General and Comparative Endocrinology 277 (2019) 66–72

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

A unique role of thyroid hormone receptor β in regulating notochord resorption during *Xenopus* metamorphosis

Keisuke Nakajima[a,b,*], Ichiro Tazawa[a], Yun-Bo Shi[b,*]

[a] Division of Embryology, Amphibian Research Center, Hiroshima University, Higashihiroshima 739-8526, Japan
[b] Section on Molecular Morphogenesis, Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD), National Institutes of Health (NIH), Bethesda, MD, USA

ARTICLE INFO

Keywords:
Metamorphosis
Xenopus laevis
Xenopus tropicalis
Thyroid hormone receptor
Tail resorption
Notochord
Matrix metalloproteinase

ABSTRACT

Tail resorption during anuran metamorphosis is perhaps the most dramatic tissue transformation that occurs during vertebrate development. Earlier studies in highly related anuran species *Xenopus laevis* and *Xenopus tropicalis* have shown that thyroid hormone (T3) receptor (TR) plays a necessary and sufficient role to mediate the causative effect of T3 on metamorphosis. Of the two known TR genes in vertebrates, TRα is highly expressed during both premetamorphosis and metamorphosis while TRβ expression is low in premetamorphic tadpoles but highly upregulated as a direct target gene of T3 during metamorphosis, suggesting potentially different functions during metamorphosis. Indeed, gene knockout studies have shown that knocking out TRα and TRβ has different effects on tadpole development. In particularly, homozygous TRβ knockout tadpoles become tailed frogs well after sibling wild type ones complete metamorphosis. Most noticeably, in TRβ-knockout tadpoles, an apparently normal notochord is present when the notochord in wild-type and TRα-knockout tadpoles disappears. Here, we have investigated how tail notochord resorption is regulated by TR. We show that TRβ is selectively very highly expressed in the notochord compared to TRα. We have also discovered differential regulation of several matrix metalloproteinases (MMPs), which are known to be upregulated by T3 and implicated to play a role in tissue resorption by degrading the extracellular matrix (ECM). In particular, MMP9-TH and MMP13 are extremely highly expressed in the notochord compared to the rest of the tail. *In situ* hybridization analyses show that these MMPs are expressed in the outer sheath cells and/or the connective tissue sheath surrounding the notochord. Our findings suggest that high levels of TRβ expression in the notochord specifically upregulate these MMPs, which in turn degrades the ECM, leading to the collapse of the notochord and its subsequent resorption during metamorphosis.

1. Introduction

Anuran metamorphosis is one of the most dramatic and best characterized developmental process that is regulated by thyroid hormone (T3). This process mimics mammalian postembryonic development, a neonatal period when plasma T3 level peaks (Tata, 1993; Shi, 1999). Anuran metamorphosis changes essential every single tissue/organ of a tadpole, from de novo development of adult organs such as the limb, to remodeling of tissues such as the intestine and brain, and finally the resorption of tadpole specific organs such as the tail and gill. These different changes are temporally coordinated to ensure the survival of the animal through the transition between two very different living conditions: from being aquatic to terrestrial (although some species like *Xenopus laevis* and *Xenopus tropicalis*, remain aquatic as frogs), from locomotion via tail to that via limbs, from oxygen intake via gills to air breathing via the lung, etc. Interestingly, all these changes are controlled by T3, which is necessary and sufficient for metamorphosis (Nakajima et al., 2005; Grimaldi et al., 2013; Sachs, 2015; Wen and Shi, 2016; Buchholz and Shi, 2018).

T3 functions by regulating gene expression via T3 receptors (TRs). TRs form heterodimer with 9-cis retinoic acid receptors (RXRs) to bind T3-response elements in T3 target genes constitutively. They can repress or activate T3-inducible genes depending on the availability of T3 (Lazar, 1993; Tsai and O'Malley, 1994; Mangelsdorf et al., 1995; Wong et al., 1995; Wong et al., 1997; Wong et al., 1998; Yen, 2001; Shi, 2009). Earlier studies by us and other have shown that in premetamorphic tadpoles when T3 concentration is low or undetectable, TRs recruit histone deacetylase-containing corepressor complexes to repress T3-inducible genes, which is important for the prevention of premature metamorphosis and ensures the tadpole to have a proper

* Corresponding authors.
E-mail addresses: kei@hiroshima-u.ac.jp (K. Nakajima), Shi@helix.nih.gov (Y.-B. Shi).

https://doi.org/10.1016/j.ygcen.2019.03.006
Received 30 January 2019; Received in revised form 22 February 2019; Accepted 5 March 2019
Available online 06 March 2019
0016-6480/ Published by Elsevier Inc.

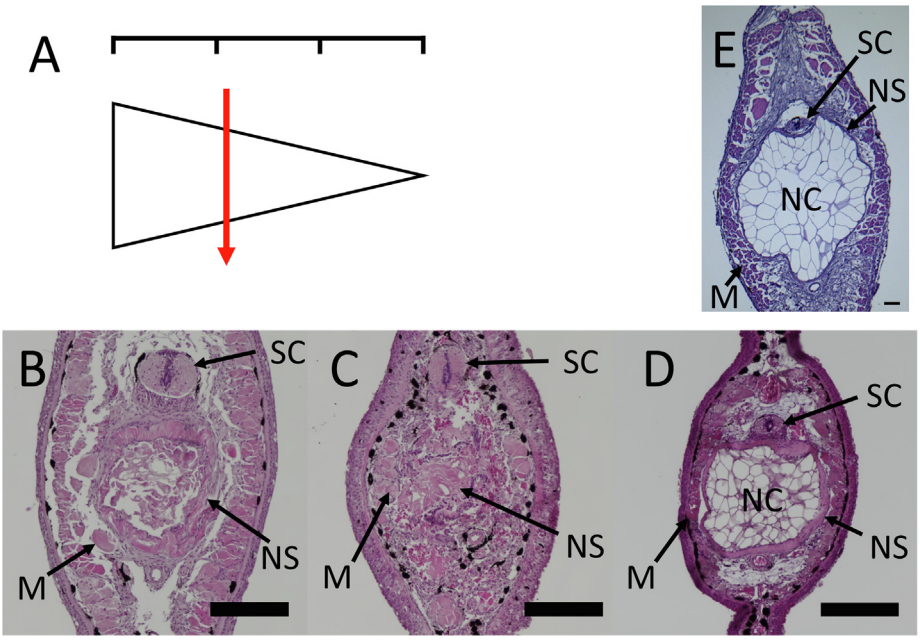

Fig. 1. Histological analysis of wild-type and TR-knockout tadpole tails at climax of metamorphosis. (A) Schematic representation of stage-63 tail sampling. A tail was sectioned at one-third of the distance from the tail base as indicated by a red arrow. (B–D) A cross sections of wild-type (B), TRα-knockout (C), and TRβ-knockout (D) stage-63 tadpole tail. Histological images are representative of three individuals examined per panel. (E) A cross section of wild type late stage-62 tadpole tail, which was sectioned about at half of the length. Sections were stained with hematoxylin and eosin. Note that the notochord in TRβ-knockout tail at stage 63 resembles that of the wild type at stage 62, showing the presence of vacuolated cells, i.e., not collapsed, compared to the notochord in the wild type or TRα-knockout tail at stage 63. Abbreviations: M, muscle; NC, notochordal cells; NS, notochord sheath; SC, spinal cord. Scale bars, 0.1 mm.

