Origin of the adult intestinal stem cells induced by thyroid hormone in *Xenopus laevis*

Atsuko Ishizuya-Oka,*,†,[1] Takashi Hasebe,* Daniel R. Buchholz,‡ Mitsuko Kajita,† Liezhen Fu,§ and Yun-Bo Shi§

*Department of Biology and †Institute of Development and Aging Sciences, Nippon Medical School, Kawasaki, Kanagawa, Japan; ‡Department of Biological Sciences, University of Cincinnati, Cincinnati, Ohio, USA; and §Laboratory of Gene Regulation and Development, National Institute for Child Health and Human Development, National Institutes of Health, Bethesda, Maryland, USA

ABSTRACT **In the amphibian intestine during metamorphosis, *de novo* stem cells generate the adult epithelium analogous to the mammalian counterpart. Interestingly, to date the exact origin of these stem cells remains to be determined, making intestinal metamorphosis a unique model to study development of adult organ-specific stem cells. Here, to determine their origin, we made use of transgenic *Xenopus* tadpoles expressing green fluorescent protein (GFP) for recombinant organ cultures. The larval epithelium separated from the wild-type (Wt) or GFP transgenic (Tg) intestine before metamorphic climax was recombined with homologous and heterologous nonepithelial tissues and was cultivated in the presence of thyroid hormone, the causative agent of metamorphosis. In all kinds of recombinant intestine, adult progenitor cells expressing markers for intestinal stem cells such as sonic hedgehog became detectable and then differentiated into the adult epithelium expressing intestinal fatty acid binding-protein, a marker for absorptive cells. Notably, whenever the epithelium was derived from Tg intestine, both the adult progenitor/stem cells and their differentiated cells expressed GFP, whereas neither of them expressed GFP in the Wt-derived epithelium. Our results provide direct evidence that stem cells that generate the adult intestinal epithelium originate from the larval epithelium, through thyroid hormone-induced dedifferentiation.—Ishizuya-Oka, A., Hasebe, T., Buchholz, D. R., Kajita, M., Fu, L., Shi, Y.-B. The origin of the adult intestinal stem cells induced by thyroid hormone in *Xenopus laevis*. *FASEB J.* 23, 2568–2575 (2009)**

Key Words: transgenic frog · organ culture · tissue recombination · gut remodeling · metamorphosis

Organ-specific adult stem cells are critical for the homeostasis of adult organs and organ regeneration. The stem cells for most mammalian organs are developed during embryogenesis when the embryo/fetus is enclosed in the uterus, making it difficult to investigate the origins of the stem cells and the mechanisms of their development. Amphibian metamorphosis shares many similarities with postembryonic development in mammals, a period encompassing a few months before and several months after birth in humans when many organs grow and mature (1, 2). Metamorphic organ remodeling thus serves as a unique model to study adult organ development. During amphibian metamorphosis, the digestive tract is remodeled extensively from the larval to the adult form for the adaptation from the aquatic herbivorous to terrestrial carnivorous life. The intestine, which has a long but simple structure with only a single fold, the "typhlosole" throughout the larval period (3), shortens and forms multiple intestinal folds during metamorphosis (4). The adult epithelium after metamorphosis has a cell renewal system along the trough-crest axis of intestinal folds (5), which mimics the mammalian crypt-villus axis (6, 7): the epithelial cells proliferate in the trough of intestinal folds, gradually differentiate as they migrate toward the tip of the intestinal folds into all cell types seen in the mammalian intestinal epithelium except for Paneth cells, that is, major absorptive cells expressing intestinal fatty acid-binding protein (IFABP) (8, 9), goblet cells, and enteroendocrine cells (10), and finally undergo apoptosis at their tip (11). These observations predict that multipotent stem cells reside in the adult epithelium of the amphibian intestine, just as in the mammalian one. However, the developmental origin of intestinal stem cells has not yet been determined for any vertebrate.

In the *Xenopus laevis* intestine before metamorphosis, all of the epithelial cells are differentiated cells including larval-type absorptive cells different from adult cells after metamorphosis, and morphologically undifferentiated cells are not detected by either light or electron microscopy (3). During metamorphosis, undifferentiated cells become detectable at stage 60 (the start of metamorphic climax) (12) as small islets between the larval epithelium and connective tissue. They are stained strongly red with pyronin Y, actively proliferate, and finally differentiate into the secondary (adult)

[1] Correspondence: Department of Biology, Nippon Medical School, 2-297-2 Kosugi-cho, Nakahara-ku, Kawasaki, Kanagawa 211-0063, Japan. E-mail: a-oka@nms.ac.jp

doi: 10.1096/fj.08-128124

0892-6638/09/0023-2568 © FASEB

epithelium (4, 5, 13). On the other hand, all of the larval-proper epithelial cells undergo apoptosis on and after stage 60 and are gradually replaced by the adult epithelial cells (11). These chronological observations indicate that the undifferentiated cells appearing at stage 60 in the *X. laevis* intestine are progenitor cells of the adult epithelium and thus include stem cells. In addition, we recently found that such undifferentiated cells express sonic hedgehog (Shh) (14, 15), Musashi-1 (Msi-1) (16), a phosphorylated form of phosphatase and tensin homolog (PTEN), and Akt (17), all of which are also expressed only in stem cells and their immediate descendants in the mammalian adult intestine and are candidate markers for stem cells of the intestinal epithelium (18–21). Therefore, in the *X. laevis* intestine, the stem cells analogous to those in the mammalian adult intestine become morphologically and immunohistochemically detectable at the start of metamorphic climax. Because amphibian metamorphosis can be easily manipulated with a single hormone, the thyroid hormone (TH) (2, 22, 23), intestinal remodeling offers an unique opportunity to study the origin and mechanisms of organ-specific adult stem cell development in a vertebrate, a poorly understood question with important implications in stem cell biology and tissue replacement/regeneration therapy.

