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Cell Interaction During Larval-To-Adult Muscle Remodeling in the Frog, *Xenopus laevis*

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1. Introduction

Amphibian metamorphosis provides an excellent model to study remodeling of the body. This phenomenon is characterized by overall remodeling of the body plan (i.e. larval body) which was once established in early embryogenesis. This metamorphic organ remodeling is induced by thyroid hormone (Gudernatsch, 1912; Kaltenbach, 1953) and a larval body is thus converted into an adult one (Ishizuya-Oka and Shi,Y.B, 2007; Miller, 1996). During this period, most of preexisted larval body organs, i.e., 'larval-specific organs' such as tail and gills degenerated (Nishikawa and Hayashi, 1995) and new 'adult-specific organs' such as fore- and hindlimbs formed (Brown et al., 2005). This cell replacement is thought to be essential for amphibian metamorphosis and deeply involved in various fundamental biological processes such as cell growth, programmed cell death, differentiation, morphogenesis and cell-cell and cell-environment interactions (Nishikawa, 1997; Shibota et.al., 2000; Shimizu-Nishikawa et al., 2002; Yamane et al., 2011). How are these remodeling-events regulated by metamorphic hormones, triiodothyronine (T_3) and thyroxine (T_4)? It will provide a great value for developmental and endocrinological research to understand the regulatory mechanism. This is because, thyroid hormone work not only for inducing amphibian metamorphosis but also for triggering metamorphosis in the fishes , such as flounder (Miwa and Inui, 1987) and conger eel (Kitajima et al., 1967), and also in sea urchin (Chino et al., 1994). The process of metamorphosis in the anuran is usually coupled with biphasic development (ancestral life-history). However, in species of direct developer such as the Puerto Rican tree frog, *Eleutherodactylus coqui*, the larval period is eliminated and the metamorphic period is juxtaposed with the embryonic period (Callery et al., 2001). In this case (direct development), interestingly, the metamorphic process is regulated by thyroid hormone (Callery and Elinson, 2000).

The reason for adopting thyroid hormone as a metamorphosis-inducing trigger by many organisms is involved in the fact that thyroid hormone is a ligand for nuclear receptors (transcription factors) and can directly cause tissue-specific gene expression changes in the same way as steroid hormone work in insect metamorphosis. Thus, in many metamorphosing organisms, their rebuilding from larval to adult body were achieved through switching of gene expression from larval to adult program by regulation of hormonal concentration and spatiotemporal expression of the receptors. For example, in developing limb buds, the expression of thyroid hormone receptor (TR)-β increases at early metamorphic period but down-regulates at metamorphic climax stage. While, in the tail, the TR-β upregulation occurs at metamorphic climax when tail shortening occurs (Yaoita and Brown, 1990).

A key feature of thyroid hormone action during metamorphosis is the multifaceted nature. The hormone promotes some tissue cells to grow and differentiate but induces the other ones to stop proliferation and degenerate (cell death). Although it is obvious that each program (growth or death) is triggered by thyroid hormone action, it still unknown how it causes the different reactions, death or live, only to a particular (larval or adult-type) cells among many TR-β-expressing cells. This is one of the most important issues in developmental biology field. In other word, it is important to understand not only the thyroid hormone actions but also the mechanisms by which some cells are programmed to commit to a specific cell fate (larval or adult type cells). It would be important to focus on cell-to-cell interactions or cell-to-cellular environments for the analysis of regulatory mechanism of cell fates. The environmental factors include nutrients, growth factors, hormones, cytokines, morphogens and extracellular matrices. There are basically two types of the target cells for such factors, i.e. adult and larval type cells. The adult type cells (or adult precursor cells) start their adult gene program by the actions from such factors while the larval type cells respond to the same factors to stop larval program and activate cell death program. In order to clarify this completely different mode of hormonal actions in organ remodeling during metamorphosis, it would be necessary to fully analyze not only hormonal response of target cells but also the interaction between target cells and surrounding cells.

Muscle remodeling also occurs during anuran metamorphosis (Nishikawa and Hayashi, 1994). It is of great interest to study muscle remodeling from larval to adult type during anuran metamorphosis from the aspect of molecular and cellular interactions. This is because, during anuran metamorphosis there are three different muscle changes, 1) degeneration of larval muscle in the tail (Kerr et al., 1974), 2) formation of new muscle (secondary myogenesis) in developing limbs, and 3) conversion of muscles from larval-type to adult-type in the trunk region (Ryke, 1953), which provide a useful system for analyzing programmed muscle cell death and initiation of adult program of myogenesis. In addition to this, it is also important from the viewpoint of evolutionary adaptation of myogenesis in the transition from fish to tetrapod trunk (Glimaldi et al., 2004). Thus, this chapter concentrates on *Xenopus laevis* muscle remodeling during metamorphosis, and in particular, the following 5 points are described in detailed. 1) Characterization of muscle contractile proteins in larval- and adult-type muscles and temporal and special progress of cell replacement by

adult muscle formation and larval muscle death (Nishikawa and Hayashi, 1994, 1995; Kawakami et al., 2009). 2) Characterization of programmed muscle cell death in the trunk and the tail of *Xenopus laevis* tadpoles (Nishikawa and Hayashi, 1995; Nishikawa et al., 1998). 3) Characterization of the differences between larval- and adult-type myoblasts and their responses to hormonal signals in vitro (Shibota et al., 2000). 4) Regulatory mechanism for muscle cell fates, death or adult differentiation, by interaction between two types of myoblasts (larval and adult types) (Shimizu-Nishikawa et al., 2002). 5) Regulatory mechanism of adult type myogenesis by interaction between myoblasts and notochord (notochord-suppression) and spinal cord cells (spinal cord-promotion) (Yamane et al., 2011). The rationale for studying a cell-to-cell interaction during larval-to-adult muscle conversion in the amphibian is that such an analysis will provide important insights into the processes occurring during the differentiation (and/or adult organogenesis) of other stem cells such as mammalian embryonic and tissue (adult) stem cells.

2. Muscle remodeling during metamorphosis of *Xenopus laevis*

In evolutionary history, amphibians are in the process of evolving from aquatic fishes into terrestrial vertebrates. There are many changes in body organs between aquatic and terrestrial species. The most obvious example of such change would be seen in the epidermal changes in the skin. The stratified and keratinized (or cornified) epidermis in whole body skin is one of the phenotypes macroevolutionaly-acquired in amphibians but not fishes and became a well-established characteristic feature of tetrapoda, i.e. terrestrial vertebrates. Since amphibians have an aquatic larval period, the skin of the larva is a fish type of non-cornified epidermis. During metamorphosis, differentiation of a terrestrial type epidermis (i.e. stratified and cornified epidermis) occurs and complete transformation of whole body skin with terrestrial-type cornified (keratinized) epidermis is achieved after metamorphosis. Thus, in the amphibian, it is a unique feature that macroevolutional (phylogenic) changes from fish to terrestrial type are replicated during metamorphic (ontogenic) changes. Other than skin, is there any organs that are converted from larval (or aquatic) to adult (or terrestrial) type during metamorphosis? Just as the skins had evolved so as to protect whole body from drying in a terrestrial environment, it would be needed for the muscles to evolve from aquatic to terrestrial type providing with increased muscle strength so as to overcome the intense gravitational force in the terrestrial environment.

From this point of view, Nishikawa and Hayashi (1994) analyzed electrophoretically the difference in profiles of muscle contractile protein between larval and adult dorsal muscles in the frog *Xenopus laevis* (Fig.1) and found that there are significant differences in isoform expression of tropomyosins (TM) and myosin heavy chains (MyHC). The isoforms of MyHC switched from larval (higher mobility on SDS-electrophoresis) to adult type (lower mobility) and adult-specific β-TM appeared during metamorphosis in addition to preexisting α-TM, resulting in the 1:1 ratio of α:β. These changes mean that adult-specific muscles are newly formed during metamorphosis. These isoforms-transitions starts quickly from stage 54 (early metamorphosis) in the hindlimb and slowly from stage 57 (mid metamorphosis) in the body (dorsal muscles). On the other hand, the regression of adult phenotypes occurs in

the tail during metamorphosis. Further immunohistochemical examination (Fig.3 A and Fig.2 B) revealed that (1)before metamorphosis, only a small number of muscle fibers at dorsomedial (DM) part of dorsal muscle expressed "adult type" muscle isoforms; and (2)during metamorphosis, the area expressing adult type isoform gradually expanded from anterior to posterior direction (axial gradient) with increase in adult type muscle fibers. Thus, we can clearly understand at a cellular level the three distinct muscular changes during *Xenopus* metamorphosis: (1) rapid adult muscle differentiation (limbs); (2) gradual muscle replacement from larval to adult type (dorsal muscles); and (3) disappearance of preexisting larval-specific muscles (tail).
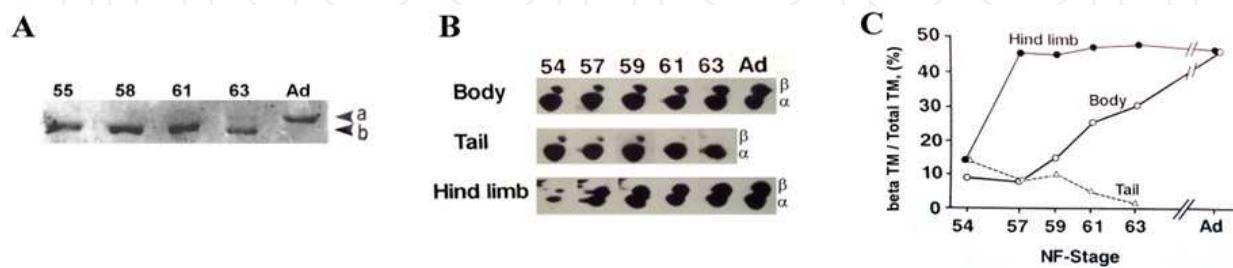

