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Molecular Study on the Transcription Factors, Nkx2 and Pax8, and Clusterin Expressed in Endocrine Cells

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学 位 論 文 要 約

Summary of Doctoral Thesis

専　　攻：　バイオサイエンス
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論文題目：内分泌細胞に発現する転写因子 Nkx2 と Pax8 およびクラスタリンに関する分子生物学的研究

Title of Thesis：Molecular Biological Study on Transcription Factors, Nkx2 and Pax8, and Clusterin Expressed in Endocrine Cells

論文要約：

Summary：

The thyroid gland is one of the endocrine organs derived from the pharyngeal endoderm. The mammalian thyroid gland consists of two types of endocrine cells, thyroid follicular cells (TFCs) and calcitonin-producing cells (C cells). TFCs are the mono-layered epithelial cells that form thyroid follicles and produce thyroid hormones (White and Portfield, 2013), whereas C cells are scattered between thyroid follicles. In contrast, these cells form different endocrine glands in non-mammalian vertebrates: TFCs form the thyroid gland and C cells form the ultimobranchial gland (UG). The structure and location of these glands are diverse among vertebrates: *e.g.* thyroid follicles are loosely distributed along the ventral aorta in many teleosts. Nevertheless, through the vertebrates, thyroid follicles work as a functional unit to produce THs, and each C cell produces calcitonin. It is therefore considered that the expression of transcription factors necessary for hormone production is conserved in TFCs and C cells through vertebrates.

The outline of the molecular machinery for thyroid hormone synthesis and secretion is well known in mammals (Kovacs and Ojeda, 2011). According to this scenario, iodide is accumulated in the follicular epithelial cells through the sodium/iodide symporter (NIS), and incorporated into tyrosyl residues within thyroglobulin (TG) by thyroid peroxidase (TPO). TPO can also catalyze the oxidative coupling of iodotyrosines, followed by storage of the iodinated TG in the colloid. The thyroid hormones are released after endocytosis of the colloid, which is regulated by thyroid-stimulating hormone. This unique phenotype of TFCs is indispensable for thyroid hormone synthesis, and maintained by the fundamental action of a specific set of transcription factors, including Pax8, Nkx2-1 (also known as TTF1, TITF1, or T/EBP), FoxE1 (also called TTF2 or TITF2), HHEX, and TAZ (Damante

et al., 2001; De Felice and Di Lauro, 2011; Di Palma et al., 2009; Kimura, 2011).

Pax8 is a member of the Pax family of vertebrate paired-domain proteins, involved in tissue differentiation and organogenesis (Blake et al., 2008; Underhill, 2012). In mammals, it has been documented that Pax8 functions as a key regulator for the maximal transcription of TG (Miccadei et al., 2002), TPO (Miccadei et al., 2002), and NIS genes (Taki et al., 2002). To our knowledge, however, there was no experimental evidence on the functional property of non-mammalian Pax8 in the thyroid gland. In the present study, we have identified two distinct Pax8 (a and b) mRNAs from the thyroid gland of the rainbow trout (*Oncorhynchus mykiss*). The mRNAs for rainbow trout Pax8a (accession number AB828387) and Pax8b (AB828388) were composed of 2734 and 3290 bases, respectively, excluding the poly(A) tail. The Pax8a protein consisted of 458 amino acid residues and contained three characteristic domains: *i.e.* the paired domain, octapeptide, and partial homeodomain. The nucleotide sequence of Pax8b mRNA was identical with that of Pax8a mRNA except for three regions. The Pax8b mRNA lacked the 5' non-coding terminus of 30 bases seen in Pax8a mRNA (region I) and a coding sequence corresponding to the position 23 to 46 of Pax8a mRNA (region II), while an addition of a 610-nucleotide sequence was found at the position 860 of Pax8b mRNA (region III). Because of a deletion of region II, Pax8b was predicted to lack an N-terminal portion of the paired domain. Additionally, an insertion of region III changed the reading frame of Pax8b mRNA, which resulted in the deletion of the C-terminal portion, corresponding to that from Asp294 to Leu458 in Pax8a. The site of this insertion corresponded to the boundary between exon 8 and exon 9 in human Pax8 gene (Poleev et al., 1995). The trout Pax8a showed 67.7%, 67.1%, 67.2%, and 76.0% amino acid similarity to human Pax8, mouse Pax8, *Xenopus* Pax8, and zebrafish Pax8, respectively. In vertebrates, Pax genes are classified into four subgroups comprising Pax1/9, Pax2/5/8, Pax3/7, and Pax4/6 (Underhill, 2012). To verify the phylogenetic position of trout Pax8s, a neighbor-joining (NJ) tree was constructed using the amino acid sequences of vertebrate Pax2/5/8 proteins. The topology of this tree indicated that both trout Pax8a and Pax8b are assigned to the cluster containing zebrafish, *Xenopus*, mouse, and human Pax8 proteins. Tissue distribution of the trout Pax8a and Pax8b mRNAs was examined by RT-PCR using specific primers. The RT-PCR analysis showed that both the Pax8a and Pax8b mRNAs were expressed mainly in the thyroid tissue and kidney. The Pax8a mRNA was also observed very weakly in the ovary and testis. Because the thyroid tissue used in the RT-PCR analysis included the ventral aorta, the expression site of trout Pax8 mRNA was examined by in situ hybridization histochemistry. Specific antisense probes that distinguish Pax8a mRNA from Pax8b mRNA could not be made due to the very high nucleotide identity between these molecules. Therefore, we utilized a cRNA probe that