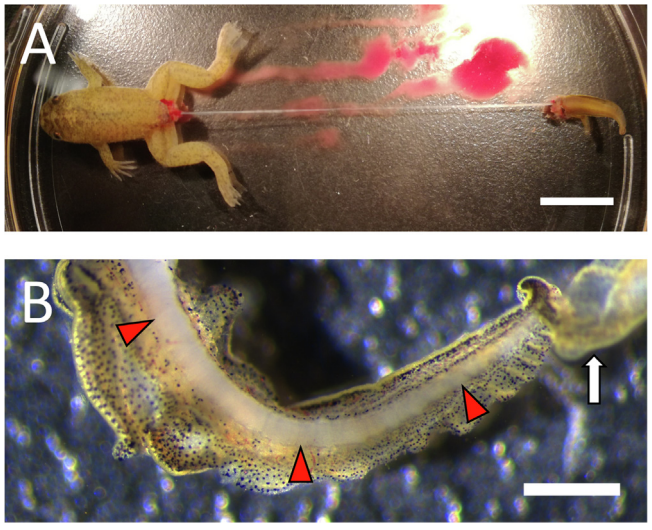

Fig. 2. Two methods for isolating notochords. (A) A representative photo of notochord preparation by method-A. The tail skin was cut circumferentially at the junction with the body (the tail base) and the tail was pulled to expose the stretched notochord, which was then isolated by cutting at the two ends of the exposed notochord. Scale bar, 10 mm. (B) A representative photo of notochord preparation by method-B. The skin and muscles of left side were peeled to the end of the tail (white arrow) to expose the notochord (red arrow heads), and notochord was then pulled out. In this case, spinal code was attached to left side that was peeled to the end of the tail (thus not visible in figure). Anterior, left; Dorsal, top. Scale bar, 1 mm.

Table 1
RNA yields from the tail at stage 63.

	Method	No.	RNA/tadpole (ng)	%RNA of Notochord/Tail
Notochord	Method-A	38	81	0.86
Notochord	Method-B	15	592	6.28
Tail without notochord	Method-B	8	9433 ± 656	
Whole Tail		5	20658 ± 687	

period of growth before initiating metamorphosis (Sachs et al., 2002; Tomita et al., 2004; Sato et al., 2007; Shi, 2009; Choi et al., 2015b; Sachs, 2015; Wen and Shi, 2015; Yen, 2015; Wen and Shi, 2016; Choi et al., 2017; Wen et al., 2017; Buchholz and Shi, 2018; Nakajima et al., 2018; Sakane et al., 2018). As T3 concentration rises later during development, T3 binds to TRs and the liganded TRs in turn release the corepressor complexes and recruit coactivator complexes to activate the target genes, leading to the transformation of different organs and tissues into the adult form (Paul et al., 2005a; Paul et al., 2005b; Paul et al., 2007; Matsuda et al., 2009; Shi, 2009; Choi et al., 2015b; Sachs, 2015; Wen and Shi, 2015; Yen, 2015; Wen and Shi, 2016; Choi et al., 2017; Wen et al., 2017; Buchholz and Shi, 2018; Nakajima et al., 2018; Sakane et al., 2018).

While extensive molecular and genetic studies in *Xenopus laevis* and *Xenopus tropicalis* have shown that TRs are both necessary and sufficient for mediating the effects of T3 on amphibian metamorphosis, a major question remains on how different organs/tissues determine when to undergo their specific changes. In this regard, tail resorption is particularly interesting. It is the most noticeable and most dramatic change during metamorphosis and takes place very late with tail length beginning to shorten only after stage 62, when plasma T3 level peaks, in *Xenopus laevis*. Earlier gene expression studies have suggested that tissue/organ-specific temporal expression of genes of the T3 signal process, including TRs, cytosolic binding proteins, deiodinases, and transporters may be responsible for the temporal coordination of metamorphosis in different organs (Shi et al., 1996; Connors et al., 2010; Choi et al., 2015a). Consistently, recent TR knockout studies in *Xenopus tropicalis* have shown that removing TRα, which is highly expressed in the limb at the onset of metamorphosis (stage 54), well before that in the tail, affects limb development dramatically but with little effect on tail resorption, whereas, removing TRβ, which has little expression in the limb but dramatically upregulated in the tail late during metamorphosis, delays tail resorption with little effect on limb development (Choi et al., 2015b; Wen and Shi, 2015; Wen and Shi, 2016; Choi et al., 2017; Wen et al., 2017; Nakajima et al., 2018; Sakane et al., 2018). Interestingly, even within the tail, TRβ knockout in *Xenopus tropicalis* has different effect on different tissues, with the most dramatic effect observed on notochord resorption (Nakajima et al., 2018). Here we have studied the potential mechanism underlying the inhibition of notochord resorption in the tail of TRβ knockout *Xenopus tropicalis* tadpoles. Our data suggest that TRβ is highly expressed in the