A: Developmental changes in MyHC (myosin heavy chain) isoform expression. The trunk dorsal muscles from tadpoles at various stages (55, 58, 61 and 63) and an adult frog (Ad) were applied to SDS-polyacrylamide gel electrophoresis (PAGE). The arrowheads (a and b) represent the positions of adult- and larval-type MyHC isoforms, respectively. B: Developmental changes of α- and β-tropomyosin (TM) expression. Muscle each from body, tail, and hindlimb were dissected from stages 54-63 tadpoles and a young frog (Ad) and applied to IEF (isoelectric focusing)/SDS-PAGE. The positions of α-TM and β-TM isoforms are indicated by α and β, respectively. C: Changes in ratios of TM isoforms during metamorphosis. The ratio (%) of β-TM to total (α and β) TM content was calculated from densitometric analysis of B and is shown on the ordinate. The abscissa shows developmental stages. The figures were modified from original paper (Nishikawa and Hayashi, 1994).

Figure 1. Expression patterns of adult type muscle contractile proteins during *Xenopus laevis* metamorphosis.

There are two possibilities for the dorsal muscle isoform conversion during metamorphosis (Fig.2A). In the hypothesis 1 (H1), the isoform transition occurs by cell replacement with anterior-posterior proliferation of adult-type myoblasts and death of preexisting larval–type fibers. On the other hands, in hypothesis 2 (H2), the switch in gene expression from larval to adult program occurs within the same cells without larval cell death. If the former (H1) is the case, myoblasts proliferation should occur with anterior-posterior gradient just before the isoform transition in dorsal muscles. Examination of this point by the assay of DNA synthetic activity (Nishikawa and Hayashi, 1994) and PCNA (proliferating cell nuclear antigen) expression (Kawakami et al., 2009) revealed that the cell proliferation activity is higher in the anterior than the posterior dorsal muscles during early metamorphosis just prior to the isoform changes.

These proliferation activities are well-matched the observations that small portions expressing β-TM (adult muscle area) appeared first at dorsomedial (DM) parts of dorsal muscles and the area gradually expanded to overall dorsal muscles (Fig.3 A, B). The DM parts correspond to the "cord" of the tadpole axial muscles which is reported by Elinson et al. (1999). The "cords" in tail portion also express adult muscle isoforms but this (β-TM$^+$) regions never increased during metamorphosis. On the other hands, in β-TM (-) regions, i.e.
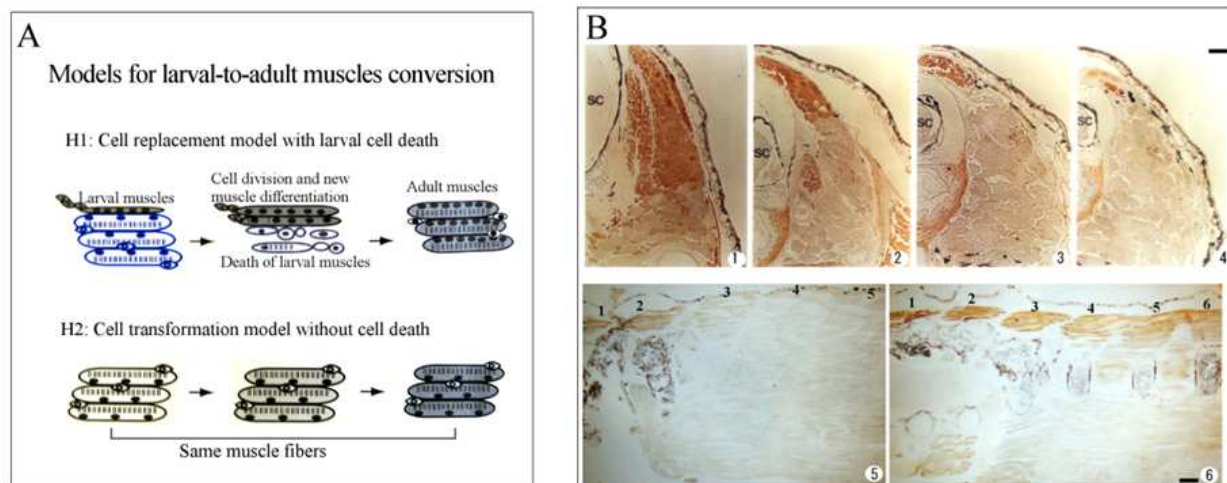
A: Models for larval-to-adult muscle conversion. In hypothesis 1 (H1), adult-type myoblast proliferation newly occur within a small portion of the muscles during early metamorphosis with a consequent increase in the number of adult muscle fibers, and also the death of preexisting larval muscle fibers occur during metamorphic climax. In hypothesis 2 (H2), all muscle cells change their gene expression from the larval program to the adult one without any cell death. B: Photomicrographs of the immunostained sections with β-TM-specific antibody. 1-4: Cross sections (from anterior to posterior) of dorsal muscles of stage 63 (climax stage) tadpole. Bar=100 μm. SC: spinal cord. 5-6: Sagittal sections of dorsal muscles at prometamorphosis; stage 59 and 61, respectively. Bar=200 μm. The number shows the arrangement of muscle segment from anterior to posterior in this order. The figures were modified from original paper (Nishikawa and Hayashi, 1994).

Figure 2. Muscle conversion models (A) and the β-TM patterns in dorsal muscle during *X. laevis* metamorphosis (B).
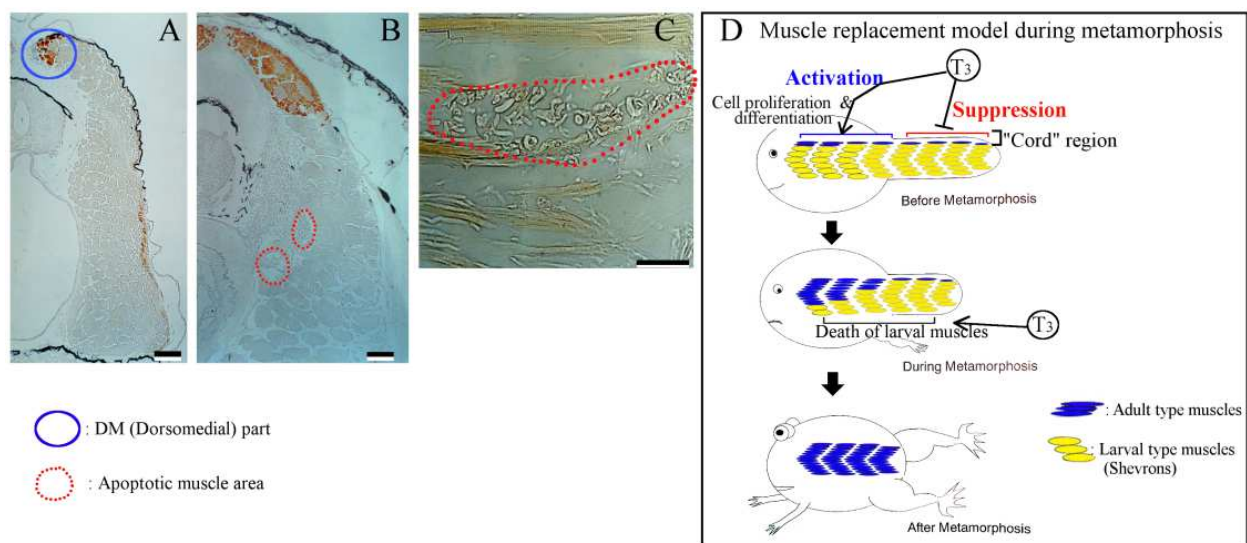
A and B: Cross sections of dorsal muscles from pre-metamorphic (A; stage 53) and metamorphic climax (B; stage 63) tadpoles. The Bars=100 μm. The brown colored areas were β-TM-positive adult muscle areas. C: Frontal section of tail muscle from tadpoles at stage 63. The apoptotic muscle fragments (sarcolytes) were β-TM-negative. The bar=50 μm. D: A schematic model of the mechanism for the muscle replacement during *Xenopus laevis* metamorphosis. Figures were reformed from the original papers (Nishikawa and Hayashi, 1994, 1995).

Figure 3. Immunohistochemical analysis of metamorphosing *X. laevis* tadpoles with β-TM (A-C) and a possible model for muscle replacement during metamorphosis (D).

larval muscle areas, many apoptotic dying muscle cells were observed (Fig.3 B, C). From these results, it has been found that the larval to adult muscle remodeling is achieved by cell replacement (H1: "cell replacement model") with new proliferation of adult myoblasts and death of preexisting larval cells, not by changing gene expression program from larval to adult one within the same cells (H2) (Nishikawa and Hayashi, 1994).