can bind to both the Pax8a and Pax8b mRNAs. The hybridization of paraffin sections with this probe exhibited that the signals for Pax8 mRNA resided in the monolayered TFCs in the adult trout. Whole mount *in situ* hybridization histochemistry was also conducted for the trout embryos 15 days postfertilization. It is known that in the zebrafish, the thyroid anlage appears in a ventral region of the midline endoderm near the heart tube (Porazzi et al., 2009; Wendl et al., 2007). Accordingly, hybridization signals for Pax8 transcripts were detected, as a single patch, at the posterior end of the ventromedial line in the pharyngeal region. Paraffin sectioning of the whole mounts revealed that the signals were confined to the thyroid primordium close to the developing ventral aorta. Transient transfection studies were carried out to elucidate the transcriptional activities of trout Pax8a and Pax8b. Initially, HeLa cells were transfected with the -713 bp rat TPO promoter-luciferase construct and with either the trout Pax8a or Pax8b-expression vectors. The luciferase activities indicated that Pax8a stimulated promoter transcription by approximately 4-fold as compared with the control vector, whereas transcription was not induced by Pax8b. In mammals, it is reported that Pax8 and Nkx2-1 could cooperatively activate the TPO gene via interplay of the enhancer and gene promoter (Miccadei et al., 2002). Therefore, we next used the 6.3-kbp human genomic fragment, which includes the TPO promoter and enhancer, to assess the effects of Pax8a and/or Pax8b on the transcriptional property of Nkx2-1. Transfection with rat Nkx2-1 showed an about 4-fold increase in luciferase activity from the 6.3-kbp TPO upstream region, agreeing well with the result by Miccadei et al., 2002. Pax8a or Pax8b alone did not increase luciferase activity, but co-transfection with Pax8a and rat Nkx2-1 induced a synergistic increase in luciferase transcription by nearly 25-fold. In contrast, Pax8b did not activate reporter transcription even when co-transfected with Nkx2-1, and rather reduced the synergistic increase in reporter activity by Pax8a and Nkx2-1. Binding sites of trout Pax8a and Pax8b were investigated by Electrophoretic mobility shift assay (EMSA) using two types of synthetic oligonucleotides: *i.e.* oligonucleotide CT (Zannini et al., 1992), and oligo B (Kikkawa et al., 1990). The former was derived from the region -64 to -41 in the rat TPO promoter, which includes a binding site for mammalian Pax8 (Francis-Lang et al., 1992; Zannini et al., 1992). The latter corresponds to a 34-bp portion in the enhancer region located approximately 5.5 kbp upstream of the human TPO gene, which binds to mammalian thyroid-specific proteins (Kikkawa et al., 1990). The EMSA using the FAM-labelled oligonucleotide probes demonstrated distinctive retarded bands representing the interaction of Pax8a with oligonucleotide CT and oligo B. These retarded bands were eliminated by addition of non-labelled oligonucleotide CT or oligo B, confirming the specific binding of Pax8a and these oligonucleotides. Similarly, shifted electrophoretic bands were detected when Pax8b

