RESEARCH ARTICLE

Reiterative expression of pax1 directs pharyngeal pouch segmentation in medaka

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ABSTRACT

A striking characteristic of vertebrate development is the pharyngeal arches, which are a series of bulges on the lateral surface of the head of vertebrate embryos. Although each pharyngeal arch is segmented by the reiterative formation of endodermal outpocketings called pharyngeal pouches, the molecular network underlying the reiterative pattern remains unclear. Here, we show that pax1 plays crucial roles in pouch segmentation in medaka (Oryzias latipes) embryos. Importantly, pax1 expression in the endoderm prefigures the location of the next pouch before the cells bud from the epithelium. TALEN-generated pax1 mutants did not form pharyngeal pouches posterior to the second arch. Segmental expression of tbx1 and fgf3, which play essential roles in pouch development, was almost non-existent in the pharyngeal endoderm of pax1 mutants, with disturbance of the reiterative pattern of pax1 expression. These results suggest that pax1 plays a key role in generating the primary pattern for segmentation in the pharyngeal endoderm by regulating tbx1 and fgf3 expression. Our findings illustrate the crucial roles of pax1 in vertebrate pharyngeal segmentation and provide insights into the evolutionary origin of the deuterostome gill slit.

KEY WORDS: Pharyngeal arch, Pharyngeal pouch, Gill slit, Segmentation, Evolution, Pax1

INTRODUCTION

The metamerism of vertebrate pharyngeal structures, such as the skeletal elements of jaws, gills and cranial nerve projections, originates from segmental development of the pharyngeal arches, which are transient embryonic structures seen in all vertebrate embryos (Graham and Richardson, 2012). The pharyngeal arches are formed from all three germ layers, and the cranial neural crest cells were traditionally thought to play a crucial role in arch segmental development (Noden, 1988). However, experimental ablation of the cranial crest cells does not affect segmental development of the reiterative endodermal outpocketings (called pharyngeal pouches) or the expression patterns of Bmp7, Fgf8, Shh and Pax1 in the pouches (Veitch et al., 1999).

Numerous studies have reported the roles of different signaling molecules and transcription factors in pouch segmentation. Retinoic acid (RA), a morphogen that globally regulates vertebrate head development, is required for posterior pouch segmentation in mice (Wendling et al., 2000), quail (Quinlan et al., 2002), zebrafish (Kopinke et al., 2006) and lamprey (Kuratani et al., 1998) (a jawless vertebrate). FGF signaling also contributes to pouch segmentation in both gnathostomes (jawed vertebrates) and lamprey (Abu-Issa et al., 2002; Crump et al., 2004; Jandzik et al., 2014). In zebrafish, pouch-specific expression of fgf3 is considered to be responsible for endodermal pouch patterning as well as subsequent chondrogenesis (David et al., 2002; Crump et al., 2004; Herzog et al., 2004). The indispensable role of Tbx1 in pharyngeal segmentation was revealed through studies using mouse and zebrafish mutants that sought to identify candidate genes involved in DiGeorge syndrome (Jerome and Papaioannou, 2001; Piotrowski et al., 2003; Xu et al., 2005). Tbx1 is expressed in the pharyngeal ectoderm, endoderm and mesoderm (Chapman et al., 1996; Vitelli et al., 2002; Piotrowski et al., 2003). In mice, although mesodermal Tbx1 is required for proper pouch development (Zhang et al., 2006), dynamic expression of Ripply3, which encodes a Tbx1 repressor, regulates the endodermal activity of Tbx1 to form pouches posterior to the second arch (Okubo et al., 2011). In zebrafish, mesodermal tbx1 drives endodermal pouch morphogenesis by upregulating the expression of wnt11r and fgf8 in a cell-autonomous manner (Choe and Crump, 2014). It was also reported that the segmental expression pattern of fgf3 is retained in the pharyngeal endoderm of tbx1 mutants and that both mesodermal and endodermal Tbx1 play roles ensuring proper pouch segmentation (Choe and Crump, 2014). Therefore, the regulatory network for the endodermal expression of tbx1 and fgf3 is crucial for generating the reiterative pattern of pharyngeal segmentation. However, we know little about the molecular mechanisms of segmental pattern formation in the pharyngeal endoderm, especially regarding regulation of the endodermal expression of tbx1 and fgf3.