In summary, we can understand the muscle remodeling in *Xenopus laevis* as "evolutional adaptation" from aquatic to terrestrial life. And the evolutional processes of terrestrial muscles seem to be replicated by the ontogenic development of anuran amphibians. In anuran amphibians, one of the most unique features in muscle development is the presence of "chevrons", larval-type muscles in axial muscles, with a fate of apoptosis triggered by T$_3$ during metamorphosis. The tadpole axial muscles are attached on both side of notochord, showing "chevron" structures in side view (Das et al., 2002). At the most dorsal and ventral parts in each chevron, there are "cord" structures that consist of two dorsal and two ventral parallel rows of slow muscle bundles connected by collagen fibers that run the length of the tail (or trunk–to-tail)(Elinson et al., 1999). These parts ("cord" muscles) persist until the very end of tail resorption and are known not to be a direct target for T$_3$-action of cell death-induction (Das et al., 2002). While the "cord" consists of slow muscle fibers, the "chevron" is composed of fast muscles. Therefore, these two muscles (chevron and cord) can be distinguished by the fast (or slow) fiber-specific antibodies such as F59 for the fast isoforms of myosin heavy chains and S46 (or S58) for the slow isoforms. The "chevron" muscles, i.e. the larval-type muscles, exist not only in the tail but also in the dorsal muscles of the tadpole trunk region and all these muscles are to die in response to T$_3$-upregulation. Interestingly, "cords" muscles, which can be considered as "adult-type" muscles by judging from their T$_3$-response, also exist both in trunk and tail regions. The adult muscle differentiation in the dorsal trunk "cords"(i.e. trunk DM parts) is to be promoted by T$_3$ while the tail "cord" region, which is thought to contribute to the tension for tail resorption, is to be destroyed finally during metamorphosis (Elinson et al., 1999; Das et al., 2002). Thus, the difference of developmental fates between tail and trunk muscles are caused by differential regulation of DM portions, activation or suppression (Fig.3 D: cell replacement model on adult muscle differentiation). Therefore, it would be important to analyze the interaction between myogenic stem cells (i. e. larval and adult myoblasts) and non-myogenic cells (or extracellular matrices) in order to understand the mechanism of the activation (or suppression) of trunk (or tail) "cord" muscles.

3. Mechanism of programmed muscle cell death and macrophage phagocytosis

Death of regenerating tails in anuran amphibians has attracted interests of many researchers and Kerr et al. are not exceptional, who define the specific term "apoptosis" for the processes of non-accidental and active cell death (Kerr et al., 1974). They observed the tail cell death in *Xenopus laevis* by electron microscopy 38 years ago and proved morphologically that the tail cell death during anuran metamorphosis is achieved by the apoptotic processes including modified formation of apoptotic bodies (i.e. sarcolytes). The sarcolytes are formed by the internal fragmentation of muscle fibers not by the usual surface budding (Fig.4 A).

Nishikawa and Hayashi (1995) examined whether the death in the trunk dorsal muscles occurs with the same processes as seen in the tail cell death and found the processes are the same between the two. The apoptotic dying muscles first appeared near the base of the tail in early climax stage of metamorphosis (stage 59) when T_3 level is quite high, and thereafter expanded in an anterior direction in dorsal body and posteriorly in the tail. The direction of area-expansion of larval cell death was thus opposite to that of adult muscle differentiation. This relationship between cell death and differentiation activities is very interesting in that cell death occurs at first in the place where the adult differentiation occurs at last. As to the signals which regulate the axial gradient in adult muscle differentiation, the involvement of the factors from the nerve (spinal cord) and the notochord cells is suggested as described below (section 6).
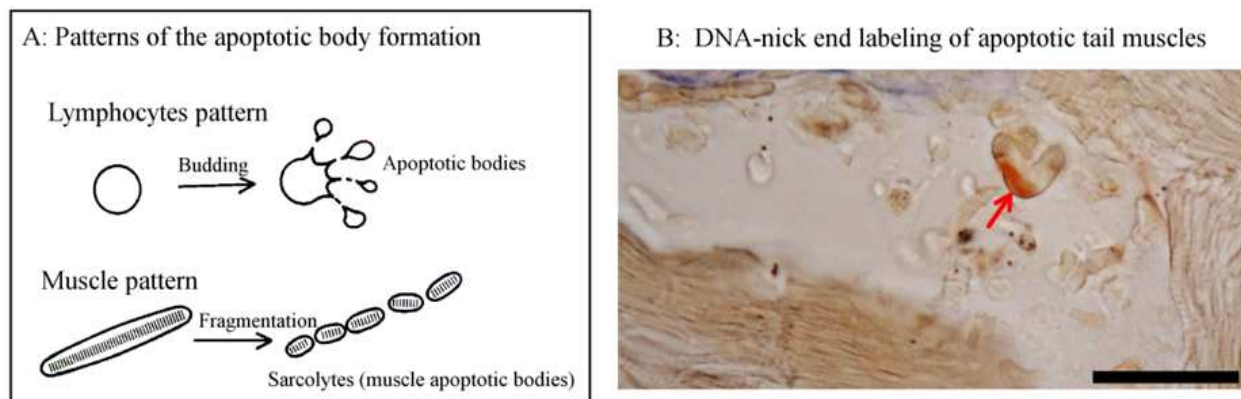
A: In case of non-muscle cells such as lymphocytes, the apoptotic bodies are formed by cytoplasmic buddings. On the other hands, in case of muscle cells, the apoptotic bodies (sarcolytes) are formed by fragmentation of muscle fibers. B: Frontal section of tail muscle from tadpoles at stage 63 was analyzed by TUNEL method (DNA-nick end labeling). There is an apoptotic muscle fragment with a TUNEL+ nucleus (a red arrow). The bar shows 50 μm. The figure was reformed from the original paper (Nishikawa and Hayashi, 1995).

Figure 4. Patterns of two different apoptotic bodies (A) and a TUNEL (DNA-nick end labeling) detection of apoptotic tail muscle cells during *Xenopus laevis* metamorphosis (B).

Oligonucleosomal DNA fragmentation is thought to be a good biochemical evidence of the apoptotic cells (Kaufmann et al., 2000). Nishikawa and Hayashi (1995) detected electrophoretically the nucleosomal DNA fragmentation in *Xenopus* tail and trunk dorsal muscles at stage 63 when the muscle degradation occurs most frequently. The apoptotic enzymes which direct the DNA fragmentation is reported as the "endonucleases" such as DNase I (Rauch et al., 1997), DNase X (Los et al., 2000), DNase II (Krieser and Eastman, 1998), CAD/DFF40 (Enari et al., 1998; Liu et al., 1997), DNase γ (Shiokawa et al., 1994; Shiokawa and Tanuma, 2001) and endonuclease G (Parrish et al., 2001). Among these endonucleases, DNase γ was originally found by Shiokawa et al. (1994) as the enzyme which is activated during apoptosis of rat thymocytes and the cDNA encoding human DNase γ was cloned (Shiokawa et al., 1998). The homolog of this gene was also cloned from *Xenopus laevis* (*xDNase γ*) and the expression was found to be upregulated in the tail during metamorphosis (Shiokawa et al., 2006). In studies with mouse C2C12 cells, it was found that DNase γ is the endonuclease responsible for DNA fragmentation in apoptosis associated with myogenic differentiation (Shiokawa et al., 2002). Furthermore, DNase γ was found to

be expressed in rat pheochromocytoma PC12 cells during naturally occurring apoptosis which associated with neuronal differentiation (Shiokawa & Tanuma, 2004). These finding suggest that DNase γ has an important function for the selective deletion of unnecessary cells that could not successfully differentiate. If we apply this idea to the metamorphic apoptosis in *X. laevis*, DNase γ might contribute to the selective deletion of unnecessary larval type cells during metamorphosis. Although the upregulation of DNase γ mRNA level occurs in metamorphosing tadpole tail of *X. laevis*, it is not determined which tissues in the tail are responsible for the upregulation of DNase γ gene. As to this point, the TUNEL analysis (Fig.4 B) suggested a possibility that apoptotic dying muscles express DNase γ. On the other hand, the possibility cannot be excluded that DNase γ is expressed in macrophages (and/or neutrophils) which phagocytose and digest the apoptotic muscle fragments. Because, it was shown that DNase γ–like activity dramatically increased in a leukocyte fraction of the liver (a major hematopoietic organ) during metamorphic climax stage in *X. laevis* (Nishikawa et al., 1997).

These are four cellular components, i.e. two types of myogenic stem cells (larval and adult myoblasts) and two types of muscle fibers (adult and larval muscles), and these are responsible primarily for the muscle conversion during *Xenopus* metamorphosis. Among these, the cells responsible to the oligonucleasomal DNA laddering are the larval muscle fibers because they shows some apoptotic features, i.e., sarcolytes formation and DNA breakage (TUNEL+ reactions)(Fig.4 B). Such muscle cell death with apoptotic DNA laddering was found to be induced dose-dependently by thyroid hormone (T_3) in tadpole tail organ cultures (Nishikawa and Hayashi, 1995). Not only death of matured larval muscles but also that of undifferentiated larval stem cells (myoblasts) is needed for the coordinated muscle conversion during metamorphosis. From this point of view, Shibota et al. (2000) examined whether the programmed cell death occurred at stem cell (i. e. myoblast) level with cell culture technique and found that larval myoblast-specific cell death was induced by thyroid hormone in a dose-dependent manner (Shibota et al., 2000: see Section 4).