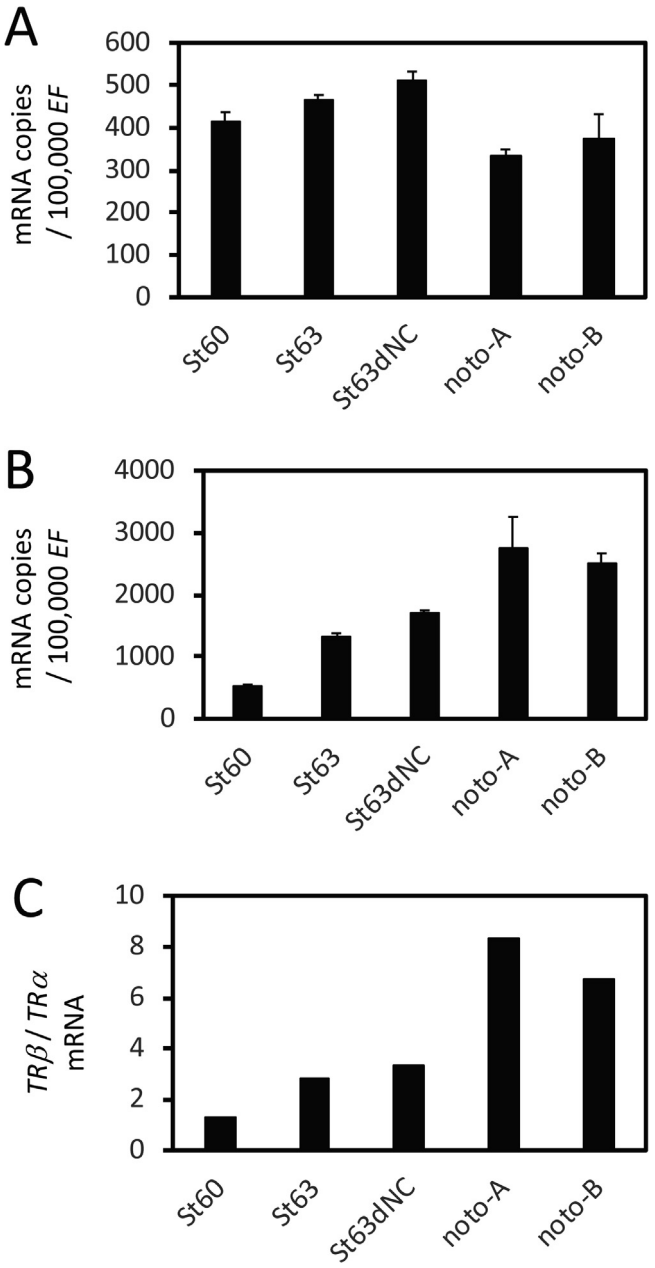

Fig. 3. TRβ is highly expressed specifically in the tail notochord at the climax of metamorphosis. The expression levels of TRα (A), TRβ (B) and ratio of TRβ to TRα (C) were determined by RT-PCR in the wild-type stage-60 whole tail (St60), stage-63 whole tail (St63), stage-63 notochord-removed-tail (St63dNC), stage-63 notochord prepared by method-A (noto-A), and method-B (noto-B), respectively. The samples included 5, 5, 8 tails and 38, 15 notochords, respectively. (A, B) The expression levels were shown as the copy number per 100,000 copies of EF1α. Data are expressed as mean ± SE of technical replications (n = 5–6).

notochord to activate the expression of several matrix metalloproteinase (MMP) genes, which in turn degrade the extracellular matrix (ECM) in/surrounding the notochord, leading to its collapse and subsequent resorption.

2. Materials and methods

2.1. Animals rearing and staging

Wild type adult *X. tropicalis* were provided by the Amphibian Research Center (Hiroshima University) through the National Bio-Resource Project of the MEXT, Japan or were purchased from NASCO. Tadpoles were staged according to Nieuwkoop and Faber (1965). To clarify the stage boundaries, stages from 58 to 60, from 60 to 62, and from 62 to 65 were determined only by the length of the forelimb, the ratio of nervus olfactorius length to the bulbus olfactorius diameter, and the ratio of tail length to body length, respectively. All animal care and treatments were done as approved based on the guidelines established by Hiroshima University for the care and use of experimental animals (approval no. G14-1) or by Animal Use and Care Committee of *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD), National Institutes of Health (NIH).

2.2. Histology

Histological analysis was done as previously described (Nakajima et al., 2018).

2.3. Notochord sample preparation

Method-A: Stage-63 tadpole were anesthetized with MS222. All operations were done in the chilled 0.6 × PBS. The skin surrounding the root of tail (the junction with the body or the tail base) and dorsal part of muscles were cut until the spinal cord was visible. Two forceps were inserted between body and tail to push the body and the tail slowly away from each other to expose the notochord. The notochord was then carefully pulled out and immersed in RNAlater RNA Stabilization Reagent (Qiagen) at 4 °C for at least 24 h.

Method-B: Stage-63 tadpole were anesthetized with MS222. All operations were done in the chilled 0.6 × PBS. Cut the skin surrounding a root of tail. Fix the tadpole to an operating table by pins at a brain segment. Grab both sides of skins near dorsal fin with two forceps. Move the forceps speedily and simultaneously to diagonal back direction. When skin was pulled, usually the muscles would attach to skin. Tail would be plucked at middle and both sides of skin with muscles would be separated. At that time, notochord and spinal cord would be attached to one side of tail pieces. Sometimes they were attached to same piece, other times they were attached to different pieces. Usually, notochord would be stretched at the plucked part. After that, notochord was carefully pulled out. Both the notochord and the rest of the tail, designated as notochord-removed-tail, were immersed in RNAlater RNA Stabilization Reagent (Qiagen) at 4 °C for at least 24 h.

2.4. RNA extraction and quantitative RT-PCR

Total RNA was purified from 5 whole tails, 38 notochords (isolated with method-A) or 15 notochords (isolated with method-B), and 8 notochord-removed-tails, by using the SV Total RNA Isolation System kit (Promega, Madison, WI, USA), which includes a DNase I treatment step. The RNA concentration was measured by using a Nanodrop (Thermo). RNA was reverse transcribed with the QuantiTect reverse transcription kit (Qiagen). The products were diluted with water to 50%, and Quantitative RT-PCR (qRT-PCR) was carried out by using the SYBR Green method. The PCR primers for genes EF1α, TRα and MMP11 were described previously (Wen and Shi, 2015). The PCR primers for genes MMP2, MMP9-TH, MMP13 and MMP14 were reported in Nakajima et al. (2018). TRβ primers were forward primer 5′-TGGTGTGTGGAGA CAAGGCTAC-3′ and reverse primer 5′-TACAAGAATAGCTCGGATGG-3′. The mRNA level was quantified and normalized to that of EF1α mRNA.

2.5. In situ hybridization

A partial cDNA encoding MMP9-TH, MMP13 and MMP14 were obtained by PCR with primers 5-ATGGGTTGGCTGGGTATTTTAATAC-3 and 5-TCATCAAAGTGAGCATCTCCTTG-3 for MMP9-TH, 5-CATCCCA TTTGATGGCCCTAATGG-3 and 5-TTGACATCATCTTGTGGCAAGCGG-3

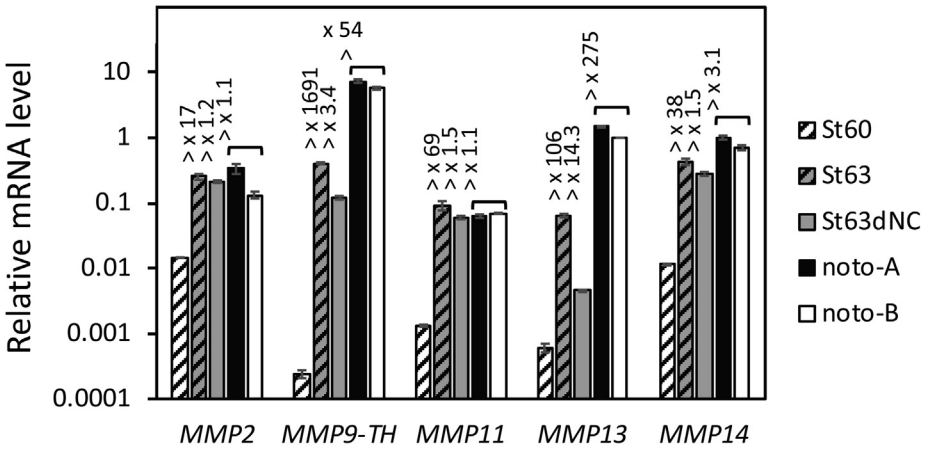

Fig. 4. Preferential high level expression of genes encoding matrix metalloproteinases (MMPs) in the tail notochord at climax of metamorphosis. The expression levels of the ECM-degrading MMP2, MMP9-TH, MMP11, MMP13 and MMP14 were determined by RT-PCR in the stage-60 whole tail (St60), stage-63 whole tail (St63), stage-63 notochord-removed-tail (St63dNC), notochord prepared by method-A (noto-A) and method-B (noto-B), respectively. The fold increase between the samples of St60 and St63, between St63dNC and St63 and between St63dNC and the average of noto-A and noto-B are indicated in the figure. Note all MMPs were upregulated at St63 compared to St60. MMP9-TH and MMP13, and to a lesser extent MMP14, were expressed at much higher levels in the notochord compared to the rest of the tail, leading to significantly lower levels of their mRNAs in St63dNC than that in the whole tail even though notochordal RNA is only a tiny fraction of the total RNA in the tail. The individual sample included 5, 5, 8 tails and 38, 15 notochords, respectively. The expression levels were shown as the copy number relative to EF1α. Data are expressed as mean ± SD of technical replications (n = 3–4).

