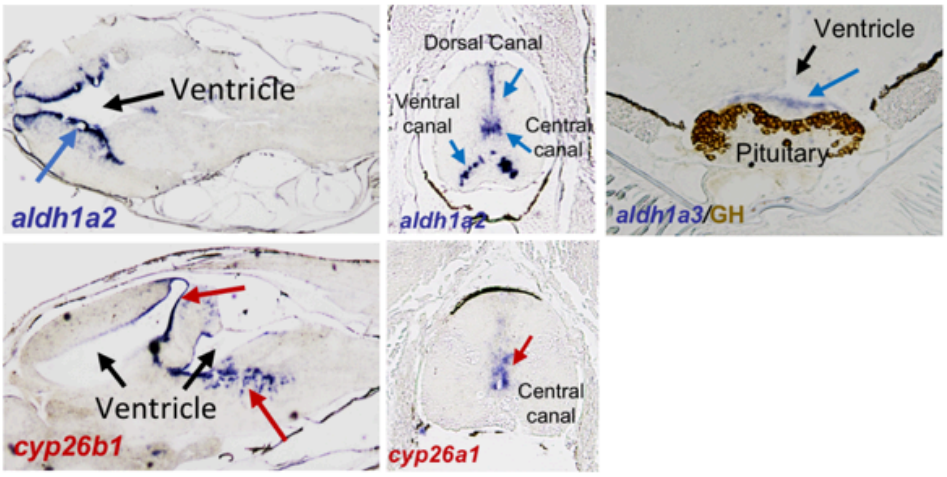

Figure 3. mRNA expression of *aldh1a*s and *cyp26*s that near brain ventricles and spinal cord canals.

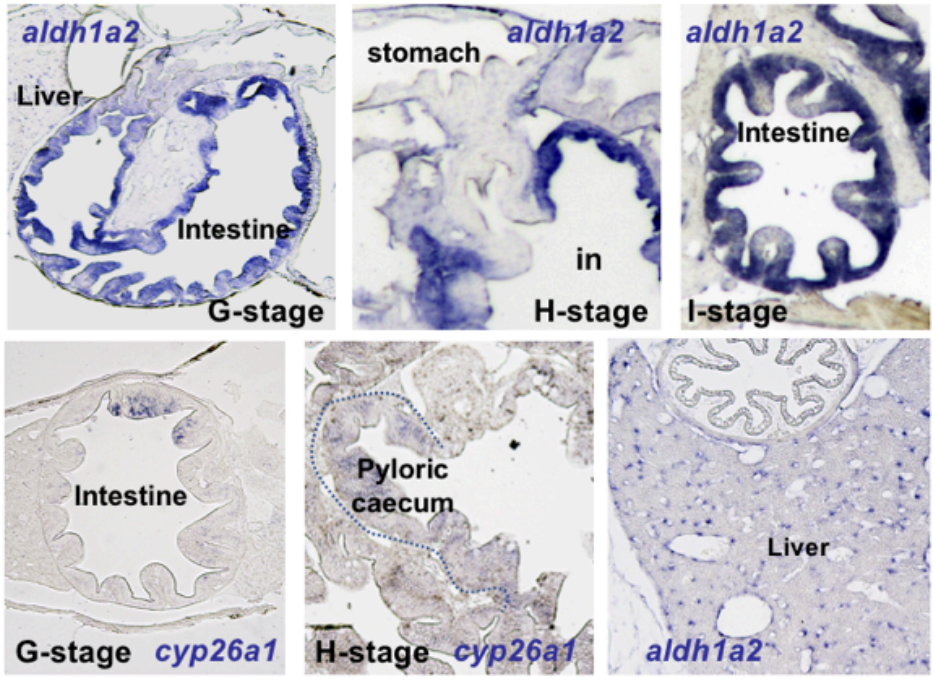

Figure 4. mRNA expression of *aldh1a2* and *cyp26a1* in internal organs.

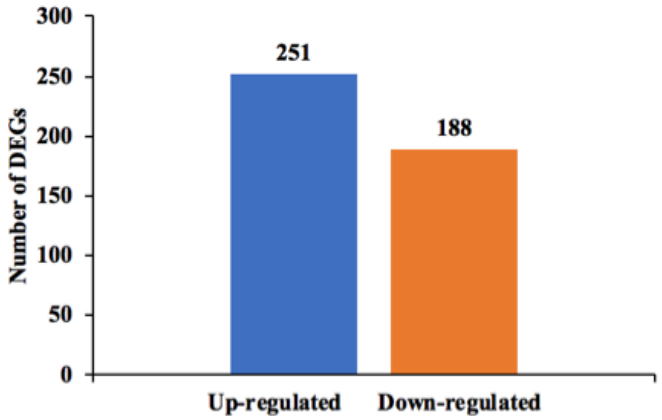

Figure 5. Statistics for the number of differential expressed genes (DEGs) affected by RA-exposure.

