Apoptosis and differentiation of *Xenopus* tail-derived myoblasts by thyroid hormone

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Abstract

The metamorphosis of anuran amphibians is induced by thyroid hormone (TH). To study the molecular mechanisms underlying tail regression during metamorphosis, we established a cell line, XL-B4, from a *Xenopus laevis* tadpole tail at a premetamorphic stage. The cells expressed myoblast markers and differentiated into myotubes in differentiation medium. XL-B4 cells expressing fluorescent proteins were transplanted into tadpole tails. At 5 days post-transplantation, fluorescence was observed in myotube-like structures, indicating that the myoblastic cells could contribute to skeletal muscle. Exposure of XL-B4 cells to the TH triiodothyronine (T_3) for several days significantly induced apoptotic cell death. We then examined an early response of expression of genes involved in apoptosis or myogenesis to T_3. Treatment of the cells with T_3 increased transcription of genes for matrix metalloproteinase-9 (MMP-9) and thyroid hormone receptor beta. Interestingly, the T_3-treatment also increased *myoD* transcripts, but decreased the amounts of *myogenin* mRNA and myosin heavy chain. Importantly, we also observed upregulation of *myoD* expression and downregulation of *myogenin* expression in tails, but not in hind limbs, when tadpoles at a premetamorphic stage were treated with T_3 for 1 day. These results indicated that T_3 could not only induce apoptosis, but also attenuate myogenesis in tadpole tails during metamorphosis.

Key Words

- myoblast
- thyroid hormone
- metamorphosis
- myogenesis
- apoptosis
- *Xenopus*

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Introduction

During the metamorphosis of anuran amphibians, tissue remodeling occurs in a dynamic and orderly manner through developmental programs (Tata 2006). For example, limb buds are generated, and the gills and tail disappear. The larval intestines are reconstructed to form the adult organs (Ishizuya-Oka 2011, Shi *et al*. 2011). All these diverse changes are believed to be controlled by triiodothyronine (T_3), an active form of thyroid hormone (TH) (Furlow & Neff 2006). In the African clawed frog *Xenopus laevis*, elimination of the gills and tail, which is accompanied by apoptotic cell death, is completed in a few days during metamorphosis (Shi *et al*. 2001). Thus, the regression of the tadpole tail is a good experimental system for studying the molecular mechanisms of programed cell death (Tata 2006). A complex of T_3 and its receptor (thyroid hormone receptor (TR)) regulate gene transcription and induce apoptosis (Brown *et al*. 1996, Sachs *et al*. 2000). Results of analyses of transgenic *X. laevis*

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expressing a dominant-negative form of Trα, one of the TR family members, indicated that TH functions not only in the elimination of the organs but also in the growth of the brain and limb buds (Schreiber *et al*. 2001, Das *et al*. 2002). However, it has remained unclear how the TR–T_3 complex could elicite the apoptotic signal during metamorphosis.

We previously isolated and analyzed death receptor (DR) members DR-Ms and tumor necrosis factor (TNF)-α receptor 1 (TNFR1), and their cognate lignads, TRAILs and TNFα, respectively, in *X. laevis* (Tamura *et al*. 2004, 2010, Ishizawa *et al*. 2006, Mawaribuchi *et al*. 2008, Ito *et al*. 2012). We found that TRAIL1/DR-Ms could enhance the transition of red blood cells from the larval to adult type during metamorphosis (Tamura *et al*. 2010, 2015). In addition, we established an endothelial cell line XLgoo from a tadpole tail at stage 55 during premetamorphosis in the species (Mawaribuchi *et al*. 2008). The cells expressing TNFR1 formed actin stress fibers and elongated in response to TNFα. Intriguingly, T_3 induced apoptosis in XLgoo cells, but TNFα partially inhibited the cell death, maybe through TNFR1.

In this study, we characterize another tadpole-tail-derived cell line, XL-B4. Because the cells expressed myoblastic cell marker genes, we confirmed that the cells could differentiate into myotubes *in vitro*. Moreover, the cells differentiated into myotubes when transplanted into tadpole tails. Interestingly, cell death and differentiation of XL-B4 cells were regulated by T_3, providing a new insights into the roles of T_3 in cell fate of myoblasts in degenerating tails during metamorphosis. Thus, the XL-B4 cells could be useful for studying the myogenesis and myotube degeneration that occur during *X. laevis* metamorphosis.