Endodermal pharyngeal pouches are not specific to vertebrates but are common in deuterostome animals, including fossil echinoderms (Clausen and Smith, 2005), as pharyngeal gill slits. Previous studies demonstrated the remarkable conservation of the expression patterns of genes governing the development of vertebrate pharyngeal pouches and the gill slits of non-vertebrate deuterostomes, clearly illustrating the homology between these structures (Holland et al., 1995; Müller et al., 1996; Wallin et al., 1996; Ogasawara et al., 1999, 2000; Lowe et al., 2003; Gillis et al., 2012). Therefore, revealing the mechanism of segmented pharyngeal pouch formation is indispensable for shedding light on not only vertebrate developmental principles but also the evolutionary origins of the vertebrate body plan (Graham et al., 2014).

In this study, we focused on pax1, which encodes a paired-box transcription factor. Conserved expression of pax1/9 homologs in...
pharyngeal gill slits provides key evidence of the homology between deuterostome gill slits and pharyngeal pouches (Holland et al., 1995; Müller et al., 1996; Wallin et al., 1996; Ogasawara et al., 1999, 2000; Lowe et al., 2003; Gillis et al., 2012). Previous reports clearly demonstrated the developmental functions of Pax1 in organogenesis of the thymus and parathyroid glands, sclerotome delineation, chondrogenesis and vertebral column formation (Wallin et al., 1996; Peters et al., 1999; Su et al., 2001; Mise et al., 2008). These studies were undertaken in mice, but no studies have elucidated the function of Pax1 in pharyngeal pouch segmentation, as the reiterative pouches are retained even in the endoderm of Pax1;Pax9 double-knockout mice (Zou et al., 2006). Previously, we unexpectedly identified a crucial function of Pax1 in pouch segmentation in medaka (Mise et al., 2008). Here, we show that the expression pattern of Pax1 is dynamic and prefigures the future location of pouches. We analyzed Pax1 mutant medaka generated by TALEN-mediated mutagenesis and reveal the indispensable functions of Pax1 in both the reiterative expression of tbx1 and fgf3 in the pharyngeal endoderm forming posterior to the second arch and in subsequent pouch segmentation.

RESULTS

Roles of Pax1 in formation of the segmental structures of the medaka pharynx

Teleost embryos develop seven pharyngeal arches. Starting from the anterior end, the first (or mandibular) arch develops Meckel’s and palatoquadrate mandibular cartilages (Fig. 1A). The second (or hyoid) arch contributes to the basihyal, ceratohyal and hyosymplectic cartilages, and the third to seventh arches give rise to a series of ceratobranchial and basibranchial cartilages (Fig. 1A). All Pax1 mutant larvae exhibited complete loss of the ceratobranchial cartilages, except for the most posterior (seventh) arch with pharyngeal teeth. The basibranchial cartilage of pharyngeal arches 3-6 was highly deformed (Fig. 1B, Table S2). Although other pharyngeal cartilages were retained, a hole in the pharyngeal arches 3-6 was highly deformed (Fig. 1B, Table S2). By contrast, the components of the mandibular arch rarely exhibited abnormalities (Fig. 1B, Table S2).

The cranial nerve is another structure that exhibits segmental organization, projecting itself into each pharyngeal arch (Fig. 1C). Of the ten cranial nerves in teleosts, cranial nerves V (trigeminal), VII (facial), XI (glossopharyngeal) and X (vagus) run into distinct pharyngeal arches (Fig. 1C). In Pax1 mutants, projections of cranial nerves IX and X were specifically suppressed, whereas projections of cranial nerves V and VII were unaffected (Fig. 1D). The innervation of cranial nerve IX was less affected than the branches of cranial nerve X, but its destination was the dorsal area of the segmental structures of the pharyngeal arches (Fig. 1C). The innervation of cranial nerve IX was less affected than the branches of cranial nerve X, but its destination was the dorsal area of the second arch (Fig. 1D, arrowhead and lower panel).

Additionally, we examined the expression of foxN1, which is normally detected in the thymus primordium (Li et al., 2007) (Fig. 1E,F). In Pax1 mutants, thymus-specific expression of foxN1 was lost (Fig. 1F). As these mutant phenotypes are often associated with pharyngeal pouch defects, we further investigated the expression pattern and function of Pax1 during pharyngeal pouch segmentation.