We have shown previously, by using the organ culture system we established, that TH can organ-autonomously induce the development of the adult epithelium in intestine isolated from *X. laevis* tadpoles at stage 57 (24). Thus, the stem cells of the adult epithelium originate from cells present in the tadpole intestine at stage 57 but not from other organs in the animal. One possibility is that the stem cells come from the differentiated larval epithelial cells. Although some previous chronological observations are consistent with this scenario (3, 25, 26), there has been no direct evidence to support it, mainly because of the lack of adequate experimental tools. In addition, recent studies showed that during organ regeneration in the mammalian intestine, bone marrow-derived stem cells can migrate and differentiate into various tissues including the epithelium of the small intestine (27, 28), raising the possibility of nonepithelial cells giving rise to the stem cells. In the *X. laevis* intestine during the early period of metamorphic climax, leukocytes such as macrophages often migrate from the connective tissue into the larval epithelium through the modified basal lamina (5), which may be remodeled by the TH up-regulated matrix metalloproteinases (29). These studies suggest that the adult stem cells arise either from differentiated larval epithelial cells through dedifferentiation or from cells present in the intestinal connective tissue at stage 57 that migrate into the larval epithelium during stages 58 to 60.

In the present study, to definitively determine the origin of the stem cells of the adult epithelium in the *X. laevis* intestine, we made use of transgenic (Tg) tadpoles constitutively expressing green fluorescent protein (GFP) for recombinant organ cultures we established previously (30). Here, we provide the first experimental evidence to demonstrate that TH-induced stem cells originate from some of the larval epithelial cells in tadpole intestine at stage 57 and form the adult intestinal epithelium.

MATERIALS AND METHODS

Animals and tissue preparation

Adult South African clawed frogs (*X. laevis*) were purchased from a commercial source. To generate Tg frogs expressing GFP, transgenesis was carried out using the restriction enzyme-mediated integration method (31, 32), and >10 tadpoles with strong GFP expression in the body were reared to adulthood. Tadpoles in the F1 generation were used in these experiments. The transgenesis vector, pDPCG, is a double promoter construct containing the ubiquitous cytomegalovirus promoter driving enhanced GFP (EGFP) throughout the body and a crystallin promoter driving GFP3 in the lens of the eye. pDPCG was derived from $pCS2^{+}$ (a gift from Dave Turner, University of Michigan, Ann Arbor, MI, USA) by *1*) PCR cloning in the β-globin 5′- and 3′-untranslated regions (UTRs) from pT7Ts (a gift from Gert Jan C. Veenstra, University of Nijmegen, Nijmegen, The Netherlands) into the *Bam*HI and *Xho*I sites, *2*) cloning in EGFP from pCGCG (33) between the 5′- and 3′-UTRs at the *Age*I and *Eco*RI sites, and *3*) PCR cloning in a crystallin:GFP3 fragment from the clone 5′δ 320γ Crys/GFP3 (34) into the Asp^{718} and *Bss*HII sites. All PCR-cloned regions were sequence verified. Wild-type (Wt) and Tg tadpoles at stage 57, when the small intestine is the longest during the larval period, were used throughout the experiments.

Organ culture

Tubular fragments were isolated from the anterior part of the small intestine just behind the bile duct junction and split open lengthwise with fine forceps. Some of them were treated with 1000 U/ml dispase (Godo, Tokyo, Japan) to separate the epithelium (Ep) from nonepithelial tissues (non-Ep), which consist mainly of the connective tissue and muscles. Each Ep was then recombined with homologous and heterologous non-Ep as shown in **Fig. 1**. Intact and recombinant intestine were cultured as described previously (24, 30). In brief, they were placed on membrane filters (Millipore, Bedford, MA, USA) on stainless steel grids in culture dishes and cultured in 60% Leibovitz-15 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% charcoal-treated FBS (Invitrogen) (CTS medium). To induce the intestinal remodeling, triiodothyronine, insulin, and hydrocortisone (Sigma-Aldrich Corp., St. Louis, MO, USA) were added to CTS medium at 20 nM, 5 μg/ml, and 0.5 μg/ml, respectively. The culture medium was changed every other day for 7 d at 26°C.

Immunohistochemical analysis for GFP, Shh, Msi-1, proliferating cell nuclear antigen (PCNA), and IFABP

Intact and recombinant intestine cultured *in vitro* were fixed with 95% ethanol at 4°C for 4 h, embedded in paraffin, and cut at 5 μm. To identify cells derived from Tg intestine, some sections were incubated with the mouse anti-GFP antibody (diluted 1:100; MBL; Nagoya, Aichi, Japan) at room temperature for 1 h. They were then incubated with Alexa Fluor

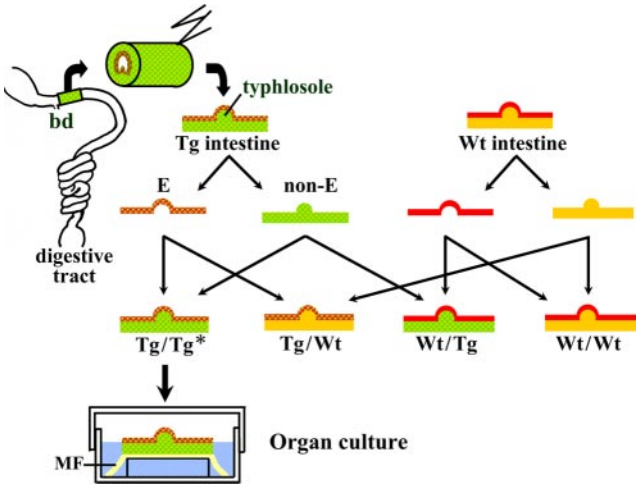

Figure 1. Schematic diagram for tissue recombination and organ culture of the *X. laevis* intestine. Tubular fragments were isolated from the small intestine just behind the bile duct junction (bd) of Tg and Wt tadpoles at stage 57, slit open lengthwise, and separated into Ep and non-Ep. Each Ep was then recombined with homologous and heterologous non-Ep. The four kinds of recombinant intestine were placed on membrane filters (MF) on grids and cultured in the medium. *Type of Ep/type of non-Ep.