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Materials and methods

Materials

The Alexa Fluor 546-conjugated anti-mouse IgG antibody was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Rabbit polyclonal anti-myosin heavy chain (MHC) antibodies (H-300, sc-20641) and anti-GAPDH antibodies (FL-335, sc-25778), and mouse monoclonal anti-α-tubulin antibodies (B-7, sc-5286) were purchased from Santa Cruz Biotechnology. Z-VAD FMK was purchased from Sigma.

Animal care and use

The Institutional Animal Care and Use Committee of Kitasato University approved all experimental procedures involving *X. laevis*.

Establishment of the XL-B4 cell line

The XL-B4 cell line was established using almost the same method as described previously (Mawaribuchi *et al*. 2008). Briefly, tail tips from *X. laevis* tadpoles at stage 55 were treated with 0.25% trypsin and 0.5% collagenase in 0.7×PBS. The cells were resuspended in 0.7×L-15 medium supplemented with 20% FCS that had been treated with AG1-X8 resin (Bio-Rad) to remove the TH, and cultured at 20 °C on a normal culture dish. Once the cells became confluent, they were passaged once a week. One cell line, named XL-B4, was recloned and characterized.

Cell culture and transfection

XL-B4 cells were grown in the medium described previously, and transfected with FuGENE HD (Roche Diagnostics). For differentiation into myotubes, the XL-B4 cells were cultured in the differentiation medium (0.7×L-15 medium supplemented with 2% horse serum (HS) that had been treated with AG1-X8 resin).

Assay of caspase activity and apoptosis

XL-B4 cells were plated at 1×10^5 cells/35 mm dish. Then, the cells were treated with TH (T_3). After 24 h, caspase-3/-7 activities were measured using the Caspase-Glo 3/7 Assay Kit (Promega). For 7 days after T_3-treatment, the TUNEL protocol was carried out using an *In Situ* Cell Death Detection kit (Roche) according to the manufacturer's instruction.

Cell viability assay

XL-B4 cells in 96-well plates were treated with T_3 for 7 days. Cell viability was measured using the CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega).

RT-PCR

Total RNA was extracted from cultured cells, tadpole tails, and hind limbs using the RNeasy Mini Kit (Qiagen). The RNA (0.5 μg) was reverse transcribed with the PrimeScript First Strand cDNA Synthesis Kit (Takara, Shiga, Japan), according to the manufacturer's instructions. The PCR was performed using a Rotor-Gene (Qiagen) with the SYBR Green Real-time PCR Master Mix (Toyobo, Osaka, Japan), and gene-specific primers (see above) as follows: 5′-AACTGCTCCGA-TGGCATGATGGATTA-3′ (forward) and 5′-ATTGCTG-GGAGAAGGGATGGTGATTA-3′ (reverse) for *myoD*, 5′-CCAGCCCTTATTTCTTTTCAGACCA-3′ (forward) and

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5′-AATCCCTGAGCCCTGTAATAAAACC-3′ (reverse) for *myogenin*. 5′-GGAAGGAGGAGATGATCAAGACT-3′ (forward) and 5′-AAGCTTCCAGGTCAACTTTATCC-3′ (reverse) for *trα*, 5′-AAAGAAAATTTTTGCCAGAGGAC-3′ (forward) and 5′-GCCTTTAAGAAGGATGATCTGGT-3′ (reverse) for *trβ*, 5′-CTCTCAGCCAAATGCAAAGT-3′ (forward) and 5′-GTTTAGGATACGATATGTGAG-3′ (reverse) for TH-inducible matrix metalloproteinase-9 (*mmp-9th*), and 5′-TCAGGCATTGAAACAGACAG-3′ (forward) and 5′-AGCTACCATATGATTTACACAGG-3′ (reverse) for *caspase-3*. As a control, *ef1α* was also amplified with specific primers: 5′-CCAGATTGGTGCTGGATATG-3′ (forward) and 5′-TTCTGAGCAGACTTTGTGAC-3′ (reverse). Statistical significance was analyzed using the paired Student's *t*-test, as appropriate. P values of <0.05 were considered to be statistically significant.

Western blotting analysis

The preparation of cell extracts and immunoblot analysis have been described previously (Ishizawa *et al.* 2006).