Expression of Pax1 prefigures the location of future pouches

In order to document the role of Pax1 in the pharyngeal endoderm, we examined a detailed temporal profile of Pax1 expression. At early stage 21 (corresponding to the 8-somite stage), Pax1 expression was observed in the lateral cells of the anterior pharyngeal endoderm (Fig. 2A). More posteriorly, expression was also observed in bilateral spots of cells (Fig. 2A). Compared with the endodermal expression of foxN1, these posterior spots seemed to mark locations for cells to bud off to form the following pouch (Fig. 2A-E). We also detected endoderm-specific pharyngeal expression of Pax1 by double-fluorescence in situ hybridization for Pax1 and other marker genes (Fig. 3). At late stage 22 (the 10-somite stage), Pax1 expression specifically marked the first and second pouches (Fig. 2F,G). Notably, other bilateral spots of Pax1 expression were detected in a posterior region relative to the second pouch (Fig. 2F, H, I, arrowheads). As the pharyngeal pouches

![Fig. 1. Roles of Pax1 in the pharyngeal arches, cranial nerve projections and thymus primordium.](image)
develop in an oblique manner along the dorsoventral axis, the signal appeared continuous from the second pouch in the ventral view (Fig. 2F,H). A lateral view clearly indicated that the posterior pax1-positive cells were separated from the pax1-positive anterior pouch cells (Fig. 2F,I, arrowheads). This posterior expression might mark cells of the nascent third pouch, as at stage 23 (the 12-somite stage) the most posterior expression of pax1 was detected in the third pouch (Fig. 2J-M, arrowheads). At stage 24 (the 16-somite stage), the bilateral spots of pax1 expression appeared just posterior to the third pouch (Fig. 2N-Q, arrows). This expression is presumed to mark the next (fourth) pouch region (Fig. 2N,P,Q, arrows). Continuous pax1-positive domains ranging over the emerging pouch were not observed at any stage. These results suggest that pax1 expression prefigures the position where the next pouch will be formed during the serial reiteration of posterior pouch development.

Reiterative development of the third and posterior pouches requires pax1

In order to determine the function of pax1 in pouch segmentation, we examined the morphology of pharyngeal pouches and arches in wild-type and pax1−/− medaka by analyzing marker gene expression patterns. First, we examined the endoderm by monitoring foxA2. In wild-type embryos at stage 23, bilayered outpocketings of endoderm epithelium were observed at the first, second and third pouches (Fig. 4A). However, in pax1 mutants, the posterior epithelium of the second pouch failed to fold, and no outpocketing was observed posterior to it (Fig. 4B). Consistently, the expression pattern of dlx2, which marks neural crest cells, showed that neural crest cells failed to segregate into segments posterior to the third arch in pax1 mutants, perhaps owing to the third pouch defect (Fig. 4C,D). The defects in pouch segmentation were more evident at stage 27 (the stage of fifth pouch formation), when pax1 mutant embryos never exhibited segmental pouches at the axial level of the third or posterior pouches (Fig. 4E,F). A few irregular slits forming posterior to the second arch were found in pax1 mutants at stage 27 (Fig. 4E,F). Additional mutants exhibited the normal anterior epithelial sheet of the second pouch, lining the second arch backward. However, these did not develop the bilayered morphology of the second pouch but rather a monolayer sheet (Fig. 4B,F,J, Fig. S2H). These abnormalities of the second pouch might be caused by the failure of the posterior half of the second pouch to develop. In concordance with the absence of
the third to fifth pouches, the segmental distribution of neural crest cells in the pharyngeal arches was disrupted in the pax1 mutants (Fig. 4G,H). In pax1 mutants, the crest cells were distributed so as to surround the unsegmented endodermal cells (Fig. 4G,H).

We also examined the expression patterns of other marker genes at stage 27. The expression of nkx2.3, which is often used as a pharyngeal pouch marker in zebrafish studies (Lee et al., 1996), showed five pairs of segmental pouches in wild-type medaka (Fig. 4I). Similar to foxA2, the expression of nkx2.3 in pax1 mutants did not show any reiterative pouch pattern, except for the first two pouches (Fig. 4I). As a mesodermal marker, we monitored the expression of tcf21, which is also known as capsulin in zebrafish (Lee et al., 2011). In pax1 mutants, the expression of tcf21 marked the mesodermal cores of the first and second arches, but the posterior expression never exhibited the reiterative distribution of the arch cores (Fig. 4K,L). The mesodermal distribution pattern was similar to that of the neural crest cells, despite being sparser (Fig. 4L,H).