568-conjugated anti-rabbit IgG (1:500; Molecular Probes, Eugene, OR, USA) or with peroxidase-labeled streptavidin (Nichirei, Tokyo, Japan) followed by 0.02% 3,3′-diaminobenzidine-4-hydrochloride (DAB) and 0.006% H_2O_2. Other sections were incubated with the following antibodies at room temperature for 1 h: the mouse anti-PCNA antibody (1:100; Novacastra, Newcastle, UK) for proliferating cells, the rabbit anti-Shh antibody (1:500) (14), and the rabbit anti-Msi-1 antibody (1:50; Abcam, Cambridge, MA, USA) for adult progenitor cells and the rabbit anti-IFABP antibody (1:500) (9) for differentiated absorptive cells. They were then visualized by sequential incubation with streptavidin-biotin-peroxidase complex and DAB/H_2O_2 as described above. There was no positive staining when the same concentration of preimmune serum was applied as the specificity control (data not shown). In addition, some sections adjacent to the ones used for immunohistochemical analysis were stained with methyl green-pyronin Y (Muto, Tokyo, Japan) for 5 min to distinguish conventionally the adult progenitor cells from the degenerating larval epithelium during the larval-to-adult intestinal remodeling. Here, the adult progenitor cells are stained red intensely because of their RNA-rich cytoplasm, whereas the larval epithelial cells undergoing apoptosis are stained much weaker both *in vivo* and in organ cultures (11, 35).

Finally, to examine the relations of Shh immunoreactivity with cell proliferation and GFP immunoreactivity, some sections were double-immunostained at room temperature for 1 h with mixtures of the anti-Shh and anti-PCNA antibodies and of the anti-Shh and anti-GFP antibodies, respectively. Similarly, to examine the relation between IFABP and GFP immunoreactivities, some other sections were double-immunostained at room temperature for 1 h with a mixture of the anti-IFABP and anti-GFP antibodies. They were then incubated with a mixture of Alexa Fluor 568-conjugated anti-rabbit IgG (1:500; Molecular Probes) and Alexa Fluor 488-conjugated anti-mouse IgG (1:500; Molecular Probes) and analyzed by fluorescence microscopy.

RESULTS

GFP is only expressed in tissue derived from Tg intestine in organ cultures

The larval intestine of Tg tadpoles consists of a single layer of the larval epithelium, the immature connective tissue that is very thin except in the typhlosole, and thin inner and outer muscles, just as in the Wt tadpole intestine (5). To check whether the larval intestine isolated from stage 57-Tg tadpoles expresses GFP *in vitro* during larval-to-adult intestinal remodeling, we cultured isolated Tg intestine in the presence of TH and immunohistochemically examined their GFP expression. In all of the tissues in the intact Tg intestine, GFP immunoreactivity was detectable throughout the cultivation, although its intensity varied somewhat in different cell types (**Fig. 2*A***). In contrast, GFP was not detected in any cells of intact Wt intestine cultured *in vitro* except for nonspecific staining of lysosome-like granules in macrophages and/or dying larval epithelial cells (Fig. 2*B*).

In recombinant intestine made of Tg Ep and Tg non-Ep (Tg/Tg), both Ep and non-Ep expressed GFP throughout the cultivation (Fig. 2*C*). In contrast, in recombinant intestine of Wt Ep and Wt non-Ep (Wt/Wt), neither Ep nor non-Ep expressed GFP except for the nonspecific staining of the lysosome-like granules (Fig. 2*D*). In recombinant intestine made of Tg Ep and Wt non-Ep (Tg/Wt), GFP expression was localized in Ep but not in non-Ep, although the intensity of GFP immunoreactivity was weak in some of the degenerat-

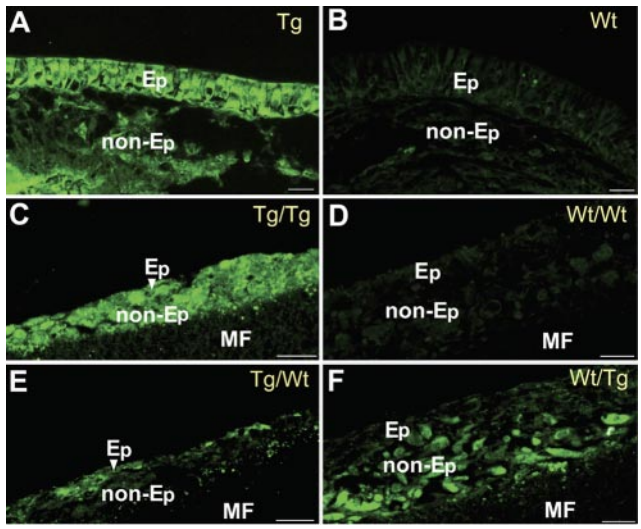

Figure 2. GFP expression is preserved in tissues derived from Tg tadpoles in recombinant organ cultures for 5 d. Cross sections were stained with anti-GFP antibody for immunofluorescent detection. *A, B*) Intact Tg (*A*) and Wt intestine (*B*) after cultivation. *C–F*) Recombinant intestine made of Tg Ep and Tg non-Ep (Tg/Tg; *C*), Wt Ep and Wt non-Ep (Wt/Wt; *D*), Tg Ep and Wt non-Ep (Tg/Wt; *E*), and Wt Ep and Tg non-Ep (Wt/Tg; *F*). GFP is expressed in Tg tissues but not in Wt tissues in all organ cultures. MF, membrane filter. Scale bars = 20 μm.

ing epithelial cells (Fig. 2*E*). On the other hand, in recombinant intestine made of Wt Ep and Tg non-Ep (Wt/Tg), it was localized in non-Ep but not in Ep (Fig. 2*F*). These results indicate that both Ep and non-Ep derived from Tg intestine retain their expression of GFP *in vitro*, whereas those derived from Wt intestine never express GFP, independent of tissues with which they are recombined.

TH induces adult epithelial development in Tg intestine as in Wt intestine

To confirm whether the adult epithelial development occurs in the Tg intestine as well as in the Wt intestine, we first chronologically observed intact intestine isolated from stage 57-Tg tadpoles and cultured in the presence of TH. Intact Tg intestine, which expressed GFP (**Fig. 3*A***), became globular in shape with the epithelium outside and the connective tissue and muscles inside by d 5. The progenitor cells of the adult epithelium were detectable on d 5 as small islets stained red with pyronin Y between the larval epithelium and the connective tissue (Fig. 3*B*). These islet cells were positive for Shh (Fig. 3*C*) and Msi-1 (Fig. 3*D*), both of which are shown to be markers for adult progenitor cells in the metamorphosing intestine (14–16). The intensity of immunoreactivity for Shh in the adult progenitor cells was stronger than that for Msi-1. The progenitor cells actively proliferated (Fig. 3*E*) and then, on d 7, differentiated into the simple columnar epithelium expressing IFABP, a marker for differentiated intestinal absorptive cells (Fig. 3*F*), but not Shh (Fig. 3*G*) and Msi-1 (Fig. 3*H*) any more. On the other hand, the larval epithelial cells, which undergo apoptosis on and after d 3 (35), were negative for Shh (Fig. 3*C*) and Msi-1 on d 5 (Fig. 3*D*) and were completely replaced by the adult epithelium on d 7. These chronological changes in the adult epithelium of intact Tg intestine are the same as those of Wt intestine reported previously (9, 14, 16).