Immunocytochemistry

XL-B4 cells were fixed with 4% paraformaldehyde in PBS, exposed to 0.2% Triton X in PBS for 30 min, and blocked with 5% skim milk in PBS for 30 min. The cells were then incubated with the anti-MHC antibody (1:200) in PBS for 2.5 h, washed three times, and incubated with Alexa Fluor 546-conjugated secondary antibody (1:1000) and 5 μg/ml Hoechst 33258 for 1 h. After being washed extensively with PBS, the cells were examined with a fluorescence microscope.

Plasmids

The enhanced green fluorescent protein (EGFP) coding sequence was amplified by PCR using the pEGFP-1 vector (Takara). The fragments were subcloned into a pEF/Myc/nuc vector (Thermo Fisher Scientific) to generate pEF/EGFP-NLS-Myc, which expressed EGFP in the nucleus. The other constructs were pDsRed2-C1 (Takara), which expresses *Discosoma sp.* red fluorescent protein (DsRed), and piGENEtRNA Pur (iGENE Therapeutics, Tokyo, Japan), which contains a puromycin-resistance gene. XL-B4 cells were transfected with all three vectors simultaneously. An expression plasmid for FLAG-tagged EGFP, pcDNA3-FLAG-EGFP, has been described previously (Tamura *et al.* 2004).

Transplantation

XL-B4 cells were transfected with pEF/EGFP-NLS-Myc, pDsRed2-C1, and piGENEtRNA pur. Two days after the transfection, the cells were selected with puromycin for an additional 2 days. The XL-B4 cells (1×10^2–10^3 cells) were directly injected into the tail muscle of each tadpole (stage 56), using an MN153 micromanipulator (Narishige, Tokyo, Japan).

Results

XL-B4 cells are derived from tadpole-tail myoblastic cells

To study intracellular signaling in an *in vitro* model of metamorphosis, we established several cell lines from *X. laevis* tadpole tails at stage 55, a premetamorphic stage. In this study, we report the characterization of one of these lines, XL-B4. PCR of genomic DNA using primers designed to distinguish genetic females (ZW) from males (ZZ) (Yoshimoto *et al.* 2008) indicated that the XL-B4 line was derived from a male tadpole (data not shown). We observed a few fused cells in confluent cultures of XL-B4 cells, but not in normal growth cultures. Because myoblasts have the potential to form myotubes through cell fusion, we examined the possibility that XL-B4 was a myoblastic cell line. In the presence of HS, myoblasts often differentiate and form myotubes *in vitro* (Lechner *et al.* 1996, Sun *et al.* 2005). The XL-B4 cells were therefore cultured in medium containing 2% HS in place of 20% FBS. After 10 days, some of the cells displayed elongated and filamentous shapes, which resembled myotubes (Fig. 1A). To test whether the myotube-like cells expressed muscle-specific MHC, fluorescence immunocytochemistry and immunoblotting with an anti-MHC antibody were performed. The antibody reacted intensely with the myotube-like cells grown in differentiation medium (Fig. 1A), and the amount of MHC was increased by the HS treatment (Fig. 1B and Supplementary Fig. 1, see section on supplementary data given at the end of this article).

We next performed a real-time RT-PCR analysis to look for the expression of *myoD* and *myogenin*, which are specifically involved in myogenesis. The results of the analysis indicated that *myoD* mRNA was more abundant in the normally cultured XL-B4 cells than that in the HS-treated cells, while *myogenin* showed higher expression in the HS-treated cells (Fig. 1C). As a control, we confirmed that *Xenopus* kidney A6 cells had no *myoD* or *myogenin* mRNA (data not shown).

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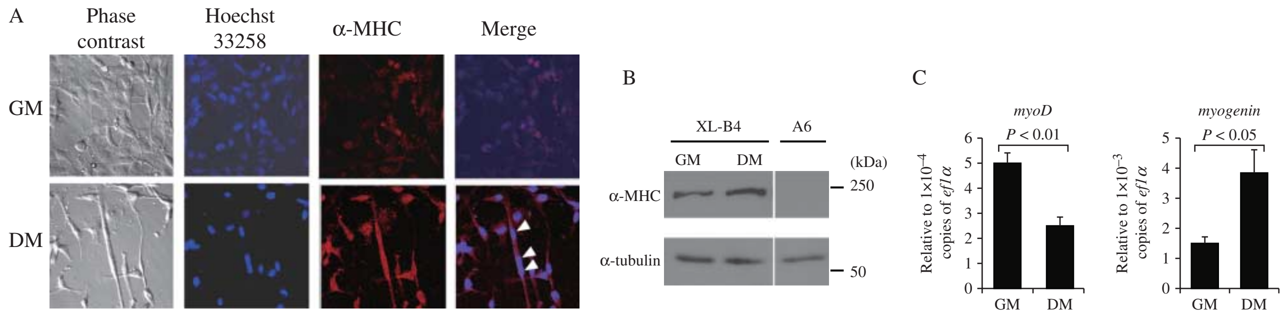