was tested for oligonucleotide CT and oligo B. Those shifted bands were also abrogated by addition of non-labelled oligonucleotide CT or oligo B. No retarded complexes were observed when the Tag polypeptide, produced from the pET32a, was mixed with oligonucleotide CT or oligo B. In addition, it was confirmed that rat Nkx2-1 can specifically bind to the oligo B, as shown previously (Mizuno et al., 1991). Collectively, the results suggest that for the trout thyroid gland, Pax8a may directly increase TPO gene expression in cooperation with Nkx2-1 while Pax8b may work as a non-activating competitor for the TPO transcription.

NKX2-1 is a member of the NK2 family that consists of transcription factors with a unique NK2 homeodomain (Bingle, 1997; Boggaram, 2009). The NK2 family members are widely distributed from invertebrates to vertebrates, and are involved in tissue differentiation and organogenesis (Stanfel et al., 2005). In vertebrates, the members of this family are designated as Nkx2 and are currently classified into ten types, Nkx2-1 to Nkx2-10, based on the order of discovery (Newman et al., 2000; Price et al., 1992; Stanfel et al., 2005). Among them, Nkx2-1 is of prime importance for the thyroid development and the regulation of thyroid-specific gene expression. The NKX2-1 gene is expressed not only in the thyroid gland, but also in the lung and embryonic diencephalon (Lazzaro et al., 1991). Nkx2-1 null mice failed to form the thyroid, lung, ventral forebrain, and pituitary, showing an essential role of NKX2-1 in the development of these organs (Kimura et al., 1996; Silberschmidt et al., 2011). NKX2-1 is also shown to function as a key regulator for the maximal transcription of TG (Sinclair et al., 1990), TPO (Francis-Lang et al., 1992), TSH receptor (Civitareale et al., 1993) and NIS genes (Endo et al., 1997). Additionally, gene expression of NKX2-3 and NKX2-5 is detected in the mouse thyroid during development (Fagman and Nilsson, 2011). For non-mammalian vertebrates, there is limited information on the Nkx2 molecules expressed in the thyroid gland. Hence, in the present study, we have carried out cDNA cloning to clarify which type of Nkx2 is expressed in the trout thyroid, and obtained three distinct cDNAs encoding three forms of Nkx2. To verify the phylogenetic position of the trout Nkx proteins, an NJ tree was constructed using the amino acid sequences of 42 Nkx family members from vertebrates. The topology of this tree indicated that all the trout Nkx proteins are assigned to the cluster including fugu, *Xenopus* and human Nkx2-4s. In addition, synteny analysis using genome data from the zebrafish, stickleback, *X. tropicalis*, and mammals confirmed that genes encoding fish Nkx2-4 proteins are the orthologues of mammalian Nkx2-4 and different from Nkx2-1 genes. Hence, we have designated the trout Nkx proteins as Nkx2-4a, Nkx2-4b, and Nkx2-4c. The mRNAs for Nkx2-4a (AB159045), Nkx2-4b (AB159044), and Nkx2-4c (AB159043) consisted of 2626, 2127, and 2573 bases, respectively, excluding the poly(A)