Another feature of the apoptotic processes is a phagocytosis by macrophages of the dying apoptotic bodies. In regenerating tail of *X. laevis*, phagocytosis of sarcolytes (muscle apoptotic fragments) was frequently observed (Kerr et al., 1974). So, do macrophages operate for the deletion of dying muscles during metamorphosis in the trunk region as well as in the tail? From this point of view, Nishikawa et al. (1998) analyzed the macrophage distribution in tadpole axial muscles (both in trunk and tail regions) using with macrophage-specific monoclonal antibodies (Ham56). The results showed that Ham56+ cells (macrophages) appeared not only in the tail but also in the trunk dorsal muscles of metamorphosing tadpoles. Electron microscopic observation revealed that the macrophages ingested the fragment of dying trunk muscle fibers in large quantities. Interestingly, macrophage number markedly increased at late metamorphic climax stage when muscle cell death most frequently occurred and decreased at the completion of metamorphosis. In other words, the distribution and change in the number of macrophages were the same as those of muscle apoptotic bodies (sarcolytes) during metamorphosis. Analysis with Western blotting suggested that Ham56 recognizes *Xenopus laevis* homologues of mouse attachmin (Tomita and Ishikawa, 1992) which is non-specific adhesion proteins in macrophages. The expression of Ham56 antigens was

found to increase with macrophage phagocytosis at the late climax stage, thus, Ham56 antigens would be essential for macrophage-dying cell interaction. Furthermore, cell culture studies with isolated *Xenopus* tail cells suggested that macrophage differentiation and its phagocytic activity were regulated by thyroid hormone (Ochi and Nishikawa, unpublished data). It still however remains unsolved how macrophages recognize the muscle cells that have a death fate (i.e. larval type muscles) or whether some signals from macrophages are needed for the induction of the first step of muscle apoptosis. As for macrophage actions for the muscle cell systems, Sonnet et al. (2006) reported that human macrophages rescue myoblasts and myotubes from apoptosis through a set of adhesion molecular systems. In this case, the signal from macrophages is a survival signal but not a death-signal. Thus, the spectrum of macrophage actions seems fairly-broad and these actions should be totally clarified in the future.

On the other hands, researchers in French (Demeneix' group) have been greatly contributed to the finding of cell death regulators for the muscle cell death during metamorphosis. The method was developed by de Luze et al. (1993) to introduce genes directly into tail muscles and using this method Sachs et al. (1997) found that somatic gene transfer with a mouse *bax* into *Xenopus* tadpole tail muscles induced apoptosis and T_3 treatment significantly increased *Bax* transcription. After that Sachs et al. (2004) further cloned a cDNA encoding *Xenopus laevis bax* (*xlbax*) and found that the gene expression increased during metamorphosis and was experimentally up-regulated by T_3-treatment. Also, overexpression and antisense experiments showed that *xlbax* is a regulator of muscle fiber death in the tail during *Xenopus* metamorphosis. In addition to the importance of Bax-regulation, Rowe (2005) clarified that the activation of caspase 9, one of the components in mitochondria-dependent cell death pathway, was pivotally involved in tail muscle apoptosis during *Xenopus* metamorphosis by using overexpression system of dominant-negative *caspase 9*. Furthermore, Du Pasquier et al. (2006) showed that developmental cell death during *Xenopus* metamorphosis involves Bid cleavage and Caspase 2 and 8 activations.

We can see from the above that cell-death signaling in the tail muscle of metamorphosing *Xenopus* tadpoles is nearly identical to that in mammalian mitochondria-dependent cell death system. However, cell death in *Xenopus* tail has distinguishing features in that it is under the control of thyroid hormone. Therefore, it would be an important subject to examine whether thyroid hormone regulates metamorphic muscle cell death not only at a muscle fiber level but also at a stem cell (myoblast) level. For such an analysis, it would be very helpful to use a primary cell culture system of isolated myoblasts. In the next section, studies with primary culture of *X. laevis* larval and adult myoblasts are described in detail (Section 4).

4. In vitro characters of two types of myogenic stem cells from *Xenopus laevis*: Differential hormonal responses in cell division, cell differentiation and programmed cell death

Are the deaths of larval muscle fibers and myoblasts induced by the thyroid hormone during anuran amphibian metamorphosis? How are the cell division and differentiation for the adult muscle stem cells (i. e. adult myoblasts) regulated by thyroid hormone? To answer these

questions, Shibota et al. (2000) established primary cell culture methods for adult- and larval-type myoblasts in the frog, *Xenopus laevis*, and examined the hormonal response in each case. In this study, frog leg and tadpole tail muscles were used for the source of adult and larval type myoblasts, each of which respectively has a life-or-death fate during metamorphosis. Generally, the trunk dorsal muscles (i.e. the axial muscles) of premetamorphic tadpoles should be used for the adult myoblast source in order to adjust temporal and spatial situations between the larval and adult type cells. However, the selective isolation of adult cells from tadpole dorsal muscles was found to be quite difficult. Because of this, the frog legs were employed as the source of adult type cells. The selective isolation method of adult stem cells from tadpole dorsal muscles was developed in a subsequent study (Shimizu-Nishikawa et al., 2002, see section 5).

It was found that there were several significant differences (1-7) in the nature of isolated cells between larval and adult-type myogenic stem cells as described below. (1) The cell size just after isolation in the larval-type (15 µm) was larger than that in the adult-type (5 µm). The size of spreading cells 1 day after inoculation was also larger in the larval type cells (30-50 µm) than in the adult type cells (5-10 µm) (Fig.5A, e and a). (2) Both types of cells could adhere to the plastic culture dish with different adhesion ratios (larval type=30-50%; adult type=50-60%). Most of attached cells (88% larval and 81% adult cells) were desmin-positive, showing the isolated cells to be myoblast-rich populations. (3) The timings of start of myotube-differentiation were quite different between larval and adult types: Myotube-formation by myoblasts fusion started on day 2 or 3 in larval but on day 4 in adult cell cultures (Fig.5A, f and c). (4) There was large difference in growth activity between larval and adult cells: The larval myoblasts increased only 2.5-fold over 6 days of culture but the adult ones 5.5-fold (Fig.5 B). (5) The cultured larval myoblasts responded to the metamorphic hormone, T_3, with decrease in the DNA synthetic activity (50%) (Fig.5 F). As the result, T_3 decreased the cell numbers (sum of myotubes and myoblasts) in larval cell cultures to 56% of those of control cultures over 6 days (Fig.5 E). On the other hands, T_3 did not have much influence on the total cell numbers in adult cultures (Fig.5 D). (6) T_3 promoted dose-dependently the differentiation of adult myoblasts into myotubes but diminished that of larval cells by half (Fig.6 A and B). (7) Death of differentiated myotubes was promoted by T_3 specifically in larval but not in adult cultures (Fig.5 C and Fig.6 C). In addition to the myotubes death, double staining with TUNEL and anti-desmin (a myoblast marker) antibody showed that death of myoblasts (desmin+ cells) was induced by T_3 specifically in larval but not adult cells (Fig.6 D). From the differences in cell sizes, the start-timing of differentiation and cell growth activity between larval and adult myogenic stem cells (1, 3 and 4), it is conceivable that adult myoblast have a more stemness phenotype than the larval myoblasts. This is to say, adult myoblasts cannot enter the myotube-forming stage without dividing many times, but in contrast, the larval myoblasts can immediately go into myotube-differentiation. From this point of view, it would be an important issue to examine differences in gene expression of early myogenic transcription factors, such as *pax3*, *myf5* and *myod*, between larval- and adult-type myoblasts for further characterization of them.