Figure 1

Characterization of tadpole-tail-derived cell line XL-B4 as myoblastic. (A) XL-B4 cells were cultured in 70% L-15 medium containing 20% thyroid hormone (TH)-depleted fetal bovine serum (growth medium (GM)) or 2% TH-depleted HS (differentiation medium (DM)) for 10 days. The cells were fixed with 4% paraformaldehyde and visualized by staining with Hoechst 33258 and an anti-myosin heavy chain (MHC) antibody followed by an anti-mouse Alexa Fluor 546 antibody. Arrowheads indicate nuclei in a multi-nuclear cell. (B) Western blot analysis of XL-B4 or A6 cell extracts was performed using the anti-MHC antibody and an anti-tubulin antibody as a control. The cell extracts were prepared from nonconfluent XL-B4 cells cultured in the GM and DM. As a negative control, extracts of *Xenopus* kidney A6 cells were also prepared. (C) RNA expression of the muscle-specific genes *myoD* and *myogenin*, or of *ef1α* as a control in XL-B4 cells or A6 cells was analyzed by real-time RT-PCR. RNA samples were from XL-B4 and A6 cells prepared as described in (B). The data represent the mean ($n=3$) and s.d.

Transplanted XL-B4 cells form a fiber-like structure in tadpole tails

We next examined whether the myoblastic XL-B4 cells could contribute to muscle tissues *in vivo*. Most of the surviving cells expressed both EGFP and DsRed. The green fluorescence was mostly localized to the nucleus, and the red fluorescence was observed in both the nucleus and cytoplasm. A representative fluorescence pattern is shown in Fig. 2A. We then transplanted the cells into the tail skeletal muscle of stage 56 tadpoles. At 5 days post-transplantation, we observed EGFP expression in the nuclei of cells that appeared as a line of DsRed fluorescence aligned with muscle fibers (Fig. 2B), indicating the formation of multinucleated myotubes from EGFP- and DsRed-expressing XL-B4 cells. Myotube-like structures that were only red, which appeared to be derived from Ds-Red expressing cells without the EGFP expression vector, were also observed (Fig. 2B). Fewer than ten myotube-like structures expressing EGFP and/or DsRed were observed 5 days after injection of 1×10^2–10^3 cells of the XL-B4 cells/tail. These results indicate that the tadpole-tail-derived XL-B4 myoblastic cells could contribute to skeletal muscle myotubes in the tail.

TH (T_3) induces apoptosis in XL-B4 cells

Amphibian metamorphosis is induced by high levels of T_3 in the blood (Furlow & Neff 2006). Because the XL-B4 cells

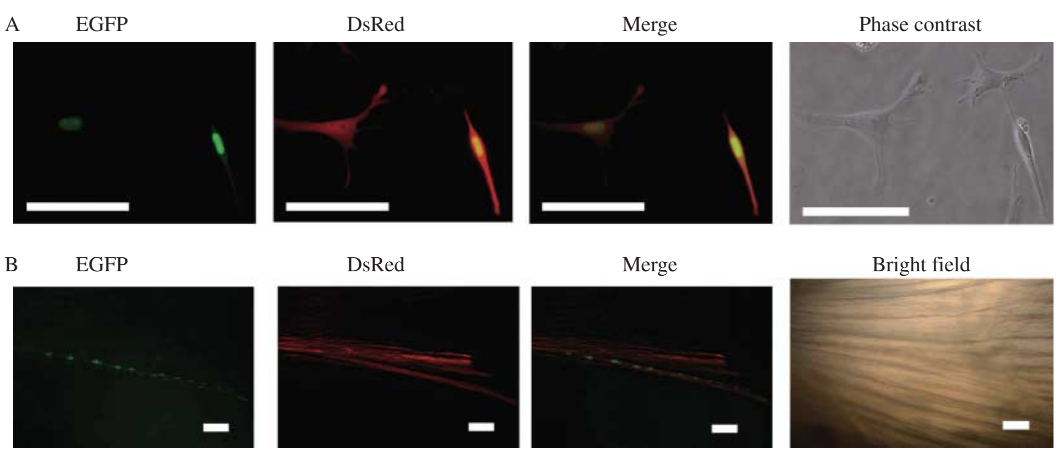

Figure 2

Transplantation of XL-B4 cells into tadpole tail. Puromycin-resistant XL-B4 cells expressing EGFP in the nucleus and DsRed in the nucleus and cytoplasm were selected. Representative cells are shown (A). XL-B4 cells selected as in (A) were injected into the tail muscle of stage 56 tadpoles. Five days post-transplantation, multinucleated myotube-like structures were observed (B). Scale bars, 50 μm.