Because of the endoderm-specific expression of pax1 in the pharynx (Fig. 3), the abnormal distribution patterns of neural crest cells and mesoderm were likely to be secondary effects of the endodermal pouch defects. These defects in pharyngeal segmentation were in complete agreement with the results of genotyping analyses of our pax1 mutant allele (n=89/89, Fig. S1C). Additionally, a pax1-specific morpholinio (Mise et al., 2008) phenocopied the pax1 mutant phenotype (Fig. S3). These results rule out the possibility that off-target effects associated with TALEN caused the pharyngeal pouch defects. We therefore conclude that pax1 plays crucial roles in the reiterative development of pharyngeal pouches forming posterior to the second arch and in the segmentation of subsequent pharyngeal pouches.

**Loss of fgf3 and tbx1 expression in the pharyngeal endoderm of pax1 mutants**

In order to elucidate the function of pax1 in pouch segmentation, the regulatory relationships with other segmentation genes known for their function in pharyngeal development were investigated. We first examined the effect of pax1 on tbx1, which is expressed in the endoderm, mesoderm and perhaps the ectoderm of the pharynx (Piotrowski et al., 2003). At stage 24, when pax1 expression was detected in the nascent fourth pouch endoderm (Fig. 2N-Q), tbx1 was also expressed in the pharyngeal pouches and the posterior endoderm (Fig. 5A,C, arrowhead). In pax1 mutants, the expression of tbx1 in the third pouch and the fourth pouch region was dramatically reduced, although expression in the anterior pouches was unaffected (Fig. 5F). Comparison of the expression patterns of tbx1 and endodermal foxA2 (Fig. 5E,J) and mesodermal tcf21 (Fig. 5B,D,G,I) revealed a clear endoderm-specific reduction in tbx1 expression (Fig. 5F,H). At stage 27, endodermal expression of tbx1 was detected in five pairs of pharyngeal pouches in wild-type embryos (Fig. 5K,L,O). However, except for the first and anterior half of the second pouch, almost no endodermal expression of tbx1 was observed in pax1 mutants (Fig. 5R,S,V). Notably, mesodermal expression of tbx1 and tcf21 in the pax1 mutants was stable, indicating that the decline in tbx1 expression in the mutant embryos was endoderm specific (Fig. 5S,T,V,W). These results suggest that the segmental expression of tbx1 in pharyngeal pouches requires Pax1.

We also examined the expression of fgf3, as the skeletal pattern of pax1 mutant medaka was almost identical to that of fgf3-deficient zebrafish (David et al., 2002; Herzog et al., 2004). In zebrafish, the expression of fgf3 in the pharyngeal endoderm of tbx1 mutants is not affected, although they lack pouch segmentation (Choe and Crump, 2014). At stage 23 in the wild type, expression of fgf3 was detected in endodermal cells of the first, second and third pouches as well as in the midbrain-hindbrain boundary, and fgf3 expression in the first and third pouches was much weaker than in the second pouch (Fig. 6A). In pax1 mutants, almost no fgf3 expression was observed in the pharyngeal pouches, including the anterior pouches (Fig. 6B, asterisks and brackets). This pock-specific disruption in fgf3 expression was also observed in pax1 mutants at stage 27 (Fig. 6D, asterisks and brackets), suggesting that pax1 is necessary for activation of the segmental expression of fgf3 in the pharyngeal pouches. These results are consistent with our observation of skeletal defects in pax1 mutant larvae and with the skeletal phenotypes associated with the zebrafish fgf3 morphant and mutant (David et al., 2002; Herzog et al., 2004). Importantly, our data suggest that reiterative expression of pax1 is crucial for the segmental expression of both tbx1 and fgf3 in the endoderm.

In zebrafish, wnt11r is reported to be expressed in the pharyngeal mesoderm in a segmental manner, and its signaling initiates the epithelial destabilization of the endoderm to form pouches (Choe et al., 2013; Choe and Crump, 2014). We therefore examined whether wnt11r regulates the reiterative expression of pax1 in medaka endoderm. However, except in the mandibular arch mesoderm, no expression of wnt11r was detected in the pharyngeal arches of either wild-type or pax1 mutant medaka (Fig. S4).