GFP expression of adult progenitor cells and differentiated cells in recombinant intestine

To determine the origin of the adult stem cells, we then performed tissue recombination experiments by using Tg and Wt intestine. Although recombinant intestine undergoes the same TH-induced changes as *in vivo* or intact intestine, there are fewer epithelial cells in the recombinant organ cultures compared with those in the cultures of intact intestine throughout the cultivation, probably because some of the epithelial cells degenerate when they are separated from the connective tissue and/or when they fail to properly contact the connective tissue after recombination. The height of the epithelium in the recombinant intestine is thus lower than that in the intact intestine because of the smaller cell number (24, 30). Nevertheless, in the recombinant intestine made of tissues from Tg tadpoles, the adult progenitor cells expressing Shh (Fig. 3*I*) and Msi-1 (Fig. 3*J*) became detectable on d 5 just as in the intact Tg intestine. Then, on d 7, some of the epithelial cells began to express IFABP (Fig. 3*K*), although some others remained negative for IFABP, typical of such *in vitro* induced intestinal remodeling.

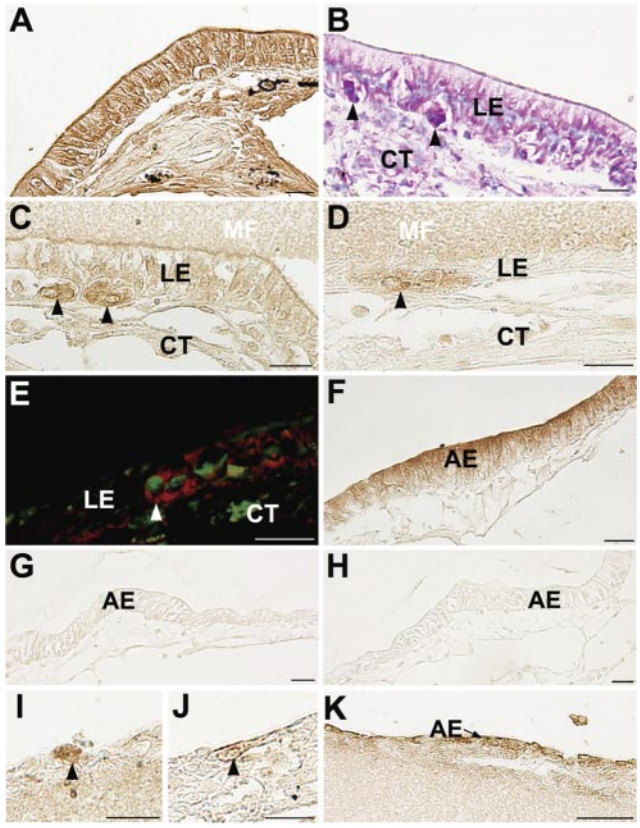

Figure 3. Development of adult intestinal epithelium in intact Tg (*A–H*) and recombinant intestine (*I–K*) cultured *in vitro*. Cross sections were immunostained with anti-GFP (*A*), anti-Shh (*C, G, I*), anti-Msi-1 (*D, H, J*), and anti-IFABP (*F, K*) antibodies, stained with methyl green-pyronin Y (*B*) or double-stained with anti-Shh (red) and PCNA (green) antibodies (*E*). In intact Tg intestine positive for GFP (*A*), adult progenitor cells become detectable on d 5 as small islets (arrowheads) stained red with pyronin Y between the larval epithelium (LE) and the connective tissue (CT) (*B*) and are positive for Shh (*C*) and Msi-1 (*D*). They proliferate (*E*) and then, on d 7, differentiate into the adult epithelium (AE) positive for IFABP (*F*), but negative for Shh (*G*) and Msi-1 (*H*). In any recombinant intestine, cells positive for Shh (*I*) and Msi-1 (*J*) are also detectable on d 5, although the intensity of Msi-1 immunoreactivity is weaker than that of Shh. Then, on d 7, the simple squamous epithelium positive for IFABP is also identified, indicative of the formation of differentiated epithelial cells (*K*). MF, membrane filter. Scale bars = 20 μm.

To determine whether the adult progenitor cells originate from the larval epithelium or the other tissues of the tadpole intestine at stage 57, we immunohistochemically examined GFP expression of the adult progenitor cells in each combination of recombinant intestine cultured for 5 d. Because the intensity of immunoreactivity for Shh (Fig. 3*I*) in the progenitor cells was stronger than that for Msi-1 (Fig. 3*J*), we used Shh as a marker to detect the adult progenitor cells in the recombinant intestine. Double immunofluores-

cence labeling with anti-Shh and anti-GFP antibodies showed that the adult progenitor cells positive for Shh always expressed GFP in Tg/Tg (**Fig. 4*A***) and Tg/Wt intestine (Fig. 4*C*; **Table 1**). In contrast, the adult progenitor cells positive for Shh never expressed GFP in the recombinant intestine when the Ep was derived from Wt animals, independent of the origin of the non-Ep (Fig. 4*B, D*; Table 1). In Wt/Tg intestine, although cells positive for GFP were rarely observed in the epithelial region, such GFP-positive cells never expressed Shh.

It is expected that the adult epithelium is formed by the differentiation of the proliferating adult progenitor/stem cells. To investigate whether the Shh-positive adult progenitor cells above give rise to the differentiated adult epithelial cells, double immunofluorescence labeling with anti-IFABP and anti-GFP antibodies was carried out on the recombinant intestine after culturing for 7 d in the presence of TH. The results showed that the differentiated adult cells positive for IFABP always expressed GFP in Tg/Tg (**Fig. 5*A***) or Tg/Wt intestine (Fig. 5*C*; Table 1). In contrast, the differentiated adult cells positive for IFABP never expressed GFP in Wt/Wt (Fig. 5*B*) and Wt/Tg intestine (Fig. 5*D*; Table 1). Thus, the adult stem cells and differentiated epithelial cells expressed GFP whenever the epithelium in the recombinant organ culture was derived from Tg animals but not whenever the epithelium was derived from the Wt animals, indicating that both the adult stem cells and differentiated epithelial cells are derived from the epithelium of stage 57 tadpoles.