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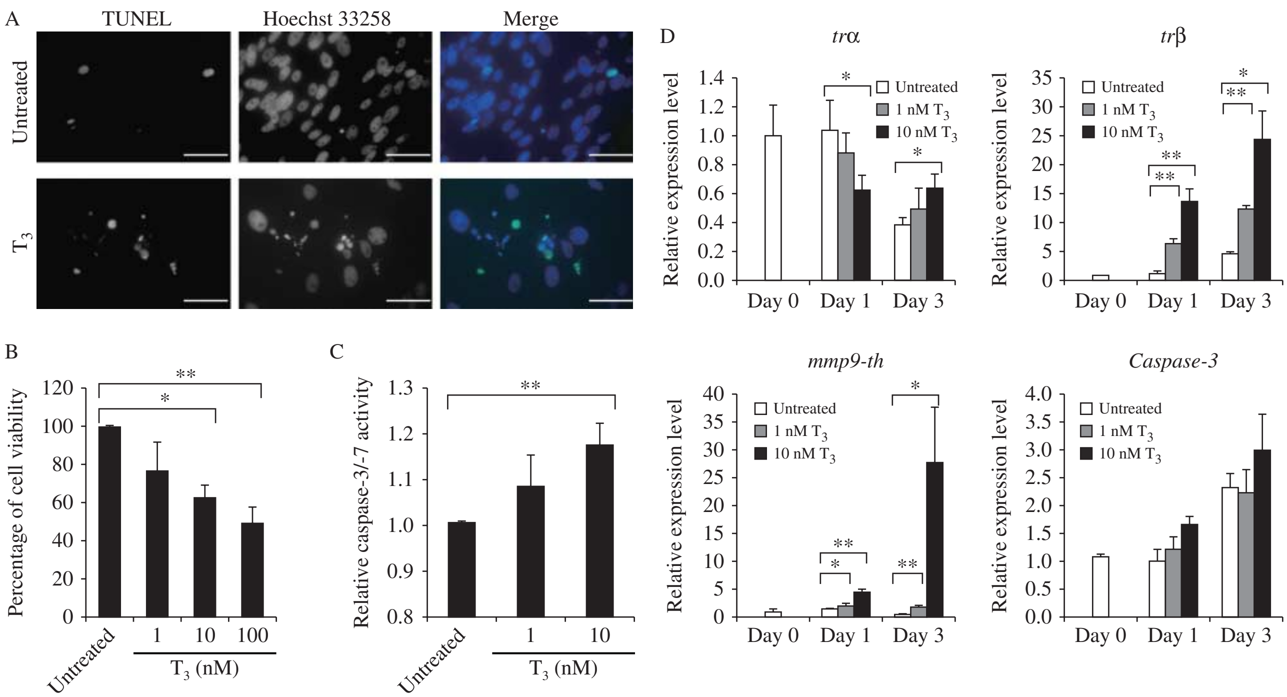

Figure 3

Thyroid hormone (T_3)-induced apoptosis in myoblast XL-B4 cells. (A) XL-B4 cells were cultured in the presence or absence of 10 nM T_3 for 7 days. The cells were then fixed, stained with Hoechst 33258 and TUNEL. Scale bars, 50 μm. (B) The viability of the cells treated with T_3 for 7 days was quantified using the CellTiter-Glo luminescent cell viability assay. Cell viability is shown as the percentage of the value obtained using untreated cells. The data represent the mean ($n=3$) and s.d.; $*P<0.05$ and $**P<0.01$ compared with untreated. (C) The activity of caspase-3/-7 in the cells treated with T_3 for 1 day was quantified using the Caspase-Glo 3/7 Assay Kit. The data represent the mean ($n=4$) and s.d.; $**P<0.01$ compared with untreated. (D) XL-B4 cells were treated with T_3 for 1 and 3 days, and then the RNAs were isolated. Real-time RT-PCR analysis for several genes was performed using specific primer pairs (see 'Materials and methods' section). *mmp-9th*, TH-inducible matrix metalloproteinase-9; *trα* and *β*, *Xenopus* thyroid hormone receptor α and β; *ef1α*, *Xenopus* elongation factor 1α. The data represent the mean ($n=3$) and s.d.; $*P<0.05$ and $**P<0.01$ compared with untreated.