Another essential difference (5-7) between larval and adult cells was found to be the difference in T_3 responses (Table 1). It was thus evident that the conversion of a larval to adult myogenic system during metamorphosis becomes possible through totally specific

control of cell division, cell differentiation and programmed cell death at a precursor cell level by T_3. In studies using with a myoblast cell line (Yaoita and Nakajima, 1997) cloned from *Xenopus laevis* tadpole tail muscles, it was found that myoblast cell death was induced by T_3 with increasing level of *caspase 3* expression. Considering this and Rowe's results (Rowe et al., 2005), there must be up-regulation of caspases 3 and 9 in the primary cultures of the larval myoblasts isolated from *Xenopus* tadpole tail.
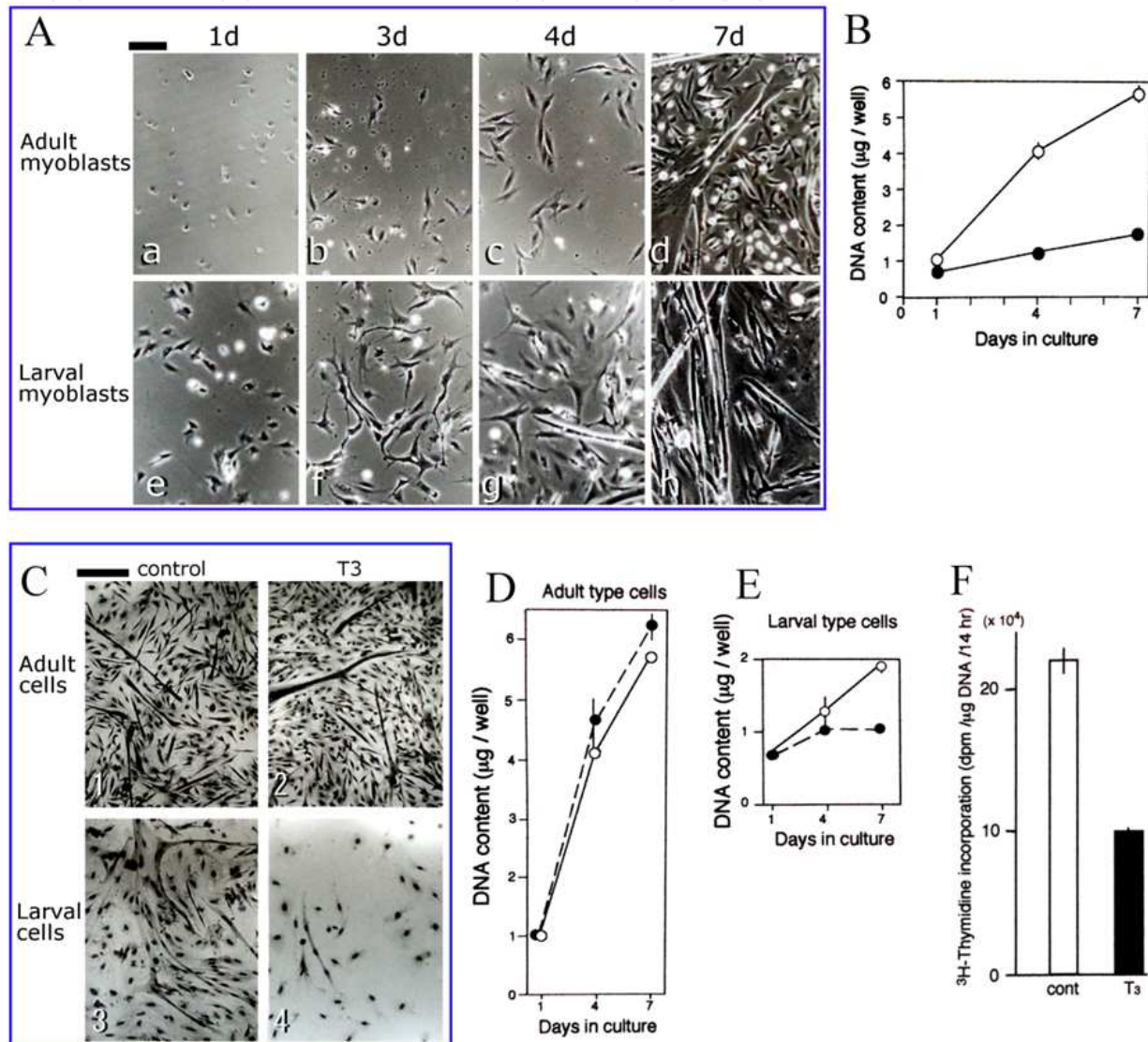
A: Phase contrast photomicrographs (Microscope: IX70, Olympus) of cultured adult (a-d) and larval myoblasts (e-h) at each culture days (1d–7d). The bar=100 μm. B: Growth curves of adult (○) and larval myoblasts (•). The cell growth activity was determined by the DNA content of total cells/culture-well. C: Photomicrographs of cultures of adult (1-2) and larval myoblasts (3-4) with (2 and 4) or without T_3 (10^{-8} M) (1 and 3). T_3 was added to the cultures at the first day of culture. Cells were fixed with 70% ethanol and stained with hematoxylin and eosin at 7th day. The bar=100 μm. D and E: The effect of T_3 on the DNA content in adult (D) and larval (E) myoblast cultures. ○: without T_3; •: with T_3 (10^{-8} M). F: The effect of T_3 on the DNA synthetic activity of larval myoblast cultures. The DNA synthetic activity was determined by measuring the ³H-thymidine incorporation into cultured cells. □: without T_3; ■: with T_3 (10^{-8}M). The figures (A, B, D-F) were reformed from the original paper (Shibota et al., 2000).

Figure 5. Primary culture of adult and larval myoblasts (A and B) and the effect of thyroid hormone (T_3) on cell growth (C-F).

As noted above, there were shown to be essential differences between larval and adult type myoblasts each isolated from tadpole tail and leg muscles. Also in the trunk dorsal muscles during metamorphosis, there should be two different (larval and adult) myogenic stem cells with a life-or-death fate. Do these cells really exist within trunk dorsal muscles? If this is the case, is there a possibility that they form heterokaryon myotubes ("chimeric fate" myotubes) with different two types of cells other than myotubes consisting only of larval or adult cells? In such chimeric myotubes, which fates, a death-fate or a life-fate, possibly be selected in response to T_3? In the next section, the study about cell interaction mechanism which regulates the myotubes fate (life-or-death) in the dorsal muscle during metamorphosis of *X. laevis* was described in detail (Shimizu-Nishikawa et al., 2002).
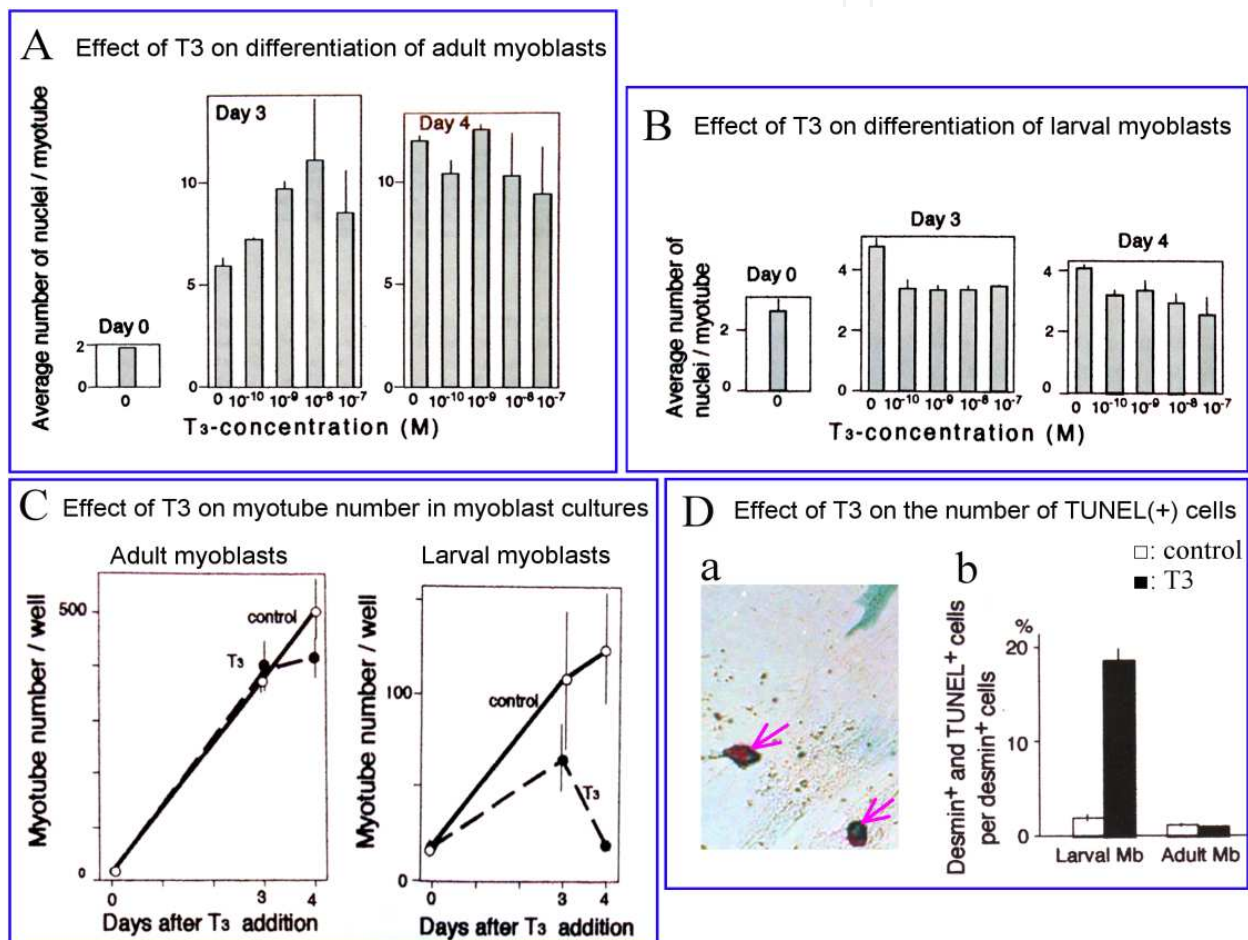
A and B: Effect of T_3 on differentiation of adult (A) and larval (B) myoblasts. Adult and larval myoblasts were cultured for 8 or 7 days, respectively. Various concentration of T_3 (10^{-10} - 10^{-7} M) were added to the cultures at 4th (A) or 3rd (B) day. Myonuclei number/myotube was determined at 0, 3 or 4th day after T_3 addition by measuring the numbers of myotubes and myonuclei within each well. The average number of myonuclei/myotube was indicated in the ordinate. C: Effect of T_3 on myotube number in adult and larval myoblast cultures. Each myoblasts were cultured for 8 or 7 days. T_3 (10^{-8} M) was added to the culture at 4th (adult cells) or 3rd (larval cells) day. The myotube number was determined at 0, 3 or 4th day after T_3 addition. ○: without T_3; ●: with T_3. D: Effect of T_3 on the number of TUNEL+ cells in myoblast cultures. a: Photomicrograph of TUNEL+ (red) and anti-desmin+ (blue) cells (arrows) in a larval cell culture. b: The larval and adult myoblasts were cultured for 7 days, fixed with formaldehyde and stained doubly with anti-desmin antibody and the TUNEL. Doubly stained cells within each well were counted and indicated in the ordinate. □: without T_3 (control); ■: with T_3 (10^{-8} M). The figures were reformed from the original paper (Shibota et al., 2000).