TABLE 1. *GFP expression of adult progenitor cells and absorptive cells in recombinant intestine in vitro*

	Recombinant intestine[a]			
Cell type	Tg/Tg	Wt/Wt	Tg/Wt	Wt/Tg
Adult progenitor cells[b]	7/7	0/4	7/7	0/9
Adult absorptive cells[c]	5/5	0/6	7/7	0/5

[a]Type of Ep/type of non-Ep. [b]Intestine samples in organ cultures possessing adult progenitor cells double positive for Shh and GFP/total samples after 5 d in culture. [c]Intestine samples in organ cultures possessing adult absorptive cells double positive for IFABP and GFP/total samples after 7 d in culture.

DISCUSSION

The larval-to-adult epithelial remodeling in the *X. laevis* intestine has been well studied at the cellular level by light and electron microscopy (5, 13). The total dependence of this process on TH and the apparent *de novo* development of adult epithelial stem cells make this a unique and highly valuable model system to study the mechanism underlying the development of tissue-specific adult stem cells. Thus, it is not surprising that the origin of progenitor cells of the adult epithelium has attracted considerable interest from the standpoint of the stem cell biology. However, so far it has eluded experimental determination, mainly because of the difficulty of identifying and monitoring adult progenitor cells. Here by making use of Tg tadpoles constitutively expressing GFP for recombinant organ culture experiments, we have provided, for the first time, definitive evidence that the origin of the progenitor/stem cells as well as that of the finally differentiated adult epithelial cell of the frog intestine is the premetamorphic tadpole intestinal epithelium.

Our recombination experiments with Tg and Wt intestine indicated that both the adult progenitor cells and the differentiated adult absorptive cells express GFP when the epithelium is derived from Tg intestine but never express GFP when the epithelium is derived from Wt intestine. This finding, together with the spatial and temporal appearance of the progenitor and differentiated epithelial cells in the organ cultures, indicate that the adult progenitor/stem cells are derived from the larval epithelial cells at latest by stage 57 (before metamorphic climax) and subsequently differentiate into absorptive epithelial cells of the frog intestine. Although we cannot completely eliminate the possibility that some adult epithelial cells might be derived from cells outside of the tadpole epithelium but migrated into the epithelial layer, our failure to detect any GFP-positive adult epithelial cells when the epithelium in the recombinant organ cultures was derived from Wt animals indicates that such non-epithelium-derived adult progenitor cells, if they exist, are very few.

Among other amphibian organs that undergo larval-to-adult remodeling during metamorphosis, the skin is

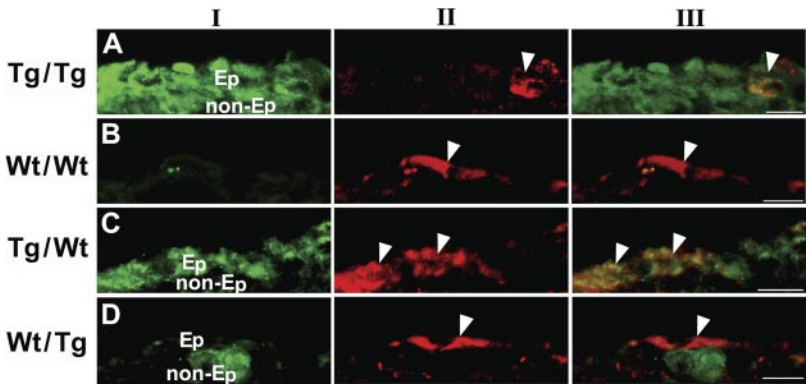

Figure 4. Analysis of GFP and Shh expression in recombinant intestine cultured for 5 d reveals that adult epithelial stem cells are derived from the tadpole epithelium. Cross-sections were double-stained with anti-GFP (I; green) and anti-Shh (II; red) antibodies. Column III panels represent the merged images of panels I and II. Recombinant intestinal sections include those made of Tg Ep and Tg non-Ep (Tg/Tg; *A*), Wt Ep and Wt non-Ep (Wt/Wt; *B*), Tg Ep and Wt non-Ep (Tg/Wt; *C*), and Wt Ep and Tg non-Ep (Wt/Tg; *D*). Adult progenitor cells positive for Shh (arrowheads) express GFP in Tg/Tg (*A*) and Tg/Wt intestine (*C*) but not in Wt/Wt (*B*) and Wt/Tg intestine (*D*). Scale bars = 10 μm.

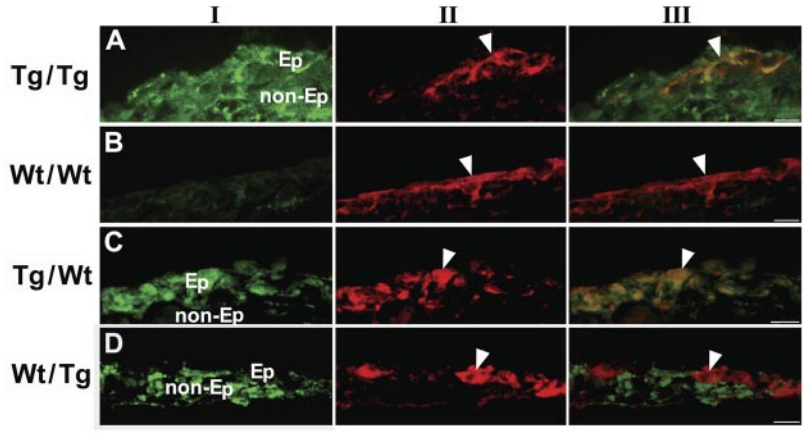

Figure 5. Analysis of GFP and IFABP expression in recombinant intestine cultured for 7 d shows that differentiated adult epithelial cells are derived from the tadpole epithelium. Cross-sections were stained with anti-GFP (I; green) and anti-IFABP (II; red) antibodies. Column III panels represent the merged images of panels I and II. Recombinant intestinal sections include those made of Tg Ep and Tg non-Ep (Tg/Tg; *A*), Wt Ep and Wt non-Ep (Wt/Wt; *B*), Tg Ep and Wt non-Ep (Tg/Wt; *C*), and Wt Ep and Tg non-Ep (Wt/Tg; *D*). Differentiated absorptive cells positive for IFABP (arrowheads) express GFP in Tg/Tg (*A*) and Tg/Wt intestine (*C*) but not in Wt/Wt (*B*) and Wt/Tg intestine (*D*). Scale bars = 10 μm.