were derived from tadpole tail during premetamorphosis, we examined whether T_3 induced apoptosis in these cells. The cells were treated with T_3 for 7 days, and apoptotic cells were assessed by TUNEL staining. We observed many floating cells, which had apoptotic features exhibiting nuclear condensation or fragmentation (Supplementary Fig. 2A). Most of the adherent cells were TUNEL-positive (Fig. 3A). As expected, T_3-treatment decreased the viability of XL-B4 cells in a dose-dependent fashion (Fig. 3B). Comparison of T_3-treatment for 3 and 7 days indicated that the proportion of apoptotic cells increased with the incubation time (Supplementary Fig. 2B). Next, we examined whether T_3-treatment induced caspase-3/-7 activity in the XL-B4 cells. Treatment with 10 nM T_3 for 1 day significantly induced activation of caspase-3/-7 (Fig. 3C). In addition, the pan-caspase inhibitor Z-VAD-FMK substantively suppressed the T_3-induced apoptosis (Supplementary Fig. 2C), even at about tenfold higher concentration of T_3 (100 nM) than that of endogenous plasma at the climax of metamorphosis (Leloup & Buscaglia 1977). These results indicated that T_3 could induce apoptosis of XL-B4 cells, a characteristic that could reflect T_3-driven tail degeneration during metamorphosis.

TH enhances the mRNA expression of *trβ*, and *mmp-9th* in XL-B4 cells

We next investigated the effect of T_3 on the transcription of several metamorphosis- and apoptosis-related genes by RT-PCR analysis. We selected genes for two TRs (TRα and TRβ) and *mmp-9th* as a metamorphosis-related gene, and *caspase-3* as an apoptosis-related gene. T_3-treatment for 1 and 3 days upregulated *trβ* transcription (Fig. 3D). The *trα* expression was slightly down- or upregulated by T_3-treatment for 1 or 3 days respectively. In contrast, in cells cultured for 3 days, regardless of the presence or absence of T_3, the amount of *caspase-3* mRNA increased. With respect to other caspase genes, *caspase-7* and *caspase-10* mRNAs appeared to be constantly expressed in the T_3-treated cells (Supplementary Fig. 3). The *mmp-9th*

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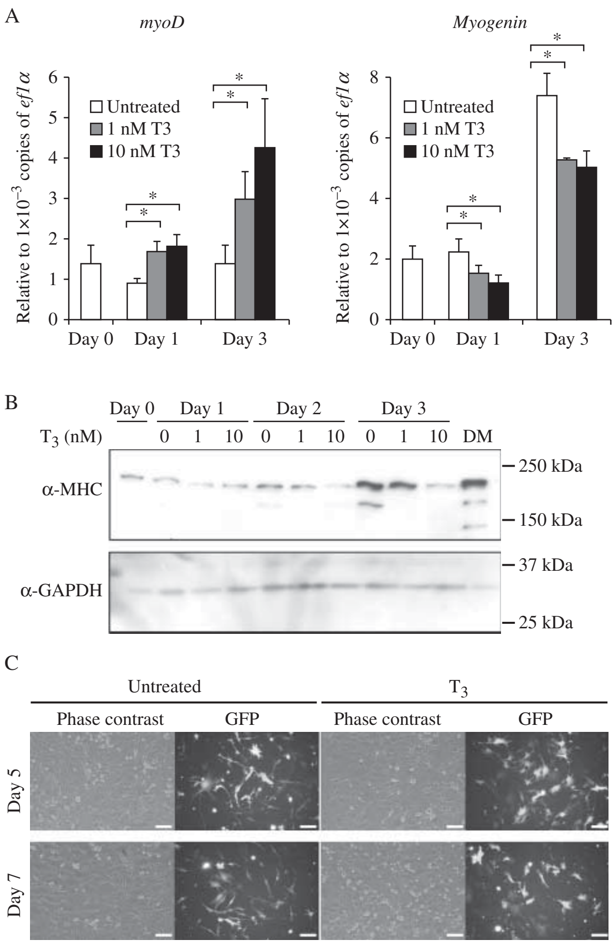