Figure 6. Effect of T_3 on differentiation and death of *Xenopus* adult and larval myogenic cells in vitro.

	Myogenic system	
	Larval-type	Adult-type
Replication of myoblasts	↓	n.e.
Myoblast differentiation	↓	↑
Myoblast death	↑	n.e.
Myotube death	↑	n.e.

↓: suppression; ↑: induction or promotion; n.e.: no effect. In the presence of T_3, larval type myogenic system of *Xenopus laevis* totally ceases and converts into adult type myogenic system during metamorphosis. The table was reformed from the original paper (Shibota et al., 2000).

Table 1. Comparison of T_3 responsiveness between larval and adult myogenic systems.

5. Interaction between larval- and adult-type myogenic precursor cells during metamorphosis: Regulation of cell death fate and adult muscle differentiation

5.1. Differential distribution of larval and adult myoblasts

It was expected that there should be two types of myoblasts (larval- and adult-type muscle stem cells) in trunk dorsal muscles because both larval and adult-type muscles coexist within the same regions. As a first indirect approach for proving the real existence of different two types of myogenic precursor cells in the same trunk muscle, Shimizu-Nishikawa et al. (2002) compared the enhancement of cell death activity in response to T_3 among three parts of the muscles, i.e., tadpole trunk, tail and limb muscles, each of which has a different muscle fate (Fig.7 A).

The results showed that the TUNEL$^+$ dying myoblasts were induced by T_3 strongly in the tail cells (10-fold induction; from 1.3% to 13%) and moderately in the trunk cells (2-fold; 2.5% to 5%), but not in the limb cells (1% to 1%). The value of cell death induction in trunk cells was between those of tail and limb cells, suggesting the possibility that two types of myogenic stem cells (i.e. T_3-inducible and non-inducible cells) are mixed in the trunk muscles. As the second approach, for further direct evidence, isolation of two types (larval and adult) of myoblasts from the trunk dorsal muscles was tried on the basis of their physical natures. The cells dissociated with enzyme-digestion from tadpole trunk dorsal muscles were pre-cultured for three days in high-serum (growth promoting) medium, harvested with trypsin-digestion and used for two-steps cell isolation with a percoll-density gradient centrifugation (a buoyant density-sensitive method) and an albumin-unit gravity sedimentation (a size-sensitive method)(Fig.7 B). The results showed that two different cell types, large (Lg) and small (Sm) cells, were isolated from dorsal muscle at 1:1 (Sm : Lg) ratio. For comparison, the result from the fractionation of the tail muscle cells with the same procedures showed that the two types of cells (Sm and Lg) were also obtained from the tail muscle but their ratio was 5 : 1 (Fig.7 C). Cultivation of these cells (Sm and Lg) revealed that Lg-cells could grow rapidly and T_3 decreased the number of Sm-cells but not that of Lg-cells (Fig.7 D). These results suggest that Lg-cells are the adult myoblasts and the Sm-cells are
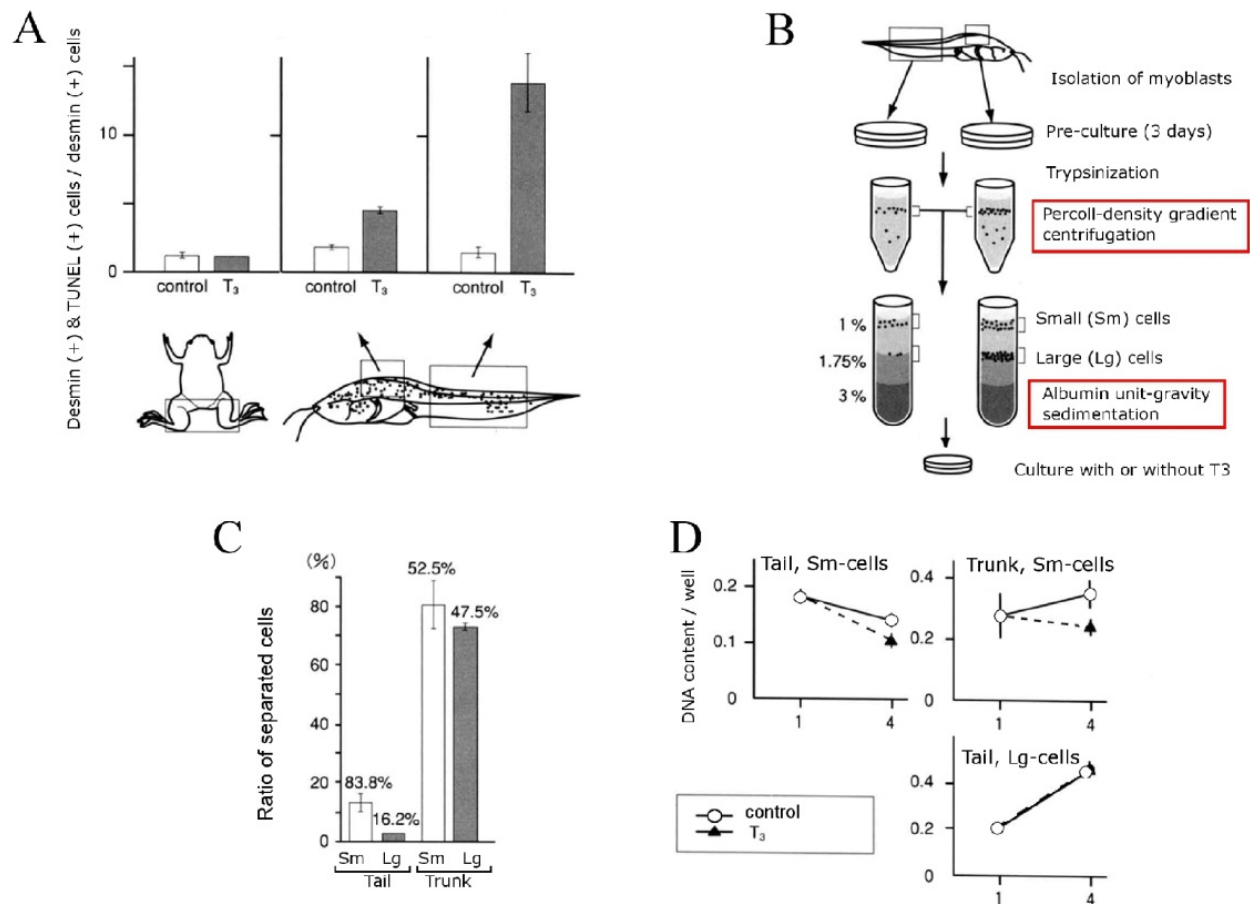
A: Comparison of T₃-mediated cell death enhancement among different parts of muscles by examination with TUNEL reaction. Myoblasts isolated each from adult frog hind limb muscles, tadpole trunk dorsal or tail muscles were cultured for 1 day and then incubated for 7 days with (shaded column) or without (open column; control) T_3. T_3 induced the death of larval myoblasts in both the tail and trunk but had no effect on adult myoblasts. B: The experimental procedure for separation of myoblasts. C: The cell number after the final separation was indicated in the y-axis. The ratio (%) of each cell fraction to total trunk or tail cells was presented at the top of each error bar. D: Effect of T_3 on the separated cells. Cells were cultured with or without T_3. T_3 was added at the first day of culture. The relative cell number was estimated by DNA content/well at the indicated days. Sm: Small-size cells. Lg: Large-size cells. ○: control. ▲: T_3 (10^{-8} M). The figures were reformed from the original papers (Shimizu-Nishikawa et al., 2002).