the only one whose adult progenitor cells have been identified in the larval tissue (36, 37). In the *X. laevis* skin, "larval basal cells" at stage 58 have the potential to differentiate into adult cells, whereas the other cells are larval-proper cells and are lost during metamorphosis. Therefore, the larval basal cells are progenitor cells of the adult epidermis. There is a growing body of evidence based on organ culture and immunohistochemical studies that these larval basal cells originate from "basal skein cells," which are easily distinguished both morphologically and immunohistochemically from the other larval-proper epidermal cells as early as at stage 45 (37–39). This finding suggests that the progenitor cells for the adult stem cells of the amphibian skin are predetermined in the tadpoles, independent of TH. In contrast, in the larval epithelium of the *X. laevis* intestine, essentially all of the epithelial cells are differentiated as larval type and yet mitotically active, although they proliferate very slowly throughout pre- and prometamorphosis (3, 11). Undifferentiated cells and/or stem cells are not morphologically identified in the epithelium until stage 59 or later (40, unpublished results). In addition, our previous immunohistochemical studies failed to identify any cells expressing detectable Shh (14) or Msi-1 (16) in the *X. laevis* larval epithelium at stage 57 as used for the present study. Therefore, we conclude that the larval epithelial cells, although differentiated at stage 57 to serve the functions of the tadpole intestine, dedifferentiate into the progenitor/stem cells during stages 58–60 (**Fig. 6**), when the levels of TH increase rapidly in the plasma (41). Such a mechanism is also supported by the fact that the larval intestinal epithelial cells, although differentiated, are capable of proliferation *in vitro* (42), thus only requiring the repression of the expression of differentiated epithelial genes for dedifferentiation. Similarly, TH-induced dedifferentiation of the larval cells has also been implicated in the *X. laevis* exocrine pancreas (43), although adult stem cells of this organ have not yet been identified. If this is indeed the mechanism during the amphibian organ remodeling, the next question to address is whether all or a limited number of the larval differentiated epithelial cells have a potency to dedifferentiate into the stem cells and how TH induces some of the epithelial cells to become stem cells.

Earlier studies using clonogenic assays have shown that during mammalian intestinal regeneration in which the stem cells are experimentally removed by radiation, partially differentiated epithelial cells, called as "epithelial transit cells," dedifferentiate into stem cells and regenerate the intestinal absorptive epithelium (44, 45). In this regard, the tadpole intestinal epithelial cells, which are differentiated but mitotically active, may resemble these epithelial transit cells. Thus, the ability to reverse from the differentiated state to the stem cell state may be a characteristic of epithelial cells conserved in the mammalian and amphibian intestine. In the mammalian intestine, it is generally established that the microenvironment known as the "stem cell niche" plays important roles in epithelial development and/or regeneration through the control of the stem cells (46–48). However, its molecular mechanisms remain mostly unknown. More importantly, the identifi-

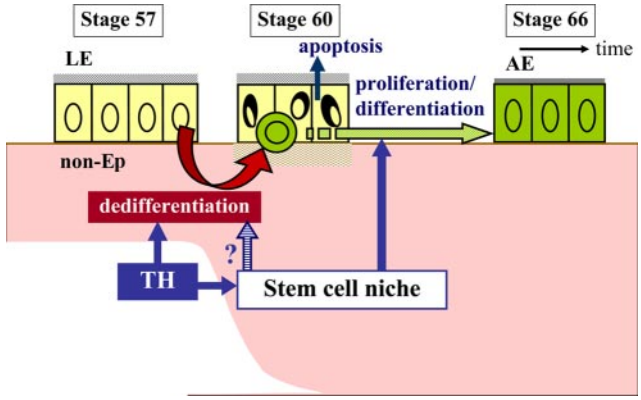

Figure 6. Schematic illustration showing the development of intestinal stem cells during TH-induced amphibian remodeling. The stem cells of the adult epithelium originate from the larval epithelium (LE) but not from the surrounding non-Ep. This process takes place as some of the larval epithelial cells, which are differentiated but retain the mitotic activity, dedifferentiate into the stem cells (S) by stage 60, and it is induced directly by TH and/or indirectly through the stem cell niche formed in the presence of TH. Thereafter, the stem cells form the adult absorptive epithelium (AE) analogous to the mammalian one under the influence of niche.

cation of niche factors that induce the epithelial transit cells into the stem cells is urgently needed for regenerative medicine and/or cancer therapy. In the amphibian intestine, we have shown previously that epithelial-connective tissue interactions are essential for the development of the adult epithelium during remodeling (30). What is unique to the amphibian intestinal model is that TH can induce all of the processes leading to the complete development of the adult intestine. Therefore, the niche factors involved in these processes are expected to be among the TH response genes, many of which have been identified in the *X. laevis* intestine by various PCR-based subtractive differential screens (49–51) and more recently by a cDNA array (52). Our identification of the origin of the adult stem cells should facilitate future analysis of the functions of potential niche factors in the induction of some differentiated cells to dedifferentiate into the stem cells.

In summary, organ-specific adult stem cells are critical for the homeostasis of adult organs and organ repair and/or regeneration. Unfortunately, it has been difficult to investigate the origins of the stem cells and the mechanisms of their development, especially in mammals. Intestinal remodeling during frog metamorphosis offers a unique opportunity for such studies. Our study here using Tg frogs provides the first conclusive experimental evidence that TH induces the differentiated larval epithelial cells to become the adult stem cells in the *X. laevis* intestine. This paves the way for use of the amphibian intestinal model as a unique model to study the molecular mechanisms leading to the induction of stem cells from the differentiated cells. Such studies will be further enhanced by the recent advances in functional analyses of TH response genes using Tg frog technology (32, 33). Undoubtedly, the findings from the frog model will not only improve our basic understanding of organ-specific adult stem cell development but also yield valuable information for regenerative medicine of the digestive tract. FJ

We thank Dr. Dave Turner (University of Michigan, Ann Arbor, MI, USA) and Dr. Gert Jan C. Veenstra (University of Nijmegen, Nijmegen, The Netherlands) for kind gifts of plasmids. This work was supported in part by the Japan Society for the Promotion of Science Grants-in-Aid for Scientific Research (C) (grant 20570060 to A.I.-O.) and in part by the Intramural Research Program of the National Institute of Child Health and Human Development, U.S. National Institutes of Health (to Y.-B.S.).