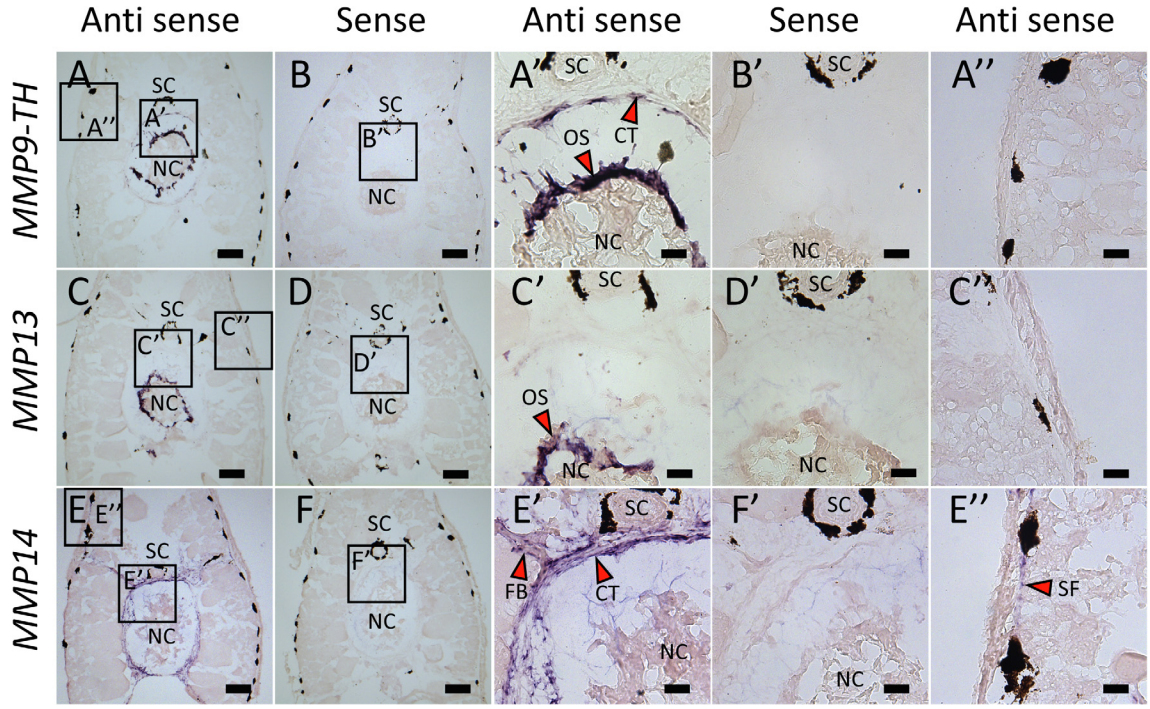

Fig. 5. Distinct localizations of MMP9-TH, MMP13 and MMP14 expression in the tail at the climax of metamorphosis. Cross sections of the tail at stage 62 were hybridized with antisense MMP9-TH (A, A′, A″), MMP13 (C, C′, C″) or MMP14 (E, E′, E″) probes or their sense probes (B, B′, D, D′, F, F′). Dark purple deposits indicate the sites of probe binding. Black pigments in some areas, e.g., spinal cord (SC), are melanin (see B, D, F). Note that MMP9-TH mRNA is expressed in the outer sheath cells (OS) and weakly in the connective tissue sheath (CT) surrounding the notochord (NC). MMP13 mRNA is expressed in the outer sheath cells, but not in the connective tissue sheath. MMP14 mRNA is expressed in the connective tissue sheath, fibroblast (FB, E′) and subepidermal fibroblast (SF, E″) but not observed in the outer sheath cells. Boxed areas in the panel A-F are magnified in panel A′-F′, A″, C″ and E″. Scale bars, 0.2 mm (A-F) or 0.05 mm (A′-F′, A″, C″ and F″).

for MMP13 and 5-TGTTTATTTCCCCAGGCCTGGCTT-3 and 5-AATGTG ATGTCCTTGTGCTGCCAC-3 for MMP14. The PCR product was inserted into pCR-Blunt II-TOPO vector (Invitrogen). The plasmids were linearized to synthesize sense and antisense probes either with T3 or T7 RNA polymerase by using digoxigenin (DIG) RNA Labeling Mix (Roche Applied Science, Indianapolis, IN). Tail fragments were isolated from tadpole at stage 62 and fixed in MEMFA followed by cryosectioning. Tissue sections were prepared at 20 μm and subjected to *in situ* hybridization (ISH). ISH was performed by using sense or antisense probes as follows. Briefly, tissue sections were washed with PBS 10 min prior to proteinase K treatment (5 μg proteinases K/ml PBS) for 5 min at 37 °C, washed two times with PBS for 1 min, post-fixed with 4% paraformaldehyde (PFA) in PBS for 20 min at room temperature, washed twice with PBS for 1 min, prehybridized with hybridization buffer (Gregorieff and Clevers, 2015), and hybridized with DIG–labeled RNA probe (1 μg/ml) in hybridization buffer, overnight at 65 °C. After hybridization, slides were washed twice with washing buffer 1 (50% formamide, 5 × SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 1% SDS) for 30 min at 60 °C and then washed three times with washing buffer 2 (50% formamide, 2 × SSC) for 30 min at 60 °C. Following three washes with TBS–0.1% Tween–20 (TBST) for 5 min at room temperature, the slides were immersed in 0.5% blocking reagent (cat. no. 1096176; Roche Applied Science) in TBST for 1.5 h at room temperature, incubated with alkaline phosphatase (AP)-conjugated anti–DIG Fab fragment (cat. no. 1093274; Roche Applied Science) diluted 1:2,000 with TBST containing 0.5% blocking reagent at 4 °C overnight, and then washed three times with TBST containing 2 mM levamisole for 20 min, followed by rinsing with AP buffer (0.1 M

Tris–HCl pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$) containing 2 mM levamisole for 5 min. Color development was performed by immersing slides in the reaction buffer (AP buffer supplemented with 2 mM levamisole, 50 fold diluted NBT/BCIP Stock Solution (Roche)) in the dark until the desired intensity of the hybridization signal was obtained. After washes with AP buffer for 15 min, the reaction was terminated by immersing the slides in 4% PFA in PBS for 30 min followed by two washes with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) for 20 min. The sections were dried and covered with Permount (Fisher Scientific) and a coverslip.