Figure 4
Effects of thyroid hormone (T_3) on the expression of myogenic markers in XL-B4 cells. XL-B4 cells were plated at 2×10^5 cells/35 mm dish. After 24 h, the cells were cultured in the presence or absence of 1 or 10 nM T_3 for 1 or 3 days. (A) Real-time PCR was performed for *myoD* and *myogenin* mRNAs from the XL-B4 cells under the above conditions by using gene-specific primer pairs (see 'Materials and methods' section). The data represent the mean ($n=3$) and s.d.; $*P<0.05$ compared with untreated. (B) Western blot analysis of the XL-B4 cell extracts cultured under the above conditions and in the DM was performed using the anti-MHC antibody and an anti-GAPDH antibody as a control. (C) XL-B4 cells were transfected with pcDNA3 FLAG-EGFP. Then, the cells were treated with 10 nM T_3. After 5 or 7 days, the cells were observed with phase-contrast microscopy (left) and fluorescent microscopy (right). Scale bars, 100 μm.

expression was gradually induced by T_3-treatment for 1–3 days (Fig. 3D). This upregulation of the gene by T_3 was not observed in *X. laevis* kidney-derived A6 cells, even at 100 nM T_3 (Supplementary Fig. 3).

TH attenuates the transcription of *myogenin* and the expression of MHC in XL-B4 cells

We next examined whether T_3-treatment might affect the expression of early and late myogenetic marker genes, *myoD* and *myogenin*, respectively, in XL-B4 cells, by real-time PCR analysis. Treatment with both 1 and 10 nM T_3 for 1–3 days significantly enhanced *myoD* transcripts as compared with no treatment (Fig. 4A, left). In contrast, the expression level of *myogenin* in untreated XL-B4 cells increased after 3 days in culture. Interestingly, the expression of *myogenin* in the T_3-treated cells was significantly lower than that in the untreated cells (Fig. 4A, right). Results of western blotting analysis indicated that the expression level of MHC was increased after 3 days in the growth media, similarly to *myogenin* mRNA. However, T_3 attenuated MHC expression in culture for 3 days in a T_3-dose-dependent manner (Fig. 4B). Next, we examined whether T_3 was involved in differentiation in the XL-B4 cells, because myoblastic cells could acquire an elongated shape (Burattini *et al*. 2004). To define the cell shape easily, the cells were transfected with an expression vector for EGFP. When the XL-B4 cells were cultured without T_3 for 5 and 7 days, most of the cells became elongated. On the other hand, T_3-treatment for 5 and 7 days barely induced elongation of the cells (Fig. 4C).

TH positively and negatively influences *myoD* and *myogenin* expression, respectively, in tadpole tails

To examine whether T_3 could regulate the expression of *myoD* and *myogenin* in not only the myoblastic XL-B4 cells, but also muscular tissues *in vivo*, we carried out real-time PCR for these two genes using RNA from the tails and hind limbs of the tadpoles. The amount of *myoD* and *myogenin* transcripts in the tails was increased and decreased, respectively, by the T_3-treatment (Fig. 5). In contrast, the

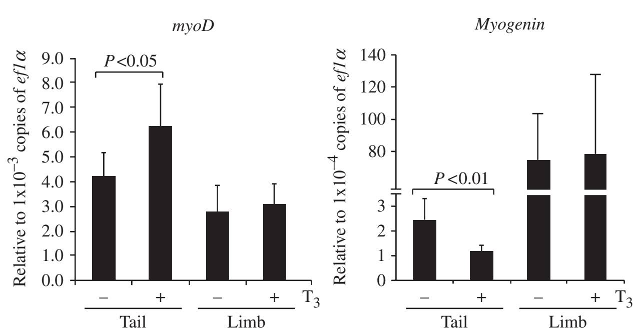

Figure 5
Effects of thyroid hormone (T_3) on the expression of *myoD* and *myogenin* in tadpole tails and hind limbs. Tadpoles (stage 57) were treated with 10 nM T_3 for 1 day. Real-time PCR was performed for *myoD* and *myogenin* mRNAs from the tails and hind limbs of the tadpoles by using gene-specific primer pairs (see 'Materials and methods' section). The data represent the mean ($n=7$) and s.d.

expression levels of both the mRNAs in the hind limbs showed no significant differences between the T_3-treated and untreated tadpoles. In addition, we should mention that *myogenin* showed more than about 30-fold higher expression in the hind limbs than in the tails, regardless of whether tadpoles was treated with T_3 or not.