**Loss of the reiterative pattern of pax1 expression in the pax1 mutant**

The mutant phenotypes described above indicate that pax1 plays a key role in establishing the primary reiterative pattern in the pharyngeal endoderm. We therefore examined the self-regulation of pax1 expression during pharyngeal pouch segmentation. Although the expression of pax1 in wild-type and pax1 mutant embryos was equivalent at stage 23, remarkably, the reiterative pattern of expression changed to a continuous pattern in the pax1 mutant while retaining independent expression in the first pouch (Fig. 7A,B). In pax1 mutants, the expression of pax1 in the lateral endoderm remained continuous posterior to the second arch at stage 27 (Fig. 7C,D). Examination of a horizontal section of the pax1 mutant embryo clearly showed the continuous expression of pax1 in the lateral endoderm where the posterior pouches failed to form (Fig. 7F).
We quantified apoptotic cells in pax1 mutants to determine whether the continuous expression of pax1 was due to cell death in the endoderm. At stage 26, TUNEL-positive cells were found in the pharyngeal regions of both wild-type and pax1 mutant embryos; however, no significant increase in the number of TUNEL-positive cells in the pharyngeal region was detected in the pax1 mutants (Fig. 7G-I). Notably, continuous pax1 expression extended to the posterior pharynx in the pax1 mutants, where the fifth pouch forms in wild-type embryos (Fig. 7C,D). The distance from the second to the posterior end of the fifth pouch did not differ significantly between wild-type and pax1 mutant embryos (Fig. 7J). These results indicate that pax1 regulates its own reiterative expression pattern and that signals for the activation of pax1 transcription might be constitutively active, with a progression to the posterior pharynx.

We also examined the expression pattern of pax9, a pax1 paralog. Expression of pax9 was detected throughout the whole area of the pharyngeal endoderm posterior to the second arch in both wild-type and in pax1 mutant embryos (Fig. S5). Therefore, of two pax1/9 cognates in medaka, the segmental expression pattern is specific to pax1.

**DISCUSSION**

**Impact of pax1 on pharyngeal segmentation and derivatives**

Our analysis of pax1 mutant medaka revealed the significant roles of pax1 in the development of the pharyngeal derivatives and pouches. The severe defects in the gill cartilages and the cranial nerve branches are thought to result from the loss of fgf3 in the pharyngeal endoderm. In zebrafish, both the fgf3 morphant and mutant cause defects in the ceratobranchial cartilages, as seen in the pax1 mutant medaka (David et al., 2002; Herzog et al., 2004). In zebrafish, fgf3 is also thought to be required for cranial nerve development, as fgf3 knockdown causes the loss of epibranchial placodes (Nechiporuk et al., 2005). Therefore, defects in the cartilages and cranial nerves in pax1 mutant medaka are generally thought to be due to the loss of...
endodermal fgf3. In addition to the function of pax1 in the pouch-
specific activation of fgf3, our results revealed another role of pax1 in
the activation of endodermal tbx1 segmental expression. Furthermore,
pax1 mutant medaka failed to develop the segmental pouches posterior to the second arch. Although considerable evidence demonstrates the requirement for tbx1 and fgf3 in pouch segmentation, the regulatory network of these genes is poorly
understood. We propose a model in which the reiterative expression of pax1 initiates segmental pouch formation by regulating tbx1 and fgf3 expression in the pharyngeal endoderm (Fig. 8). Importantly, pax1 is reiteratively expressed in the nascent pouch endoderm, and the segmental expression pattern of pax1 depends on the activity of Pax1 protein. Therefore, the reiterative pattern of pax1 expression is probably the primary pattern for pharyngeal segmentation.

Fig. 5. Pax1 is required for endodermal expression of tbx1. (A–J) Expression of tbx1 (A,C,F,H), tcf21 (B,D,G,I) and foxA2 (E,J) in the pharyngeal regions of wild-type (A–E) and pax1 mutant (F–J) embryos at stage 24. (C–E,H) Transverse sections around the axial level of the developing fourth pouch (arrowhead in A). Endodermal expression of tbx1 was specifically reduced in pax1 mutant embryos, whereas mesodermal expression was retained (F,H, n=7). (K–W) Expression of tbx1 (K,L,O,R,S,V), tcf21 (M,P,T,W) and foxA2 (N,Q,U) in the pharyngeal regions of wild-type (K–Q) and pax1 mutant (R–W) embryos at stage 27. (K,R) Upper and lower panels show left side and ventral views, respectively. (L–N,S–U) Horizontal sections of the pharyngeal region. (O–Q,V,W) Transverse sections around the axial level of the pharynx posterior to the otic vesicle. In pax1 mutants, endodermal expression of tbx1 was specifically reduced posterior to PP2, whereas mesodermal expression was retained (R,S,V, n=18). Asterisks and brackets indicate regions affected by the loss of pax1. OV, otic vesicle; PA, pharyngeal arch; PP, pharyngeal pouch; me, mesoderm; en, endoderm; NT, neural tube. Scale bars: 50 μm, except 100 μm in K,R.
In the pharyngeal endoderm of the pax1 mutant, we also observed loss of expression of foxN1, a gene normally expressed in cells of the thymus primordium (Li et al., 2007), indicating that pax1 is indispensable for thymus development in medaka. Although mouse Pax1 is necessary for proper development of the thymus epithelium, it is not sufficient for thymus development, as Foxn1 expression was observed in Pax1 single-mutant mice (Su et al., 2001). Even though indirect effects of the pax1 mutation on pouch defects must be considered, our results nevertheless shed light on the significant roles of pax1 in the development of the pharyngeal derivatives and pouches in medaka.