3. Results

3.1. TRα and TRβ knockout tadpoles have distinct tail resorption phenotypes during Xenopus tropicalis metamorphosis

Recent knockout studies in *Xenopus tropicalis* have shown that TRα and TRβ knockout has distinct effects on metamorphosis (Choi et al., 2015b; Wen and Shi, 2015; Wen and Shi, 2016; Choi et al., 2017; Wen et al., 2017; Nakajima et al., 2018; Sakane et al., 2018). Of interest is that at the climax of metamorphosis, TRα knockout had little effect on the rate of metamorphic progression, i.e., no change in the time needed to go from early climax of stage 58 to the end of metamorphosis (stage 66) when compared to wild type animals. On the other hand, TRβ knockout led to dramatic developmental delay in tail resorption, and to a lesser extent, in head narrowing due to gill resorption and shortening of olfactory nerves although other metamorphic changes such as the development of the limbs were normal (Nakajima et al., 2018). Examination of the tail during metamorphosis revealed that the notochord of the TRβ knockout tadpoles, when compared that in the wild type or TRα knockout animals, was resistant to resorption. The notochord in the TRβ knockout tadpoles at stage 63 was not collapsed compared to wild-type and TRα knockout tadpoles (Fig. 1A–D). Vacuolated cells, which occupy a large volume of tail at late stage 62 (Fig. 1E), were still remaining in the knockout tadpoles at stage 63 and the notochordal sheath also appeared healthy at stage 63 (Fig. 1D). However, muscle cells were fewer and smaller compared to wild type (Fig. 1B) or TRα knockout (Fig. 1C) animals at stage 63 (Fig. 1D). Thus, TRβ appears to play a uniquely important role in notochord resorption.

3.2. TRβ expression is highly enriched in the notochord compared to TRα at the climax metamorphosis

To investigate why TRα and TRβ affect tail resorption differently, we analyzed their expression in the notochord vs. the rest of the tail at stage 63, when the differential effect of TRβ knockout on notochord was observed. To isolate the notochord, we used two different methods as diagramed in Fig. 2, with method-B yielding more RNA per notochord (Table 1), suggesting method-B was more efficient in separating the notochord from the rest of the tail. Thus, we kept the rest of the tail after notochord removal with method-B as the notochord-removed-tail sample.

Analysis of the mRNA levels by qRT-PCR revealed that TRα mRNA level changed little between stage 60 (prior to the onset of tail length reduction at stage 62) and stage 63 in whole tails and was similar in the whole tail or notochord-removed-tail samples (Fig. 3A), as might be expected since notochord mRNA represents only a tiny fraction of the RNA in the whole tail (Table 1). In the notochord samples, TRα mRNA level was lower than that in notochord-removed-tail, independent of the method of notochord isolation (Fig. 3A). On the other hand, TRβ mRNA level increased between stage 60 and stage 63 in whole tails and was also expectedly similar in the whole tail and notochord-removed-tail samples (Fig. 3B). Interestingly, contrary to TRα expression, TRβ mRNA level was much higher in the notochord than that in notochord-removed-tail samples, again independent of the method of notochord isolation (Fig. 3B). Furthermore, when ratio of mRNA copy numbers for TRβ and TRα was determined, an even more dramatic difference observed in the notochord, with TRβ expressed at 8-fold higher level than TRα (Fig. 3C).

3.3. Differential expression of matrix metalloproteinase (MMP) genes in the tail notochord at the climax of metamorphosis

Tissue resorption involve cell death and degradation of the surround extracellular matrix (ECM). Earlier studies have shown that a number of MMPs are upregulated in tissues involving cell death, such as the intestine and tail, during metamorphosis. Among the MMPs include MMP2, MMP9-TH, MMP11, MMP13 and MMP14 (Wang and Brown, 1993; Patterton et al., 1995; Ishizuya-Oka et al., 1996; Stolow et al., 1996; Berry et al., 1998; Fu et al., 2006; Fujimoto et al., 2006; Hasebe et al., 2006; Fujimoto et al., 2007; Hasebe et al., 2007b; Fu et al., 2009). Organ-culture and transgenic studies have shown that MMPs, at least MMP11 (stromylysin-3), are important for T3-induced cell death during metamorphosis and *Xenopus laevis* development (Su et al., 1997; Ishizuya-Oka et al., 2000; Damjanovski et al., 2001; Amano et al., 2005a; Amano et al., 2005b; Fu et al., 2005; Hasebe et al., 2007a; Mathew et al., 2009). To determine if high levels of TRβ expression may lead to preferential upregulation of MMP genes in the tail notochord in *Xenopus tropicalis*, we analyzed the mRNA levels for MMP2, MMP9-TH, MMP11, MMP13 and MMP14 by RT-PCR in the same RNA samples as used for TR expression studies in Fig. 3. In agreement with earlier studies, all these MMP genes are upregulated between stage 60 and stage 63 in whole tadpole tail, with the highest folds of change observed for MMP9-TH (1691-fold increase) and MMP13 (106 fold) (Fig. 4).

Importantly, when the expression in the notochord and the rest of the tail was analyzed, we observed that the mRNA levels for MMP9-TH and MMP13, the two most highly upregulated MMP genes, were expressed several orders of magnitude higher in the notochord compared to the rest of the tail (54 and 275 folds); and to a lesser extent, MMP14 was also preferentially expressed in the notochord (3.1 fold). This led significantly lower levels of their mRNAs in the notochord-removed-tails compared to the whole tail, despite the fact that notochordal RNA is only a tiny fraction of the total tail RNA (Table 1, Fig. 4).

The above results suggest that high levels of TRβ expression may activate the expression of MMP genes, particularly, MMP9-TH and MMP13, and to a lesser extent MMP14 in the notochord, to facilitate its resorption during metamorphosis. To investigate this possibility, we analyzed the spatial expression of these three MMPs by *in situ* hybridization in the tail at stage 62. MMP9-TH and MMP13 were highly expressed in the notochord. The MMP9-TH mRNA was expressed in the outer sheath cells of the notochord and weakly in the connective tissue sheath surrounding the notochord (Fig. 5A′ and Supplemental Fig. 1). MMP13 mRNA was highly expressed in the outer sheath cells, although not in the connective tissue sheath (Fig. 5C′). MMP14 mRNA was expressed in the connective tissue sheath, fibroblasts (Fig. 5E′) and subepidermal fibroblasts (Fig. 5E″) but not observed in the outer sheath cells. This more diffused expression pattern may explain the relatively small enrichment in MMP14 expression in the notochord compared to the rest of the tail as discovered by RT-PCR (Fig. 4).