**Figure 7.** Cell type-dependent patterns of T_3-induced myoblast death.

larval myoblasts. Thus, it was shown that the trunk dorsal muscles contain almost the same number of two different (larval and adult) myoblasts. Interestingly, there were Lg-cells (adult type myoblasts) even in the tail muscle with a small ratio (1/5 of that in the dorsal muscle). In other words, there are many adult type muscle stem (AMS) cells with high growth activity in the dorsomedial (DM) portions of the trunk dorsal muscle but, on the other hands, a small number of AMS cells in the tail DM portions. These results suggest that the life-or-death fates of trunk and tail muscles are determined primarily by the differential distribution of adult myoblasts within the muscles. On the other hand, from the fact that tail muscle does not convert to adult type even though they contain AMS cells, it is conceivable that during metamorphosis the growth and differentiation of AMS cells is specifically activated in the trunk DM portions but suppressed in the tail DM portions. As to this spatial control, Yamane et al. (2011) suggested the possibility that the trunk-specific adult

myogenesis is regulated by two cell-interactive mechanisms: a promotion by spinal cord (SP) cells and a suppression by notochord (Nc) cells (see section 6).

5.2. Interaction between adult and larval myoblasts

It is well known that myoblasts at first proliferate, then stop cell divisions and finally fuse among themselves to form multinuclear myotubes toward terminal differentiation. Unlike this, however, there is an exceptional uninuclear myotube formation without myoblast fusion during somitic myogenesis in *Xenopus* early embryonic development (Muntz, 1975, Boujelida and Muntz, 1987). In this uninuclear myotubes, surprisingly, the amitosis-like nuclear divisions occur twice to yield the multinuclear (4-nucleus) myotubes (Boujelida and Muntz, 1987). However, Muntz' observation (1975) showed that "satellite cells" (myogenic stem cells) occur after stage 40 and increase between stage 45 and 59, and the basement membrane becomes seen at stage 40. Furthermore, at stage 50-55, the muscle cells were shown to take on the appearance of adult skeletal muscle fibers, numerous small nuclei occupying peripheral patches of cytoplasm. From these observations, it is conceivable that the fusion of satellite cells with the myotomal muscle fibers at the onset of metamorphosis (stage 48-50) enable the further multinucleation of the tadpole trunk muscles (Muntz, 1975). Supporting this idea, the isolated myoblasts from *Xenopus* tadpole trunk muscles could actually fuse to themselves to form multinucleated myotubes in vitro (Shimizu-Nishikawa et al., 2002).

In the trunk dorsal muscles of *X. laevis* tadpoles, there are almost the same number of two kinds of different myoblasts (Fig.7 C). Then, do the two different types of myoblasts which either have a life or death fate fuse to form the heterokaryon myotubes with the "chimeric fates" during metamorphosis? If this is the case, how do the "chimeric myotubes" behave in response to T_3? Which fate do they choose death or life, under the T_3-influences? What mechanisms exist for the smooth transition from larval to adult myogenic system? From these points of view, Shimizu-Nishikawa et al. (2002) carried out the study about cell interactions between the larval and adult myogenic stem cells in *X. laevis* (Fig.8). In this study, at first, the myoblasts isolated from adult leg (adult type myoblasts) or larval tail muscles (larval type myoblasts) were implanted under the skin of tadpole tail (or dorsal region) in order to know whether each type (larval or adult) of myoblasts fuse preferentially with its own type rather than the other type (Fig.8 A). To distinguish two types (adult and larval type) of different myoblasts by means of the differential nuclear staining with quinacrine, the Xenograft system with two closely related *Xenopus* species was employed, and thus the myoblasts from *X. borealis* (intense and heterogeneous nuclear staining) were transplanted into *X. laevis* larvae (homogenous weak nuclear staining). The result showed that transplanted adult type myoblasts migrated into tadpole muscle area and fused with preexisting larval type muscles (or myoblasts) to form heterokaryon myotubes with both types of myonuclei. Thus, it was found that adult myoblasts was not committed to fuse with its own type (adult type) but could fuse also with different type (larval) myoblasts in vivo.
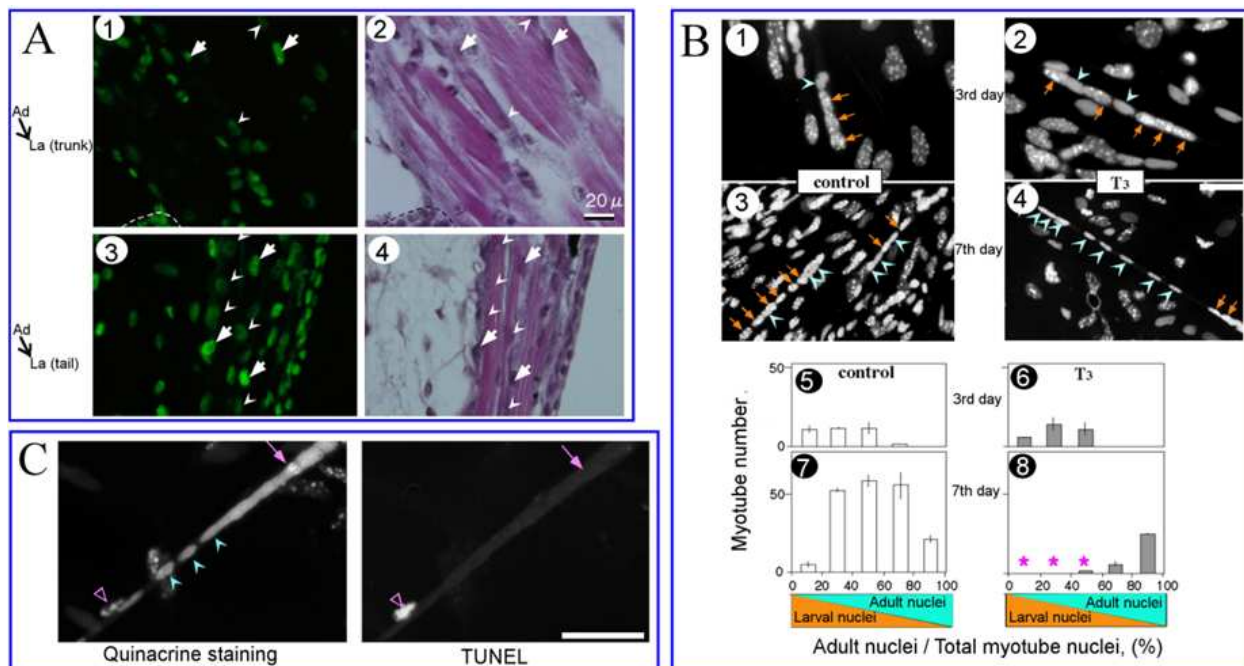
A: Formation of heterokaryon myotubes by cell transplantation. Cell aggregates (*Xenopus borealis* adult myoblasts) were transplanted subcutaneously into *X. laevis* (stage 48) trunk (1 and 2) or tail (3 and 4). Nine days after transplantation, muscle sections were made and stained with quinacrine (1 and 3) and then hematoxilin/eosin (2 and 4). The bar shows 20 μm. Nuclei of *X. borealis* show bright fluorescent spots (arrows) and can be distinguished from those of *X. laevis* (arrowheads). Dotted line (1) shows the transplanted cell aggregate. B: Heterokaryon formation in vitro and the T_3 response. Adult and larval myoblasts were co-cultured with (2 and 4: T_3) or without T_3 (1 and 3: control). 1 – 4: Fluorescent photomicrographs of the quinacrine stained nuclei of heterokaryon myotubes. The larval and adult nuclei were indicated by arrow and arrowheads, respectively. 1 and 2: Day 3. 3 and 4: Day7. The bar=50 μm. 5 – 8: Myotubes were classified by the proportion (%) of adult nuclei per total nuclei in each myotubes. The result was shown by histogram. Note that there are no heterokaryon myotubes dominated by the larval nuclei in T_3–cultures (asterisks). C: Larval nuclei do not die within the surviving heterokaryon myotubes even in the presence of T_3. Adult (*X. laevis*; arrowheads) and larval (*X. borealis*; arrows) myoblasts were co-cultured and T_3 was added 1 day after inoculation. Cells were fixed at 8th day and subjected to TUNEL reaction (left figure) and then stained with quinacrine (right figure). In adult nuclei-rich myotubes, the larval nuclei were TUNEL-negative (arrows). On the other hand, nuclei of a mononucleated larval myoblast (an open triangle) were TUNEL-positive. The bar=50 μm. The figures were reformed from the original papers (Shimizu-Nishikawa et al., 2002).

Figure 8. Formation of heterokaryon myotubes between adult and larval myoblasts.

Then, is there a possibility that the larval muscle fibers in tadpole are rescued from their T_3-mediated death by fusing with the adult type myoblasts and transform into adult muscle fibers? If this is the case, such cell-fate conversion may accelerate the speed of adult myogenesis because such tadpoles do not have to use much more energy for destroying a lot of differentiated larval muscles. So, does a fusion-mediated fate-change really occur? For the clarification of this point, it was examined using an in vitro co-culture system whether chimeric myotubes with both adult and larval myoblasts respond to T_3 to die or not (Fig.8 B). As the result, it was clarified that both larval and adult myoblasts randomly fuse to each other to make heterokaryon and the rescue from their T_3-mediated death occur only when the proportion of adult nuclei number was higher than 80 % within the myotube. Since the rescue from larval cell death thus requires incorporation of so many adult cells, the rescue of

the trunk myotubes would occur at a very low rate and most of larval type cells would usually die during metamorphosis by the action of T₃. Interestingly, an apoptotic feature (DNA fragmentation) was not observed in any larval nuclei within the surviving heterokaryon myotubes (i.e. adult nuclei ratio $\geq$ 80%). This mean that the larval nuclei were protected from apoptotic death and their death fate was converted to a life fate (Fig.8 C). However, because a lot of adult cell fusion are needed for preexisting larval muscles to increase the adult-nuclear ratio up to 80%, it is reasonable in vivo situation that adult dorsal muscle conversion by the rescue of the larval myotubes seldom occurs or it occurs only at very few fibers in the anterior portion of body axis with high growth activity. Accordingly, it would never occur in the tail portion where the growth activity of myoblasts is very low (Fig.9). In essence, adult conversion of the trunk dorsal muscles is mainly carried out by the new myotubes formation rather than the old myotubes rescue.