tail. All the Nkx2-4 proteins were predicted to conserve three characteristic domains: the tinman-like amino terminal decapeptide, NK2 homeodomain, and NK2-specific domain, and these domains were nearly identical to those of mammalian NKX2-1. The NJ tree further showed that Nkx2-4b and Nkx2-4c have close relationships, whilst Nkx2-4a is slightly distant from them. Transient transfection studies were carried out to elucidate the transcriptional activities of trout Nkx2-4 isoforms. Initially, HeLa cells were co-transfected with the -170 bp rat TG promoter-luciferase construct and with either rat NKX2-1- or trout Nkx2-4a-expression vectors, to assess the applicability of the heterologous luciferase assay system. Although the TG promoter was more efficiently activated by rat NKX2-1, trout Nkx2-4a could induce a significant increase in transcription from the promoter, confirming that this assay system can be utilized to estimate transcriptional activity of trout Nkx2-4s. The TG promoter was then transactivated with trout Nkx2-4a, Nkx2-4b, and Nkx2-4c, and their effects were compared. The luciferase activities revealed that Nkx2-4a significantly stimulated promoter transcription, comparable to Nkx2.1b, whereas only faint transcription was induced by Nkx2-4c. Binding sites of trout Nkx2-4 isoforms were investigated by EMSA using FAM-labelled synthetic oligonucleotide C (Guazzi et al., 1990). This oligonucleotide was derived from the region -83 to -60 in the rat TG promoter, which includes a binding site for mammalian NKX2-1 (Damante et al., 2001; Musti et al., 1987). The EMSA demonstrated distinctive retarded bands representing the interaction of oligonucleotide C with trout Nkx2-4a, Nkx2-4b and Nkx2-4c. These retarded bands were abrogated by addition of non-labelled oligonucleotide C, confirming the specific binding of Nkx2-4 isoforms and this oligonucleotide. No retarded complexes were observed when the Tag polypeptide, produced from the pET32a, was mixed with oligonucleotide C. In addition, it was confirmed that rat NKX2-1 can specifically bind to the oligonucleotide C, as shown previously (Guazzi et al., 1990). Tissue distribution of Nkx2-4a, Nkx2-4b and Nkx2-4c mRNAs was examined in the rainbow trout by RT-PCR using specific primers. The specificity of primers for each of the Nkx2-4 cDNAs had been confirmed by performing PCR with the Nkx2-4 clones as templates. The RT-PCR analysis showed that both Nkx2-4a and Nkx2-4b mRNAs were expressed mainly in the thyroid tissue, although their expression was also observed very weakly in the testis. On the other hand, the expression pattern of Nkx2-4c transcripts was different, since these were detected in the ovary as well as in the thyroid tissue. It was also revealed that Nkx2-4a, Nkx2-4b and Nkx2-4c mRNAs were all expressed in the thyroid tissue of a single trout, suggesting the presence of three Nkx2-4 genes in the genome of rainbow trout. The expression sites of Nkx2-4a and Nkx2-4c mRNAs were further examined by *in situ* hybridization histochemistry with DIG-labelled antisense RNA probes. The hybridization signals for

Nkx2-4a mRNA were localized to the monolayered epithelial cells of trout thyroid follicles. Likewise, Nkx2-4c mRNA was detected in these cells of the thyroid, but its signal was also observed in the granulosa cells of the ovary. Taken together, the results suggest that in the rainbow trout, Nkx2-4a and Nkx2-4b might play a major role in TG gene transcription whereas Nkx2-4c might have some functions in the ovary as well as the thyroid. On the other hand, we have found that Nkx2-1 might play a role in CT gene expression in the trout UG.

In this study, we found that clusterin (CLU) is highly expressed in the trout UG by suppression subtractive hybridization method. CLU, as known as apolipoprotein J, is disulfide linked glycoprotein (Shanan et al., 2005) and is expressed in various tissues. CLU is known as multifunctional protein, but its fundamental function is not known. Furthermore, there is no experimental report about the functions of CLU in the fish. To elucidate the function of CLU, we generated the CLU mutant fish by utilizing the Targeting Induced Local Lesions IN Genomes (TILLING) method, one of the reverse genetic methods to generate mutant fish. Additionally, we performed the high resolution melting curve analysis (HRM) to distinguish the TILLING mutants (Masuyama et al., 2012, Ishikawa et al., 2013, Okuyama et al., 2014). By HRM, heterozygous mutants can be easily distinguished from homozygous mutants, but it is difficult to separate homozygous mutants from wild-type animals because both homozygotes and wild-types have their own perfect matched DNA sequences. Therefore, we improved HRM and developed an effective, easy and rapid genotyping method to distinguish homozygotes from the others.

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