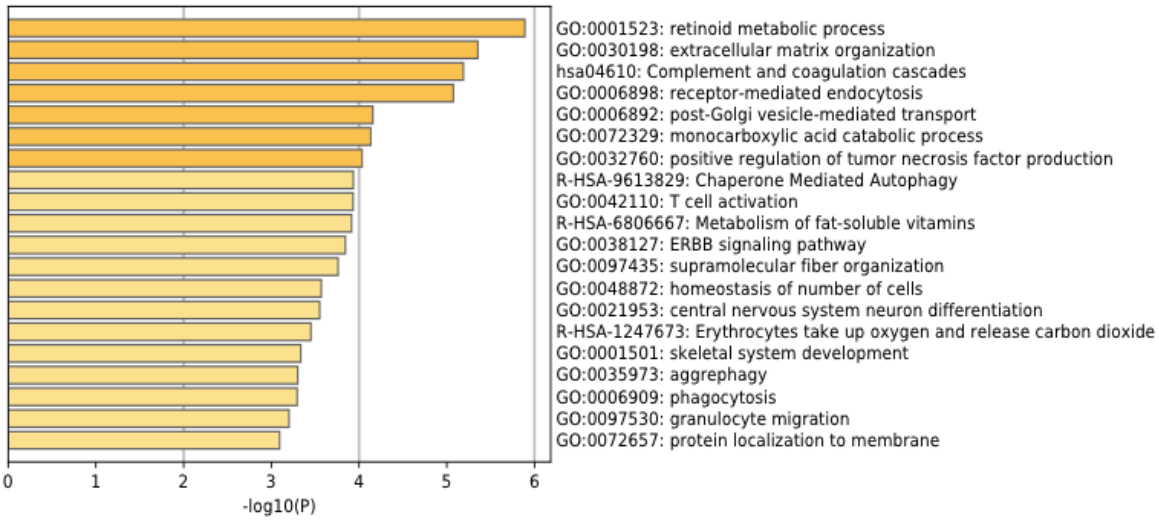

Figure 6. Summary of pathway and process enrichment.

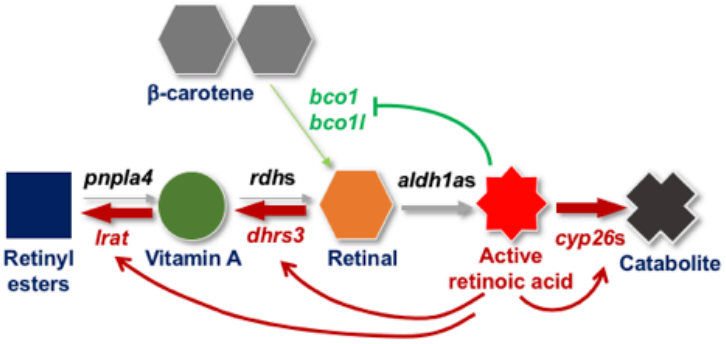

Figure 7. Scheme of the negative feedback in the RA metabolism pathway induced by RA-exposure.

Table 4. DEGs between control and RA groups, of genes encoding components that function in skeletal formation.

Gene name	Symbol	Control FPKM	RA FPKM	log2FC
runt-related transcription factor 2	*runx2*	0.09	0.49	2.15
collagen, type II, alpha 1a	*col2a1a*	11.4	3.88	-1.77
matrix metallopeptidase 9	*mmp9*	3.93	32.66	2.84
matrix metallopeptidase 13b	*mmp13b*	2.2	19.11	2.9
parathyroid hormone 3 receptor	*pth3r*	0	1.19	3.85

Table 5. DEGs between control and RA groups, of genes encoding components that function in pigmentation.