Reiterative endodermal expression of pax1 in the segmental development of pouches

Compared with segmentation of the somitic mesoderm and hindbrain, there is little information about segmentation of the pharyngeal arches (Graham et al., 2014; Choe and Crump, 2015). Even though previous studies have underscored the importance of the endoderm and mesoderm for pharyngeal segmentation, there is limited information on how segmentation is brought about in the endoderm (Graham et al., 2005; Choe and Crump, 2015). In this study, we found that medaka pax1 expression is activated reiteratively in cells where the next pouches will be formed. Additionally, we showed that endodermal pax1 plays an indispensable role in segmental pouch formation, except for the first pouch and anterior wall of the second pouch. Importantly, pax1 is required for the endodermal activation of thbx1 and fgf3 transcription, the functions of which in the development and patterning of endodermal pouches were described previously (Piotrowski et al., 2003; Crump et al., 2004; Herzog et al., 2004; Choe and Crump, 2014). The results of our TUNEL assay did not suggest an increase in apoptosis in the pharyngeal endoderm of the pax1 mutants. The size of the pharyngeal endoderm in the wild-type and pax1 mutant embryos did not differ significantly, suggesting that the loss of Pax1 does not cause developmental delay or loss of endoderm. These results indicate that pax1 is required for primary reiteration in pharyngeal pouch segmentation.

How does pax1 generate its reiterative expression pattern? The uniform pattern of pax1 expression seen in the pax1 mutant indicated that the reiterative pattern of pax1 expression in the endoderm requires Pax1 function. The transcription of pax1 is active throughout the pharyngeal endoderm in the absence of functional Pax1, suggesting that some form of negative regulation sets a pax1-negative region in the interpouch endoderm. In the vertebrate somite, Hes genes, which encode transcriptional repressors displaying an oscillatory expression pattern, play a role in the molecular clock through direct negative-feedback transcription loops (Hirata et al., 2002, 2004). Because Pax genes are basically transcription activators (Chalepakis et al., 1991; Noll, 1993), an indirect pathway might act to repress pax1 transcription. In contrast to the transient expression of Hes genes in somites through cell-autonomous repression, pax1 expression in pouches is retained after pouch segmentation and, therefore, repression of pax1 may function in a non-cell-autonomous manner (Fig. 8).

Previous studies showed that Fgf and RA signaling pathways are required for pharyngeal pouch segmentation, and phenotypes associated with Fgf and RA deficiencies are similar to the pax1 mutant phenotype in the endoderm (Wendling et al., 2000; Abu-Issa et al., 2002; Crump et al., 2004; Kopinke et al., 2006). In these previous studies, the expression patterns of pax1/9 cognates were affected, corresponding to defects in the posterior pouches caused by the lack of RA (Wendling et al., 2000). Additionally, Tbx1 reportedly modulates the dynamics of RA signaling in the developing vertebrate head by regulating Cyp26 genes, which encode RA-degrading enzymes (Roberts et al., 2006; Bothe et al., 2011). Regarding the function of Fgf signaling in pouch formation, it has been shown that its inhibition causes complete loss of the pharyngeal pouches forming posterior to the second arch (Abu-Issa et al., 2002; Crump et al., 2004). Even though the complete picture remains obscure, our results highlight the novel role of pax1 in activating the expression of thbx1 and fgf3. Therefore, the role of pax1 in pouch segmentation is probably tightly connected with the regulation of RA and Fgf signaling in the pharyngeal endoderm. Further investigations of the interplay and genetic relationships among relevant genes, including pax1 and signaling pathway genes, will contribute to a deeper knowledge of the mechanism of pharyngeal segmentation.