4. Discussion

Tail resorption is one of the most dramatic and last events to complete during anuran metamorphosis. Gene expression analyses have led to the hypothesis that the delayed upregulation of TR gene expression in the tail relative to other organs may contribute to this temporal regulation of tail resorption during metamorphosis. Here we have revealed differential notochord-specific high level expression of TRβ and MMP genes, suggesting that TRβ may affect notochord resorption via activation of MMP genes.

The effect of TRβ knockout on tail resorption is most dramatic for the notochord (Nakajima et al., 2018). During natural metamorphosis, TRα is gradually upregulated in the tail after stage 54 by a few folds.

However, the translation of TRα is repressed via repressing sequence in the 5′-UTR of TRα gene (Okada et al., 2012). TRβ is drastically upregulated and is itself a direct target gene of T3. These regulations result in a higher level of TRβ protein in the tail at the climax of metamorphosis (Eliceiri and Brown, 1994; Nakajima et al., 2012; Okada et al., 2012). Perhaps due to this drastic auto-regulation of TRβ at the climax of metamorphosis (likely in many organs), TRα knockout has little effect at the climax of metamorphosis. Likewise, the dramatic increase in TRβ expression in the tail where the change in TRα expression is relatively small, may also be the underlining reason for the significant delay in tail resorption during metamorphosis as TRα is insufficient to compensate for the loss of highly upregulated TRβ. Our findings here suggest that the differential expression of TRβ gene in the notochord is particularly important. The mRNA level of TRβ is 8 times of that for TRα in the notochord compared to the 3-fold difference for the rest of the tail. This result is consistent with the fact that TRβ expression is strongest in the notochord, the notochord sheath, the subepidermal fibroblasts, and the spinal cord as revealed by *in situ* hybridization in *Xenopus laevis* (Berry et al., 1998). Thus, one may expect that TRβ knockout preferentially reduces T3 signaling in the notochord compared to the rest of the tail, leading to major defect in notochordal metamorphosis, i.e., resorption.

How TRβ affects notochord resorption remains to be determined. Our analyses of MMPs genes suggest that the local, high levels of TRβ expression may preferentially activate MMP genes in the notochord. MMPs are ECM degrading enzymes that are implicated in regulating cell fate and tissue remodeling in development and pathogenesis. The ECM, consisting of many proteins and other macromolecules (Hay, 1991; Timpl and Brown, 1996), provides a structural support for tissues and interacts with cells through cell surface receptors (Damsky and Werb, 1992; Schmidt et al., 1993; Brown and Yamada, 1995) as well as regulating the availability of signaling molecules (Vukicevic et al., 1992; Werb et al., 1996). ECM remodeling and degradation by MMPs can thus affect cell fate and behavior (Shi et al., 1998). Consistently, our earlier studies on the metamorphosing *Xenopus laevis* intestine have shown that ECM can affect T3-induced larval epithelial apoptosis (Su et al., 1997). More importantly, our organ culture studies have demonstrated that MMP11 (stromelysin-3) is important for T3-induced larval intestinal epithelial cell death (Ishizuya-Oka et al., 2000). Transgenic overexpression of MMP11 induced precocious larval intestinal epithelial apoptosis as well as cell death in the tail in premetamorphic tadpoles, likely via the cleavage of laminin receptor (Amano et al., 2005a; Amano et al., 2005b; Fu et al., 2005; Mathew et al., 2009), supporting a role of MMPs in T3-induced cell death and tissue resorption.

The drastic, preferential expression of MMP9-TH, a gelatinase, and MMP13, a collagenase, in notochord strongly implicate a role for both in notochord resorption. It is known that cells in the notochordal sheath and/or outer sheath express type IV collagen (Nakajima and Yaoita, 2003), which is a nonfibrillar collagen and a major component of basement membrane, and a substrate of gelatinases (Nelson et al., 2000) including MMP9-TH. It is also known that the notochordal sheath is highly rich in type II collagen (Kenney and Carlson, 1978; Cheah et al., 1991; Wood et al., 1991), a major substrate of collagenases (Nelson et al., 2000) including MMP13. Furthermore, both MMP9-TH and MMP13 genes contain thyroid hormone response elements, which can be bound by TR for their induction by T3 (Fujimoto et al., 2007). Thus, it is tempting to speculate that the high level of TRβ expression in the notochord activates the expression of these MMPs in the notochord. The MMPs, in turn, cleave the collagens in the notochord sheath and thus play an essential role in the collapse of the notochord (as reflected by the absence or reduction of the vacuolated cells, Fig. 1) and its subsequent degradation during tail resorption. It would be very interesting in the future to obtain the notochord from TRβ-knockout and/or TRα and TRβ double-knockout tadpoles and compare their gene expression profiles to those in wild-type animals. Such studies should help to clarify the mechanism for upregulation of MMP13 in anterior tail of TRβ-knockout animals (Nakajima et al., 2018). A comprehensive analysis, such as RNA-Seq, should allow the identification of potentially novel genes induced by T3 in the notochord during metamorphosis. Clearly, it would also be important to determine in the future whether and how TRβ regulates the MMP genes in the notochordal tissues and if the MMPs indeed participate in notochord resorption.

Author contributions statement

KN conceived and designed the experiment; IT, performed histological analysis; KN and YS analyzed the data and prepared the manuscript; All authors participated in the manuscript preparation and approve the final version of the manuscript.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgement

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (Grant 25430089) and the intramural Research Program of NICHD, NIH, United States. We thank all members of laboratory for advices and helps, and animal care staffs for frog husbandry.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.03.006.

References

Amano, T., Fu, L., Marshak, A., Kwak, O., Shi, Y.B., 2005a. Spatio-temporal regulation and cleavage by matrix metalloproteinase stromelysin-3 implicate a role for laminin receptor in intestinal remodeling during Xenopus laevis metamorphosis. Dev. Dyn. 234, 190–200.

Amano, T., Kwak, O., Fu, L., Marshak, A., Shi, Y.-B., 2005b. The matrix metalloproteinase stromelysin-3 cleaves laminin receptor at two distinct sites between the transmembrane domain and laminin binding sequence within the extracellular domain. Cell Res. 15, 150–159.

Berry, D.L., Schwartzman, R.A., Brown, D.D., 1998. The expression pattern of thyroid hormone response genes in the tadpole tail identifies multiple resorption programs. Dev. Biol. 203, 12–23.

Brown, K.E., Yamada, K.M., 1995. The role of integrins during vertebrate development. Sem. Develop. Biol. 6, 69–77.

Buchholz, D.R., Shi, Y.B., 2018. Dual function model revised by thyroid hormone receptor alpha knockout frogs. Gen. Comp. Endocrinol.

Cheah, K.S., Lau, E.T., Au, P.K., Tam, P.P., 1991. Expression of the mouse alpha 1(II) collagen gene is not restricted to cartilage during development. Development 111, 945–953.

Choi, J., Ishizuya-Oka, A., Buchholz, D.R., 2017. Growth, development, and intestinal remodeling occurs in the absence of thyroid hormone receptor alpha in tadpoles of Xenopus tropicalis. Endocrinology 158, 1623–1633.