Gene name	Symbol	Control FPKM	RA FPKM	Fold change
* *GTP cyclohydrolase 2*	*gch2*	15.20	29.07	1.91
melanocyte inducing transcription factor a	*mitfa*	1.46	2.27	1.55
melanocyte inducing transcription factor b	*mitfb*	2.58	2.67	1.03
tyrosinase	*tyr*	2.74	2.99	1.09
tyrosinase-like	*tyrl*	0.60	0.83	1.37
L-Dopachrome tautomerase	*dct*	11.62	14.12	1.21
transcription factor Sox-10a	*sox10a*	4.34	3.79	0.87
transcription factor Sox-10b	*sox10b*	0.35	0.34	0.96

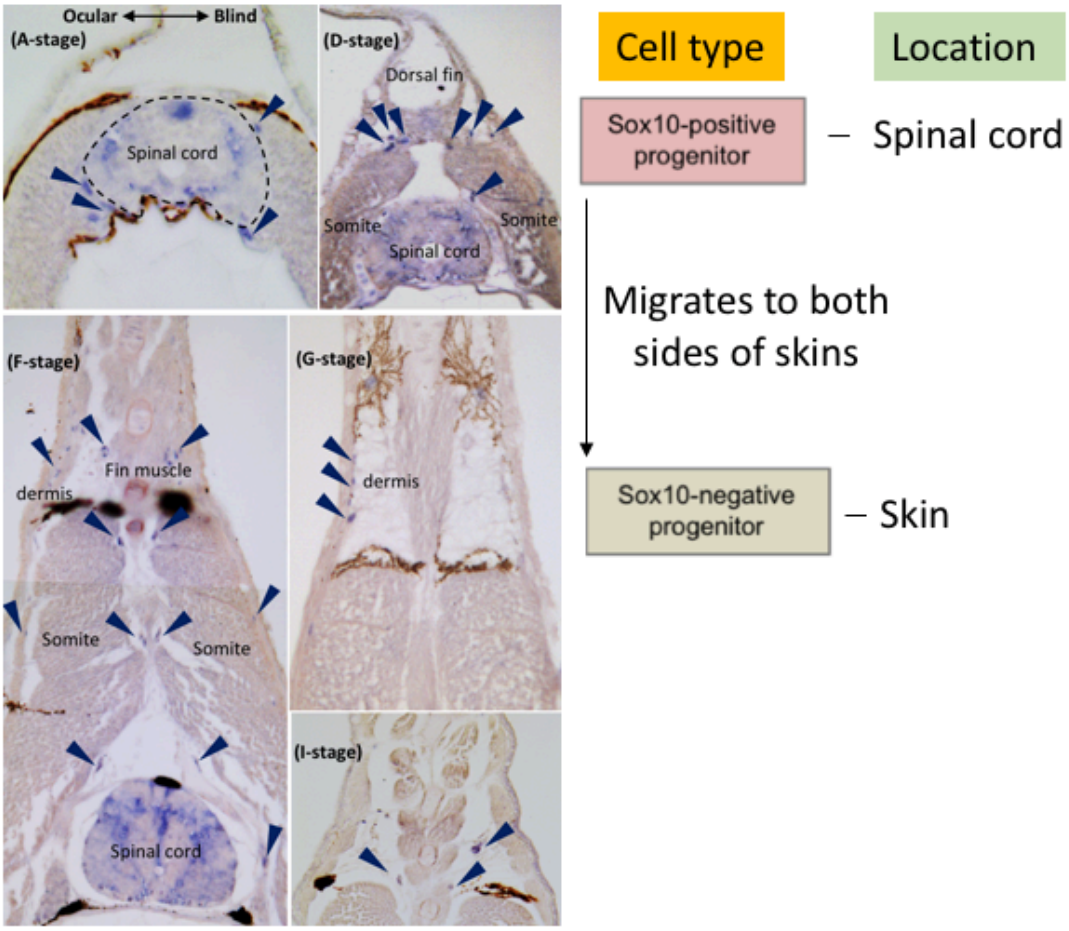

Figure 8. Sox10-positive cells in larval stages. The Sox10-posive cells is firstly detected in spinal cord and migrates to both sides of skins. From G-stage, the Sox10-positive progenitors on both sides of skins gradually become Sox10-negative. Blue arrowhead, Sox10-positive cell.