Discussion

We established several cell lines from *X. laevis* tail and have characterized two of them. One is a vascular endothelial cell line XLgoo (Mawaribuchi *et al.* 2008), and the other is a myoblastic cell line XL-B4, described in this study. In the XL-B4 cells, expression of the myotube marker MHC was observed under normal culture conditions (Fig. 1). In mammals, the rat L6 myoblastic cell line expresses MHC in both growth and differentiation media (Dekelbab *et al.* 2007). In contrast, mouse C2C12 myoblasts do not express MHC in growth medium, but they do express it in differentiation medium (Artaza *et al.* 2002). Therefore XL-B4 cells have a characteristic feature of differentiating myobast cells, as L6 cells do.

Previously, Yaoita & Nakajima (1997) established a myoblast cell line, XLT-15, from *X. laevis* tadpole tails, and showed that treatment with T_3 and a temperature shift from 20 to 25 °C induced apoptotic cell death. In this study, we showed that T_3 exposure under constant-temperature conditions could induce apoptosis and attenuate late-stage differentiation of myogenesis in XL-B4 cells (Figs 3 and 4). Therefore XL-B4 cells will be useful for classifying the gene expression and intracellular signaling in apoptosis and differentiation induced by T_3 exposure under normal conditions except for eliminating other factors, such as heat shock.

It is well known that the activation of a caspase cascade often plays a major role in apoptotic signaling (Nagata 1997, Nicholson 1999, Nakajima *et al.* 2000). T_3 is likely to induce apoptosis, mediated through caspase(s) in XL-B4 cells (Supplementary Fig. 2C), as in the T_3-sensitive endothelial line XLgoo (Mawaribuchi *et al.* 2008). It will be interesting to clarify how the caspase cascades are activated by T_3 in the XL-B4 myoblasts and XLgoo endothelial cells.

Myogenic regulatory factors (MRFs) including Myf5, MyoD, and myogenin belong to a protein family of basic-helix–loop–helix transcription factors. MRFs act sequentially during myogenesis. Myf5 enhances the transcription of *myoD*, resulting in myogenic commitment. MyoD is believed to upregulate *myogenin*, leading to differentiation of muscle cells (Buckingham & Rigby 2014). In this study, T_3-treatment enhanced and attenuated the transcription of *myoD* and myogenin, respectively, in not only the myoblastic XL-B4 cells, but also tadpole tails (Figs 4 and 5). T_3 might enhance the transcription of *myoD*, mediated through its receptor and retinoid X receptor, although *myogenin* expression could be indirectly repressed by T_3. Interestingly, the effects of T_3 were not observed in tadpole limbs, which could develop to adult limbs (Fig. 5). Taken together, these findings indicate that the transcriptional change in *myoD* or *myogenin* might be caused by T_3 in not adult-type, but larval-type myogenic cells in *X. laevis*. In addition, Hirai *et al.* (2010) indicated that MyoD could enhance apoptosis of myoblasts in mice. This finding might support the idea that T_3 maintains an undifferentiated state as myoblast by attenuating *myogenin* expression, and then assists apoptotic cell death by enhancing expression of MyoD in degenerating tails during metamorphosis. Anyway, we should clarify which cells respond to T_3 with differentiation or apoptosis in myogenic development during tail degeneration in the future.

The results of our transplantation study (Fig. 2) indicated that the larval-myoblast-derived XL-B4 cells could contribute to the skeletal muscle of the tail. In future studies, XL-B4 cells stably expressing fluorescent proteins and a dominant-positive or -negative mutant against a gene of interest could be transplanted into the tail, and the effect on myogenesis at an early tadpole stage or degradation during metamorphosis could be observed. Shimizu-Nishikawa *et al.* (2002) elucidated the specific developmental fates of larval- and adult-type muscle during metamorphosis. Transplantation of the XL-B4 cells not only into the tail but also into other tissues, such as the limb, at various stages could be useful for studying tissue remodeling.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-14-0327.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

K Tamura, S Takayama, T Ishii, S Mawaribuchi, and M Ito performed the experiments; K Tamura, S Takayama, N Takamatsu, and M Ito designed the research; and K Tamura, S Takayama, and M Ito wrote the paper.

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