Choi, J., Moskalik, C.L., Ng, A., Matter, S.F., Buchholz, D.R., 2015a. Regulation of thyroid hormone-induced development in vivo by thyroid hormone transporters and cytosolic binding proteins. Gen. Comp. Endocrinol. 222, 69–80.

Choi, J., Suzuki, K.I., Sakuma, T., Shewade, L., Yamamoto, T., Buchholz, D.R., 2015b. Unliganded thyroid hormone receptor alpha regulates developmental timing via gene repression as revealed by gene disruption in Xenopus tropicalis. Endocrinology 156, 735–744.

Connors, K.A., Korte, J.J., Anderson, G.W., Degitz, S.J., 2010. Characterization of thyroid hormone transporter expression during tissue-specific metamorphic events in Xenopus tropicalis. Gen. Comp. Endocrinol. 168, 149–159.

Damjanovski, S., Amano, T., Li, Q., Pei, D., Shi, Y.-B., 2001. Overexpression of matrix metalloproteinases leads to lethality in transgenic Xenopus Laevis: implications for tissue-dependent functions of matrix metalloproteinases during late embryonic development. Dev. Dyn. 221, 37–47.

Damsky, C.H., Werb, Z., 1992. Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. Curr. Opin. Cell Biol. 4,

772–781.
Eliceiri, B.P., Brown, D.D., 1994. Quantitation of endogenous thyroid hormone receptors alpha and beta during embryogenesis and metamorphosis in Xenopus laevis. J. Biol. Chem. 269, 24459–24465.
Fu, L., Das, B., Mathew, S., Shi, Y.B., 2009. Genome-wide identification of Xenopus matrix metalloproteinases: conservation and unique duplications in amphibians. BMC Genomics 10, 81.
Fu, L., Ishizuya-Oka, A., Buchholz, D.R., Amano, T., Matsuda, H., Shi, Y.B., 2005. A causative role of stromelysin-3 in extracellular matrix remodeling and epithelial apoptosis during intestinal metamorphosis in Xenopus laevis. J. Biol. Chem. 280, 27856–27865.
Fu, L., Tomita, A., Wang, H., Buchholz, D.R., Shi, Y.-B., 2006. Transcriptional regulation of the Xenopus laevis stromelysin-3 gene by thyroid hormone is mediated by a DNA element in the first intron. J. Biol. Chem. 281, 16870–16878.
Fujimoto, K., Nakajima, K., Yaoita, Y., 2006. One of the duplicated matrix metalloproteinase-9 genes is expressed in regressing tail during anuran metamorphosis. Develop Growth Differ 48, 223–241.
Fujimoto, K., Nakajima, K., Yaoita, Y., 2007. Expression of matrix metalloproteinase genes in regressing or remodeling organs during amphibian metamorphosis. Develop Growth Differ 49, 131–143.
Gregorieff, A., Clevers, H., 2015. *In situ* hybridization to identify gut stem cells. Curr. Protoc. Stem Cell Biol. 34 (2F.1), 1–11.
Grimaldi, A., Buisine, N., Miller, T., Shi, Y.B., Sachs, L.M., 2013. Mechanisms of thyroid hormone receptor action during development: lessons from amphibian studies. Biochim. Biophys. Acta, Mol. Cell. Res. 1830, 3882–3892.
Hasebe, T., Fu, L., Amano, T., Shi, Y.-B., 2007a. Evidence for a cooperative role of gelatinase A and membrane type-1 matrix metalloproteinase during Xenopus laevis development. Mech. Dev. 124, 11–22.
Hasebe, T., Hartman, R., Matsuda, H., Shi, Y.B., 2006. Spatial and temporal expression profiles suggest the involvement of gelatinase A and membrane type 1 matrix metalloproteinase in amphibian metamorphosis. Cell Tissue Res. 324, 105–116.
Hasebe, T., Kajita, M., Fujimoto, K., Yaoita, Y., Ishizuya-Oka, A., 2007b. Expression profiles of the duplicated matrix metalloproteinase-9 genes suggest their different roles in apoptosis of larval intestinal epithelial cells during Xenopus laevis metamorphosis. Dev. Dyn. 236, 2338–2345.
Hay, E.D., 1991. Cell biology of extracellular matrix. Plenum Press, New York.
Ishizuya-Oka, A., Li, Q., Amano, T., Damjanovski, S., Ueda, S., Shi, Y.-B., 2000. Requirement for matrix metalloproteinase stromelysin-3 in cell migration and apoptosis during tissue remodeling in Xenopus laevis. J. Cell Biol. 150, 1177–1188.
Ishizuya-Oka, A., Ueda, S., Shi, Y.-B., 1996. Transient expression of stromelysin-3 mRNA in the amphibian small intestine during metamorphosis. Cell Tissue Res. 283, 325–329.
Kenney, M.C., Carlson, E., 1978. Ultrastructural identification of collagen and glycosaminoglycans in notochordal extracellular matrix in vivo and in vitro. Anat. Rec. 190, 827–849.
Lazar, M.A., 1993. Thyroid hormone receptors: multiple forms, multiple possibilities. Endocr. Rev. 14, 184–193.
Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., 1995. The nuclear receptor superfamily: the second decade. Cell 83, 835–839.
Mathew, S., Fu, L., Fiorentino, M., Matsuda, H., Das, B., Shi, Y.-B., 2009. Differential regulation of cell type specific apoptosis by stromelysin-3: a potential mechanism via the cleavage of the laminin receptor during tail resorption in Xenopus laevis. J. Biol. Chem. 284, 18545–18556.
Matsuda, H., Paul, B.D., Choi, C.Y., Hasebe, T., Shi, Y.-B., 2009. Novel functions of protein arginine methyltransferase 1 in thyroid hormone receptor-mediated transcription and in the regulation of metamorphic rate in Xenopus laevis. Mol. Cell. Biol. 29, 745–757.
Nakajima, K., Fujimoto, K., Yaoita, Y., 2005. Programmed cell death during amphibian metamorphosis. Semin. Cell Dev. Biol. 16, 271–280.
Nakajima, K., Fujimoto, K., Yaoita, Y., 2012. Regulation of thyroid hormone sensitivity by differential expression of the thyroid hormone receptor during Xenopus metamorphosis. Genes Cells 17, 645–659.
Nakajima, K., Tazawa, I., Yaoita, Y., 2018. Thyroid hormone receptor alpha- and beta-knockout Xenopus tropicalis tadpoles reveal subtype-specific roles during development. Endocrinology 159, 733–743.
Nakajima, K., Yaoita, Y., 2003. Dual mechanisms governing muscle cell death in tadpole tail during amphibian metamorphosis. Dev. Dyn. 227, 246–255.
Nelson, A.R., Fingleton, B.M., Rothenberg, M.L., Matrisian, L.M., 2000. Matrix metalloproteinases: biologic activity and clinical implications. J. Clin. Oncol. 18, 1135–1149.
Nieuwkoop, P.D., Faber, J., 1965. Normal Table of Xenopus laevis. North Holland Publishing, Amsterdam.