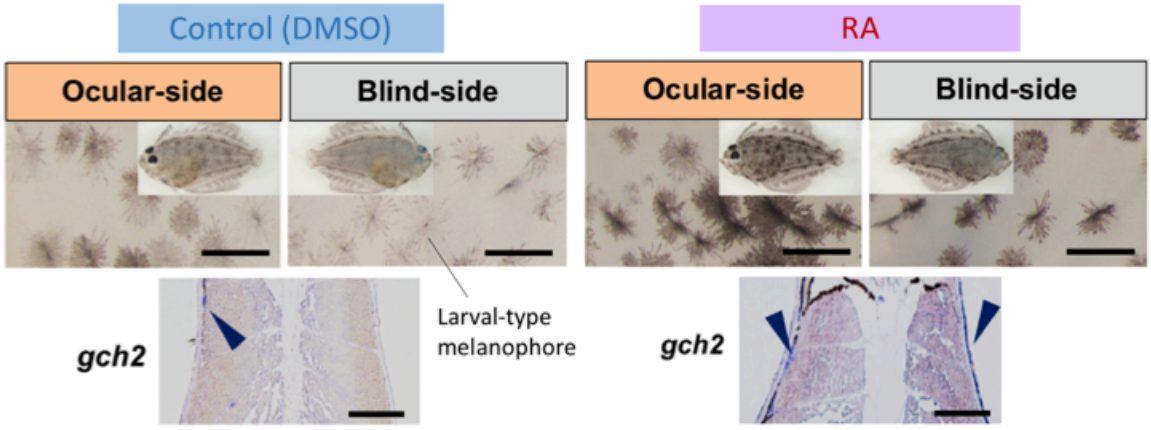

Figure 9. Larval-type melanophores and *gch2* mRNA expressions in both sides of skins in control and RA-treated flounders after 3 days treatment.

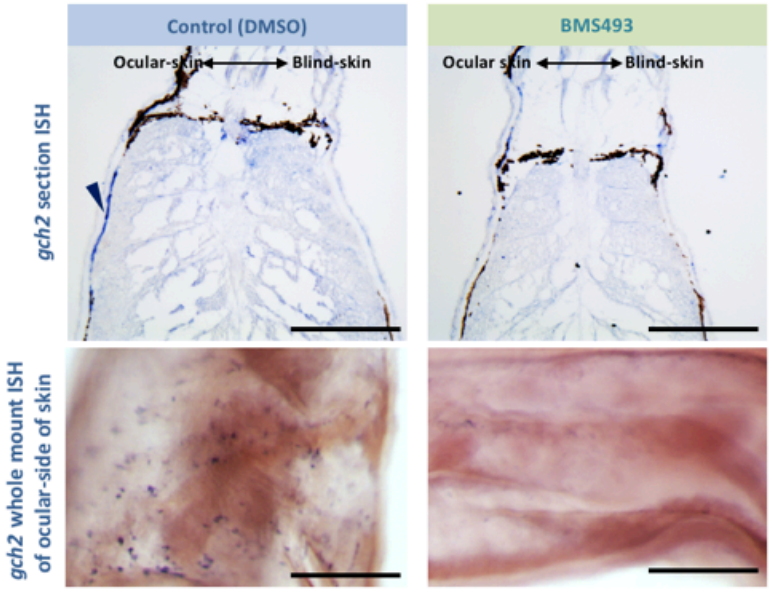

Figure 10. Suppression of *gch2*-positive chromatoblast differentiation at ocular-side skin by an RAR antagonist (BMS493).

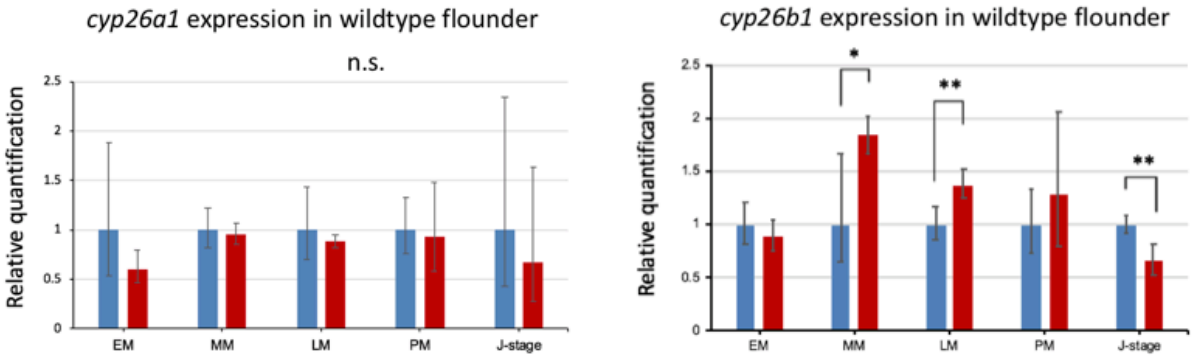

Figure 11. c*yp26a1* and *cy26b1* expression in metamorphosing larvae. Blue column, ocular-skin. Red column, blind-skin EM, early metamorphosis; ISH, in situ hybridization; MM, mid-metamorphosis; LM, late metamorphosis, PM, post-metamorphosis.