Evolution of the mechanism of pharyngeal segmentation: from gill slits to pharyngeal arches

That pax1 mutant medaka exhibit serious pouch defects is rather surprising, given that Pax1 knockout mice reportedly show minimal defects in pharyngeal segmentation and, even in Pax1/Pax9 double-homozygous mutant mice, no defects in pharyngeal segmentation have been reported (Su et al., 2001). This discrepancy in Pax1 knockout phenotypes between mice and medaka might be due to evolutionary changes in the genetic hierarchy of pax1/9 cognates,
tbx1, and other genes, and as such might constitute an example of developmental system drift (True and Haag, 2001). Previous studies in mouse reported that Pax1/Pax9 regulate later organogenesis, such as that of the thymus and parathyroid gland, and palate skeletogenesis, rather than pouch segmentation (Peters et al., 1998, 1999; Su et al., 2001). The evolution of the pharyngeal derivatives seems to be closely related to adaptation to a terrestrial lifestyle. During vertebrate evolution, degeneration of the posterior gill skeleton and a reduction in pouch number are evident, and a pouch-derived parathyroid gland would be necessary for control of calcium homeostasis in the terrestrial as opposed to aquatic environment (Okabe and Graham, 2004; Graham and Richardson, 2012).

Regarding the function of Pax1/Pax9 in pouch development in mice, one can consider the functional transition of Pax1/Pax9 from segmentation to later organogenesis, such as that of the thymus. Accompanying such functional transition, alternative factors might have been recruited to the pouch segmentation regulatory network. Ripply3, which encodes a repressor of Tbx1, is a conceivable candidate, as this gene is expressed in the mouse pharyngeal endoderm in a similar fashion to medaka pax1, and its function is necessary for the segmentation of the third and posterior pouches (Okubo et al., 2011). Interestingly, the pouch defects that we found in pax1 mutant medaka are almost identical to those seen in Ripply3 mutant mice. We could not identify the sequence of ripply3 in the whole-genome databases of medaka (Kasahara et al., 2007), stickleback (Jones et al., 2012), cod (Star et al., 2011), platyfish (Schartl et al., 2013), tilapia (http://ensembl.org/Oreochromis_niloticus/Info/Index), Amazon molly (http://ensembl.org/Poecilia_formosa/Info/Index) or puffer fish (Aparicio et al., 2002). In medaka, only one Ripply gene, annotated as ripply2, was found in the genome, but its expression was detected in paraxial mesoderm and not the pharynx (Fig. S6). However, we found a ripply3 gene in the genome of the following fish species: coelacanth (Amemiya et al., 2013), spotted gar (http://ensembl.org/Lepisosteus_oculatus/Info/Index), cavefish (McGaugh et al., 2014), rainbow trout (Berthelot et al., 2014) and zebrafish (Kettleborough et al., 2013). Considering the phylogenetic relationships among fish species (Nair et al., 2012), loss of the ripply3 gene is likely to have occurred.