Okada, M., Nakajima, K., Yaoita, Y., 2012. Translational regulation by the 5'-UTR of thyroid hormone receptor alpha mRNA. J. Biochem. 151, 519–531.
Patterton, D., Hayes, W.P., Shi, Y.B., 1995. Transcriptional activation of the matrix metalloproteinase gene stromelysin-3 coincides with thyroid hormone-induced cell death during frog metamorphosis. Dev. Biol. 167, 252–262.
Paul, B.D., Buchholz, D.R., Fu, L., Shi, Y.-B., 2005a. Tissue- and gene-specific recruitment of steroid receptor coactivator-3 by thyroid hormone receptor during development. J. Biol. Chem. 280, 27165–27172.
Paul, B.D., Buchholz, D.R., Fu, L., Shi, Y.-B., 2007. SRC-p300 coactivator complex is required for thyroid hormone induced amphibian metamorphosis. J. Biol. Chem. 282, 7472–7481.
Paul, B.D., Fu, L., Buchholz, D.R., Shi, Y.-B., 2005b. Coactivator recruitment is essential for liganded thyroid hormone receptor to initiate amphibian metamorphosis. Mol. Cell. Biol. 25, 5712–5724.
Sachs, L.M., 2015. Unliganded thyroid hormone receptor function: amphibian metamorphosis got TALENs. Endocrinology 156, 409–410.
Sachs, L.M., Jones, P.L., Havis, E., Rouse, N., Demeneix, B.A., Shi, Y.-B., 2002. N-CoR recruitment by unliganded thyroid hormone receptor in gene repression during Xenopus laevis development. Mol. Cell. Biol. 22, 8527–8538.
Sakane, Y., Iida, M., Hasebe, T., Fujii, S., Buchholz, D.R., Ishizuya-Oka, A., Yamamoto, T., Suzuki, K.T., 2018. Functional analysis of thyroid hormone receptor beta in Xenopus tropicalis founders using CRISPR-Cas. Biol. Open 7.
Sato, Y., Buchholz, D.R., Paul, B.D., Shi, Y.-B., 2007. A role of unliganded thyroid hormone receptor in postembryonic development in Xenopus laevis. Mech. Dev. 124, 476–488.
Schmidt, J.W., Piepenhagen, P.A., Nelson, W.J., 1993. Modulation of epithelial morphogenesis and cell fate by cell-to-cell signals and regulated cell adhesion. Sem. Cell Biol. 4, 161–173.
Shi, Y.-B., 1999. Amphibian Metamorphosis: From Morphology to Molecular Biology. John Wiley & Sons Inc, New York.
Shi, Y.-B., 2009. Dual functions of thyroid hormone receptors in vertebrate development: the roles of histone-modifying cofactor complexes. Thyroid 19, 987–999.
Shi, Y.-B., Li, Q., Damjanovski, S., Amano, T., Ishizuya-Oka, A., 1998. Regulation of apoptosis during development: input from the extracellular matrix. Intern. J. Mol. Med. 2, 273–282.
Shi, Y.-B., Wong, J., Puzianowska-Kuznicka, M., Stolow, M., 1996. Tadpole competence and tissue-specific temporal regulation of amphibian metamorphosis: roles of thyroid hormone and its receptors. BioEssays 18, 391–399.
Stolow, M.A., Bauzon, D.D., Li, J., Sedgwick, T., Liang, V.C., Sang, Q.A., Shi, Y.B., 1996. Identification and characterization of a novel collagenase in Xenopus laevis: possible roles during frog development. Mol. Biol. Cell 7, 1471–1483.
Su, Y., Shi, Y., Stolow, M., Shi, Y.-B., 1997. Thyroid hormone induces apoptosis in primary cell cultures of tadpole intestine: cell type specificity and effects of extracellular matrix. J. Cell Biol. 139, 1533–1543.
Tata, J.R., 1993. Gene expression during metamorphosis: an ideal model for post-embryonic development. BioEssays 15, 239–248.
Timpl, R., Brown, J.C., 1996. Supramolecular assembly of basement membranes. BioEssays 18, 123–132.
Tomita, A., Buchholz, D.R., Shi, Y.-B., 2004. Recruitment of N-CoR/SMRT-TBLR1 corepressor complex by unliganded thyroid hormone receptor for gene repression during frog development. Mol. Cell. Biol. 24, 3337–3346.
Tsai, M.J., O'Malley, B.W., 1994. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Ann. Rev. Biochem. 63, 451–486.
Vukicevic, S., Kleinman, H.K., Luyten, F.P., Roberts, A.B., Roche, N.S., Reddi, A.H., 1992. Identification of multiple active growth factors in basement membrane Matrigel suggests caution in interpretation of cellular activity related to extracellular matrix components. Exp. Cell Res. 202, 1–8.
Wang, Z., Brown, D.D., 1993. Thyroid hormone-induced gene expression program for amphibian tail resorption. J. Biol. Chem. 268, 16270–16278.
Wen, L., Shi, Y.B., 2015. Unliganded thyroid hormone receptor alpha controls developmental timing in Xenopus tropicalis. Endocrinology 156, 721–734.
Wen, L., Shi, Y.B., 2016. Regulation of growth rate and developmental timing by Xenopus thyroid hormone receptor alpha. Dev. Growth Differ. 58, 106–115.
Wen, L., Shibata, Y., Su, D., Fu, L., Luu, N., Shi, Y.-B., 2017. Thyroid hormone receptor α controls developmental timing and regulates the rate and coordination of tissue specific metamorphosis in Xenopus tropicalis. Endocrinology 158, 1985–1998.
Werb, Z., Sympson, C.J., Alexander, C.M., Thomasset, N., Lund, L.R., MacAuley, A., Ashkenas, J., Bissell, M.J., 1996. Extracellular matrix remodeling and the regulation of epithelial-stromal interactions during differentiation and involution. Kidney Int. Suppl. 54, S68–S74.
Wong, J., Patterton, D., Imhof, D., Guschin, D., Shi, Y.-B., Wolffe, A.P., 1998. Distinct requirements for chromatin assembly in transcriptional repression by thyroid hormone receptor and histone deacetylase. EMBO J. 17, 520–534.
Wong, J., Shi, Y.-B., Wolffe, A.P., 1997. Determinants of chromatin disruption and transcriptional regulation instigated by the thyroid hormone receptor: hormone-regulated chromatin disruption is not sufficient for transcriptinal activation. EMBO J. 16, 3158–3171.
Wong, J., Shi, Y.B., Wolffe, A.P., 1995. A role for nucleosome assembly in both silencing and activation of the Xenopus TR beta A gene by the thyroid hormone receptor. Genes Dev. 9, 2696–2711.
Wood, A., Ashhurst, D.E., Corbett, A., Thorogood, P., 1991. The transient expression of type II collagen at tissue interfaces during mammalian craniofacial development. Development 111, 955–968.
Yen, P.M., 2001. Physiological and molecular basis of thyroid hormone action. Physiol. Rev. 81, 1097–1142.
Yen, P.M., 2015. Unliganded TRs regulate growth and developmental timing during early embryogenesis: evidence for a dual function mechanism of TR action. Cell Biosci. 5, 8.