REFERENCES

1. Tata, J. R. (1993) Gene expression during metamorphosis: an ideal model for post- embryonic development. *Bioessays* **15,** 239–248
2. Shi, Y.-B. (1999) *Amphibian Metamorphosis: from Morphology to Molecular Biology*, John Wiley & Sons, New York
3. Marshall, J. A., and Dixon, K. E. (1978) Cell proliferation in the intestinal epithelium of *Xenopus laevis* tadpoles. *J. Exp. Zool.* **203,** 31–40
4. McAvoy, J. W., and Dixon, K. E. (1977) Cell proliferation and renewal in the small intestinal epithelium of metamorphosing and adult *Xenopus laevis*. *J. Exp. Zool.* **202,** 129–138
5. Shi, Y.-B., and Ishizuya-Oka, A. (1996) Biphasic intestinal development in amphibians: embryogenesis and remodeling during metamorphosis. *Curr. Top. Dev. Biol.* **32,** 205–235
6. Bjerknes, M., and Cheng, H. (1981) The stem-cell zone of the small intestinal epithelium. III. Evidence for columnar, enteroendocrine, and mucous cells in the adult mouse. *Am. J. Anat.* **160,** 77–91
7. Cheng, H., and Bjerknes, M. (1985) Whole population cell kinetics and postnatal development of the mouse intestinal epithelium. *Anat. Rec.* **211,** 420–426
8. Shi, Y.-B., and Hayes, W. P. (1994) Thyroid hormone-dependent regulation of the intestinal fatty acid-binding protein gene during amphibian metamorphosis. *Dev. Biol.* **161,** 48–58
9. Ishizuya-Oka, A., Ueda, S., Damjanovski, S., Li, Q., Liang, V. C.-T., and Shi, Y.-B. (1997) Anteroposterior gradient of epithelial transformation during amphibian intestinal remodeling: immunohistochemical detection of intestinal fatty acid-binding protein. *Dev. Biol.* **192,** 149–161
10. McAvoy, J. W., and Dixon, K. E. (1978) Cell specialization in the small intestinal epithelium of adult *Xenopus laevis*. *J. Anat.* **125,** 155–169
11. Ishizuya-Oka, A., and Ueda, S. (1996) Apoptosis and cell proliferation in the *Xenopus* small intestine during metamorphosis. *Cell Tissue Res.* **286,** 467–476
12. Nieuwkoop, P. D., and Faber, J. (1967) *Normal Table of* Xenopus laevis *(Daudin),* North-Holland Publishing, Amsterdam
13. Hourdry, J., and Dauca, M. (1977) Cytological and cytochemical changes in the intestinal epithelium during anuran metamorphosis. *Int. Rev. Cytol.* **5**(Suppl.), 337–385
14. Ishizuya-Oka, A., Ueda, S., Inokuchi, T., Amano, T., Damjanovski, S., Stolow, M., and Shi, Y.-B. (2001) Thyroid hormone-induced expression of sonic hedgehog correlates with adult epithelial development during remodeling of the *Xenopus* stomach and intestine. *Differentiation* **69,** 27–37
15. Hasebe, T., Kajita, M., Shi, Y.-B., and Ishizuya-Oka, A. (2008) Thyroid hormone- upregulated hedgehog interacting protein is involved in larval-to-adult intestinal remodeling by regulating sonic hedgehog signaling pathway in. *Xenopus laevis*. *Dev. Dyn.* **237,** 3006–3015
16. Ishizuya-Oka, A., Shimizu, K., Sakakibara, S., Okano, H., and Ueda, S. (2003) Thyroid hormone-upregulated expression of Musashi-1 is specific for progenitor cells of the adult epithelium during amphibian gastrointestinal remodeling. *J. Cell Sci.* **116,** 3157–3164
17. Ishizuya-Oka, A., and Shi, Y.-B. (2007) Regulation of adult intestinal epithelial stem cell development by thyroid hormone during *Xenopus laevis* metamorphosis. *Dev. Dyn.* **236,** 3358–3368
18. De Santa Barbara, P., van den Brink, G. R., and Roberts, D. J. (2003) Development and differentiation of the intestinal epithelium. *Cell. Mol. Life Sci.* **60,** 1322–1332
19. Kayahara, T., Sawada, M., Takaishi, S., Fukui, H., Seno, H., Fukuzawa, H., Suzuki, K., Hirai, H., Kageyama, R., Okano, H., and Chiba, T. (2003) Candidate markers for stem and early progenitor cells, Musashi-1 and Hes1, are expressed in crypt base columnar cells of mouse small intestine. *FEBS Lett.* **535,** 131–135
20. Potten, C. S., Booth, C., Tudor, G. L., Booth. D., Brady, G., Hurley, P., Ashton, G., Clarke, R., Sakakibara, S., and Okano, H. (2003) Identification of a putative intestinal stem cell and early lineage marker, Musashi-1. *Differentiation* **71,** 28–41
21. He, X. C., Zhang, J., Tong, W. G., Tawfik, O., Ross, J., Scoville, D. H., Tian, Q., Zeng, X., He, X., Wiedemann, L. M., Mishina, Y., and Li, L. (2004) BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt-β-catenin signaling. *Nat. Genet.* **36,** 1117–1121
22. Dodd, M. H. I., and Dodd, J. M. (1977) The biology of metamorphosis. In *Physiology of Amphibia*, Vol. 3 (Lofts, B., ed) pp. 467–599, Academic Press, New York
23. Kikuyama, S., Kawamura, K., Tanaka, S., and Yamamoto, K. (1993) Aspects of amphibian metamorphosis: hormonal control. *Int. Rev. Cytol.* **145,** 105–148
24. Ishizuya-Oka, A., and Shimozawa, A. (1991) Induction of metamorphosis by thyroid hormone in anuran small intestine cul-