**Fig. 7.** Pax1 is required for the reiterative pattern of pax1 expression in the pharyngeal endoderm. (A,B) Expression pattern of pax1 in wild-type (A) and pax1 mutant (B) embryos at stage 23. In pax1 mutants, the reiterative pattern of pax1 expression was disturbed, showing a continuous pattern posterior to PA2 (B, n=8). (C-F) Expression pattern of pax1 in wild-type (C,E) and pax1 mutant (D,F) embryos at stage 27. In pax1 mutants, the reiterative pax1 pattern observed in the wild-type embryos (C,E) was changed to a continuous pattern (D,F, n=8). (E,F) Horizontal sections of wild-type and pax1 mutant embryos stained with pax1 probe. (G,H) DAPI staining and TUNEL signals (arrowheads) in wild-type (G) and pax1 mutant (H) embryos at stage 26. White dotted lines delineate endodermal regions. (I) Quantification of apoptotic cells (TUNEL signals) in the pharynx of wild-type (n=14) and pax1 mutant (n=8) embryos in each region of the endoderm (endo, P=0.776), other regions (non-endo, P=0.207) and total (P=0.598) at stage 26. (J) Quantification of the size of the pharyngeal endoderm at stage 27. In wild type, the distance from the anterior epithelium of PP2 to the posterior epithelium of PP5 was measured (x_w, n=12). Because PP3-5 were not formed, the distance from the anterior epithelium of PP2 to the posterior limit of pax1 expression was measured in pax1 mutant embryos (x_m, n=9). x_w and x_m were not significantly different (P=0.413). Data represent mean±s.e.m. OV, otic vesicle; PP, pharyngeal pouch; en, endoderm; n.s., not significant. Scale bars: 100 μm in A-F; 50 μm in G,H.
This results in no endodermal expression of $pax1/9$ mutants, negative regulation of endoderm patterns gives rise to the segmental pharyngeal pouches. In a non-cell-autonomous manner. Reiteration of these bifacial ($pax1$ and $tbx1/10$) expression in the pharyngeal pouches (purple). Downstream targets of $Pax1$ transcription to create the interpouch endodermal regions (orange) in a non-cell-autonomous manner. Reiteration of these bifacial ($pax1$ and $tbx1/10$) expression patterns gives rise to the segmental pharyngeal pouches. In $pax1$ mutants, negative regulation of $pax1$ expression by $Pax1$ has no effect (pale purple). This results in no endodermal expression of $tbx1$ and $fgf3$ and continuous expression of $pax1$ in the posterior pharynx, whereas expression in $pp1$ and the anterior wall of $pp2$ is almost normal. $OV$, otic vesicle; $PP$, pharyngeal pouch.

**Staining**

Whole-mount skeletal staining with Alcian Blue (Sigma, A5268) was performed using a modified protocol (Yasutake et al., 2004). Details are provided in the supplementary Materials and Methods.

Whole-mount immunostaining of medaka embryos was performed as previously described (Sakai et al., 2007). Neural axons were visualized using an anti-acetylated tubulin monoclonal antibody (Sigma, T6793; 1:800) and Alexa 546 rabbit anti-mouse IgG secondary antibody (ThermoFisher, A-11060; 1:600). Images were acquired using a TCS SP8 inverted confocal laser scanning microscope (Leica).

**TUNEL assay**

The mean number of apoptotic cells, as determined by TUNEL assay, in the pharyngeal region was calculated and subject to statistical analysis as detailed in the supplementary Materials and Methods.

**Fig. 8. Model of the genetic networks regulating pouch segmentation.**

In wild-type embryos, functional $Pax1$ specifically activates $tbx1$ and $fgf3$ expression in the pharyngeal pouches (purple). Downstream targets of $Pax1$ inhibit $pax1$ transcription to create the interpouch endodermal regions (orange) in a non-cell-autonomous manner. Reiteration of these bifacial ($pax1/pax1^-$) endoderm patterns gives rise to the segmental pharyngeal pouches. In $pax1$ mutants, negative regulation of $pax1$ expression by $Pax1$ has no effect (pale purple). This results in no endodermal expression of $tbx1$ and $fgf3$ and continuous expression of $pax1$ in the posterior pharynx, whereas expression in $pp1$ and the anterior wall of $pp2$ is almost normal. $OV$, otic vesicle; $PP$, pharyngeal pouch.

**Materials and Methods**

**Medaka**

Mature adult wild-type medaka were kept in fresh water in plastic aquaria under artificial reproductive conditions (10 h dark, 14 h light; 26°C). Developmental stages were determined as previously outlined (Iwamatsu, 2004). This study was performed in accordance with the Guidelines for Animal Experimentation of the National Institutes of Natural Sciences, with approval of the Institutional Animal Care and Use Committee of the National Institutes of Natural Sciences.

**TALEN-mediated mutagenesis of $pax1$ and morpholino knockdown**

The medaka $pax1$ mutant was established using the TALEN method (Joung and Sander, 2013). The TALENs were designed in the paired domain of the $pax1$ gene and constructed as previously reported (Ansai et al., 2014). A medaka $pax1$ mutant with a 7 bp deletion in the paired domain was obtained. Details of TALEN-mediated mutagenesis of $pax1$ and fish genotyping are shown in Fig. S1. The $pax1$ and control morpholinos are described in the supplementary Materials and Methods.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

K.O. and H.W. conceived of and designed the research; K.O., K.I. and T.M. undertook the experiments and K.O. confirmed and analyzed the data; K.I. and A.K. generated $pax1$ mutant medaka by TALEN; K.O., S.T. and H.W. wrote and edited the manuscript.

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Supplementary information
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