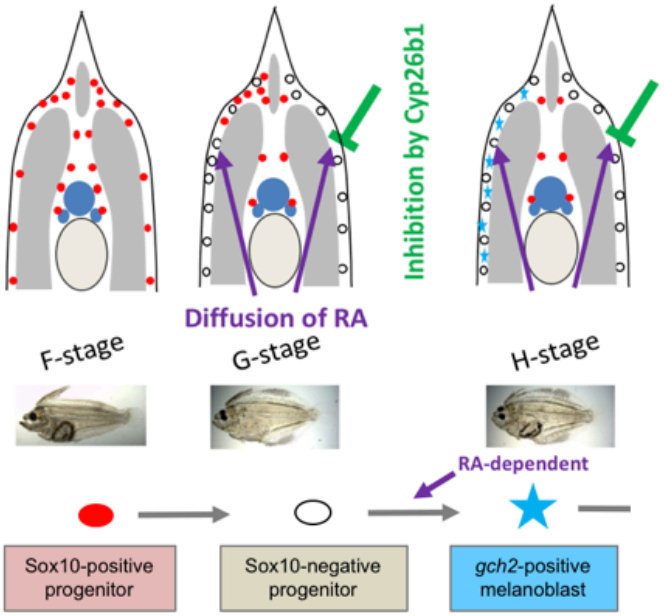

Figure 12. Scheme of the metamorphic chromatophore differentiation process and the developmental regulatory system for ocular-side-specific pigmentation in flounders during metamorphosis.

論文審査の結果の要旨及び担当者

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学位論文題目	Study on developmental roles of retinoic acid and its regulation in metamorphosing Japanese flounder larvae (変態期ヒラメ仔魚におけるレチノイン酸とその代謝の発生学的機能に関する研究)

論 文 審 査 の 結 果 の 要 旨

ヒラメは東アジアにおいて商業的に最も養殖されている異体類である。また、仔魚期においては他の魚類と同様に左右対称であるものの、変態期に眼球の移動や有眼側特異的な色素胞分化などにより左右非対称な成魚の体制となるため、非対称性に関する基礎生物学的な研究のモデルとしても興味深い。ヒラメの増養殖において無眼側皮膚の異常黒化や骨格異常が頻繁に生じ、市場における価値の下落や資源保護のための放流において生存率の低下の原因となっている。ビタミン A はヒラメの発生における必須栄養素であるが、これら異常との関連が指摘されてきた。本研究は、ビタミン A の誘導体であるレチノイン酸がヒラメ変態における色素胞分化や骨形成に担う役割について詳細に解析を行ったものである。

本研究ではまず、レチノイン酸の代謝およびシグナル伝達に関連する遺伝子に着目し、レチノイン酸の合成酵素 Aldh1a、分解酵素である Cyp26、受容体としてシグナルを伝える Rar の発現を調べた。変態期を通して *aldh1a2* が腸や肝臓において強く発現していることを明らかにし、変態におけるレチノイン酸の主たる産生に関与している可能性を指摘した。また、レチノイン酸シグナル伝達が色素胞分化において重要な役割を担うことを示す証拠として、変態期において有眼側、無眼側のどちらの皮膚にも *rar* が検出されることを見出した。

ついで本研究では、レチノイン酸浸漬による比較トランスクリプトーム解析を行い、レチノイン酸シグナルにより制御される遺伝子を網羅的に単離した。レチノイン酸代謝の負のフィードバックと細胞外マトリックスへの影響を明らかにし、特に、*mmps* の発現と *col* ファミリー遺伝子の発現減少がレチノイン酸暴露で生じる脊椎骨融合を引き起こす可能性を指摘した。さらに、レチノイン酸処理した無眼側皮膚の異常黒化は、無眼側皮膚での *gch2* 陽性色素芽細胞の増加に関連している可能性を指摘した。

更に本研究では、化学物質暴露実験により、過剰なレチノイン酸が無眼側において色素胞分化を亢進し、レチノイン酸シグナルの阻害により有眼側の色素芽細胞の分化が抑制されることを見出し、色素胞分化においてレチノイン酸シグナルが非常に重要であることを示唆した。さらに、レチノイン酸の分解酵素である *cyp26b1* の無眼側皮膚での発現上昇が、レチノイン酸レベルを低下させ、左右非対称な色素胞分化を生じさせている可能性を指摘した。

以上のように本研究は、ヒラメ仔魚の変態においてレチノイン酸が色素胞分化や骨形成に担っている役割について解析し、レチノイン酸シグナル伝達と左右非対称な変態に関係する様々な遺伝子との間に強い関連性が存在することを見出した。これらの知見は、ヒラメを含む異体類のみならず、様々な魚種の養殖における体色異常、骨形成異常を防止するための技術開発に大きく寄与するものと考えられる。

以上のことから審査員一同は、博士（農学）の学位を授与するに値するものと認定した。