tured organotypically in vitro. *In Vitro Cell. Dev. Biol.* **27A,** 853–857
25. Amano, T., Noro, N., Kawabata, H., Kobayashi, Y., and Yoshizato, K. (1998) Metamorphosis-associated and region-specific expression of calbindin gene in the posterior intestinal epithelium of *Xenopus laevis* larva. *Dev. Growth Differ.* **40,** 177–188
26. Schreiber, A. M., Cai, L., and Brown, D. D. (2005) Remodeling of the intestine during metamorphosis of *Xenopus laevis*. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 3720–3725
27. Krause, D. S., Theise, N. D., Collector, M. I., Henegariu, O., Hwang, S., Gardner, R., Neutzel, S., and Sharkis, S. J. (2001) Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell* **105,** 369–377
28. Okamoto, R., Yajima, T., Yamazaki, M., Kanai, T., Mukai, M., Okamoto, S., Ikeda, Y., Hibi, T., Inazawa, J., and Watanabe, M. (2002) Damaged epithelia regenerated by bone marrow-derived cells in the human gastrointestinal tract. *Nat. Med.* **8,** 1011–1017
29. Shi, Y.-B., Fu, L., Hasebe, T., and Ishizuya-Oka, A. (2007) Regulation of ECM remodeling and cell fate determination by matrix metalloproteinase strmoelysin-3 during thyroid hormone-dependent postembryonic development. *Pharmacol. Ther.* **116,** 391–400
30. Ishizuya-Oka, A., and Shimozawa, A. (1992) Connective tissue is involved in adult epithelial development of the small intestine during anuran metamorphosis in vitro. *Roux Arch. Dev. Biol.* **201,** 322–329
31. Kroll, K. L., and Amaya, E. (1996) Transgenic *Xenopus* embryos from sperm nuclear transplantations reveal FGF signalling requirements during gastrulation. *Development* **122,** 3173–3183
32. Buchholz, D. R., Tomita, A., Fu, L., Paul, B. D., and Shi, Y.-B. (2004) Transgenic analysis reveals that thyroid hormone receptor is sufficient to mediate the thyroid hormone signal in frog metamorphosis. *Mol. Cell. Biol.* **24,** 9026–9037
33. Fu, L., Buchholtz, D., and Shi, Y.-B. (2002) Novel double promoter approach for identification of transgenic animals: a tool for in vivo analysis of gene function and development of gene-based therapies. *Mol. Reprod. Dev.* **62,** 470–476
34. Offield, M. F., Hirsch, N., and Grainger, R. M. (2000) The development of *Xenopus tropicalis* transgenic lines and their use in studying lens developmental timing in living embryos. *Development* **127,** 1789–1797
35. Ishizuya-Oka, A., Li, Q., Amano, T., Damjanovski, S., Ueda, S., and 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
36. Yoshizato, K. (1989) Biochemistry and cell biology of amphibian metamorphosis with a special emphasis on the mechanism of removal of larval organs. *Int. Rev. Cytol.* **119,** 97–149
37. Yoshizato, K. (2007) Molecular mechanism and evolutional significance of epithelial-mesenchymal interactions in the body- and tail-dependent metamorphic transformation of anuran larval skin. *Int. Rev. Cytol.* **260,** 213–260
38. Suzuki, K., Utoh, R., Kotani, K., Obara, M., and Yoshizato, K. (2002) Lineage of anuran epidermal basal cells and their differentiation potential in relation to metamorphic skin remodeling. *Dev. Growth Differ.* **44,** 225–238
39. Utoh, R., Shigenega, S., Watanabe, Y., and Yoshizato, K. (2003) Platelet-derived growth factor signaling as a cue of the epithelial-mesenchymal interaction required for anuran skin metamorphosis. *Dev. Dyn.* **227,** 157–169
40. Marshall, J. A., and Dixon, K. E. (1978) Cell specialization in the epithelium of the small intestine of feeding *Xenopus laevis* tadpoles. *J. Anat.* **126,** 133–144
41. Leloup, J., and Buscaglia, M. (1977) La triiodothyronine: hormone de la metamorphose des amphibiens. *C. R. Acad. Sci.* **284,** 2261–2263
42. Su, Y., Shi, Y., Stolow, M. A., and 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
43. Mukhi, S., Mao, J., and Brown, D. D. (2008) Remodeling the exocrine pancreas at metamorphosis in *Xenopus laevis*. *Proc. Natl. Acad. Sci. U. S. A.* **105,** 8962–8967
44. Potten, C. S. (1998) Stem cells in gastrointestinal epithelium: numbers, characteristics and death. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **353,** 821–830
45. Potten, C. S., Booth, C., and Pritchard, D. M. (1997) The intestinal epithelial stem cell: the mucosal governor. *Int. J. Exp. Pathol.* **78,** 219–243
46. Blau, H. M., Brazelton, T. R., and Weimann, J. M. (2001) The evolving concept of a stem cell: entity or function? *Cell* **105,** 829–841
47. Mills, J. C., and Gordon, J. I. (2001) The intestinal stem cell niche: there grows the neighborhood. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 12334–12336
48. Spradling, A., Drummond-Barbosa, D., and Kai, T. (2001) Stem cells find their niche. *Nature* **414,** 98–104
49. Shi, Y.-B., and Brown, D. D. (1993) The earliest changes in gene expression in tadpole intestine induced by thyroid hormone. *J. Biol. Chem.* **268,** 20312–20317
50. Amano, T., and Yoshizato, K. (1998) Isolation of genes involved in intestinal remodeling during anuran metamorphosis. *Wound Repair Reger.* **6,** 302–313
51. Shimizu, K., Ishizuya-Oka, A., Amano, A., Yoshizato, K., and Ueda, S. (2002) Isolation of connective tissue-specific genes involved in *Xenopus* intestinal remodeling: thyroid hormone up-regulates Tolloid/BMP-1 expression. *Dev. Growth Evol.* **212,** 357–364
52. Buchholz, D. R., Heimeier, R. A., Das, B., Washington, T., and Shi, Y.-B. (2006) Pairing morphology with gene expression in thyroid hormone-induced intestinal remodeling and identification of a core set of TH-induced genes across tadpole tissues. *Dev. Biol.* **303,** 576–590

Received for publication December 23, 2008.
Accepted for publication February 26, 2009.