Then, not involving the rescue mechanism, another mechanism which promotes the adult myoblasts differentiation should be needed in order to make efficiently the adult muscles in dorsal muscle region. In order to know whether such promotion of adult myogenesis involves some kinds of cell-cell interactions, experiments with "separated co-culture" of two types of (adult and larval) myoblasts were conducted (Shimizu-Nishikawa et al., 2002). In this experiment, adult (frog leg muscle) and larval (tadpole tail muscle) type myoblasts were separately inoculated in the two different areas in the same culture dish in order to avoid a direct adult-to-larval cell interaction ("separated co-culture") and their differentiation activity was compared with that in control cultures with either one of the two types by counting myotube and myotube-nuclei numbers within each areas. In this "separated co-culture" system, two types of myoblasts can communicate only through culture medium but through direct cell-to-cell interactions. The result clearly showed that differentiation of adult myoblasts into myotubes was promoted by larval myoblasts but that of larval myoblasts was not affected by adult cells (Fig.10 A).
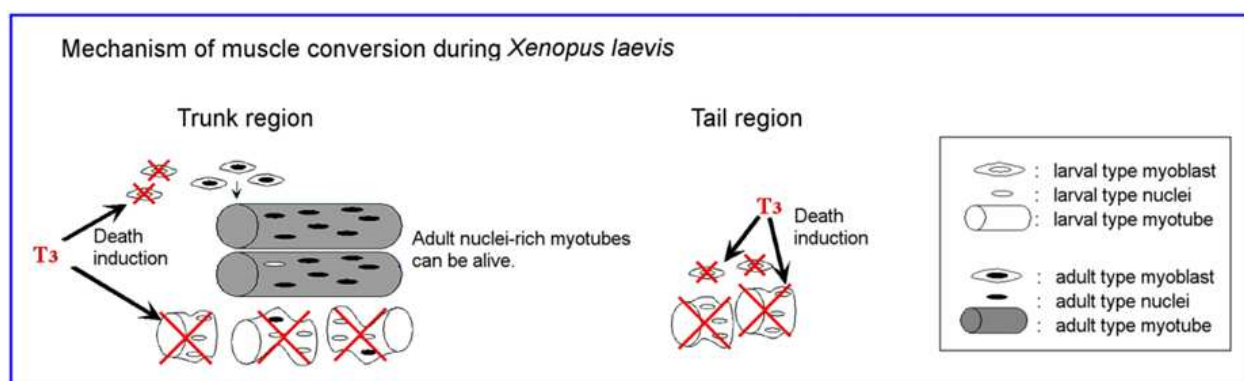
In trunk region, many adult myonuclei-rich myotubes (adult nuclei ratio< 80%) are formed and survive under the presence of T₃ while the pre-existed larval myonuclei-rich myotubes and larval myoblasts are to die by T₃. In tail region, however, adult myonuclei-rich myotubes are not formed and T₃ induces the death of larval myoblasts and larval myotubes. The figures were reformed from the original papers (Shimizu-Nishikawa et al., 2002).

Figure 9. A model for myotubes formation during *Xenopus laevis* metamorphosis.
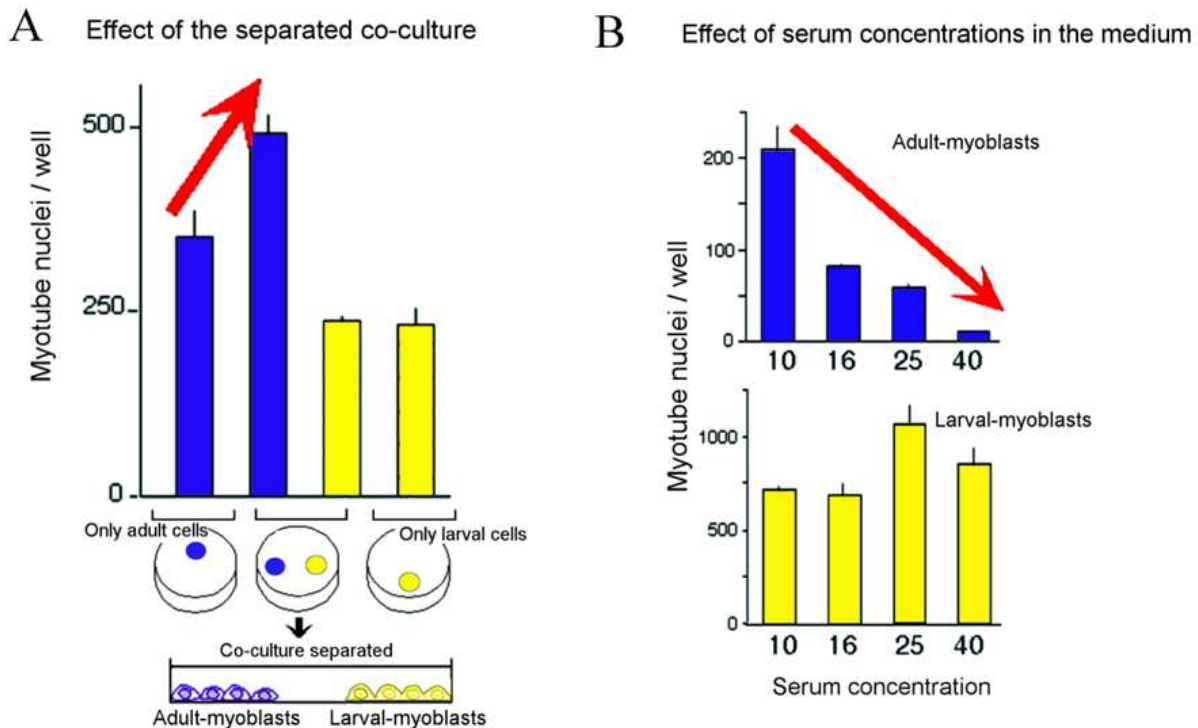
A: Effect of separated co-culture. Adult and larval myoblasts were cultured as in schematic drawings. Each type of myoblasts (3 x 10⁴ cells) was inoculated into an area (5-mm diameter) in a culture plate. The number of nuclei in myotubes was analyzed at third day of culture. B: Effect of serum concentration on myoblast differentiation. Each adult and larval myoblast was cultured in culture medium with different concentration of serum. Differentiation (number of nuclei in myotubes) was analyzed at 10th (adult) or 8th (larval) day of culture. The figures were reformed from the original papers (Shimizu-Nishikawa et al., 2002).

Figure 10. Effects of separated co-culture and serum concentration on myoblast differentiation.

This effect should be caused by some humoral factors which released from larval myoblasts but not by a direct cell-to-cell contact, because it occurred at a certain distance in the "separated" areas. So, it was examined whether the activity which promotes adult differentiation was observed in conditioned medium (CM). The result indicated that the activity was found only in a larval myoblast CM but not in an adult myoblast CM, suggesting that larval cell secreted a factor(s) for adult muscle differentiation. This putative factor was found to be in the retentate (R) fraction with molecular weight (MW) more than 10,000 through ultra filtration (MW 10,000 cut-off). The ultra filtration also revealed the inverse activity that inhibits the adult myoblast differentiation in the R fraction of control culture medium, suggesting that control medium (maybe serum components in medium) intrinsically contains a factor(s) which antagonizes with the factor(s) in L-CM to inhibit the adult myoblast differentiation. Thus, in order to examine if such inhibitory factor is from serum components of the culture medium, each adult and larval myoblasts were cultured in various conditions with different fetal calf serum (FCS) concentrations and their differentiation (myotube formation) activities were measured (Fig.10 B). As a result, differentiation of adult myoblasts was found to be suppressed dose-dependently by FCS but that of larval cells not to be affected. Taken from these results, it is conceivable that adult differentiation promoting factor(s) being released from larval cells functions through antagonistic regulation of the adult differentiation-inhibitory factor(s) in the control medium

(i.e. serum). It was suggested from a work with mouse myogenic cells (Cusella-DeAngelis et al., 1994) that the differentiation inhibitory factor(s) in serum could possibly be some kind of molecules related to TGF-β. Because, interestingly, TGF-β dose-dependently suppressed the differentiation of mouse fetal (but not embryonic) myoblasts in the same way as FCS dose-dependently suppressed that of *Xenopus* adult (but not larval) myoblasts. It still remains as an important question to be solved whether modulation of TGF-β activity is responsible for the specific promotion of adult myoblast differentiation in dorsal muscles during *Xenopus* metamorphosis.

In summary, it was found for the first time that the *Xenopus* larval myoblasts that have a death-fate under T$_3$ accept not only their death but also a big role as a cooperator for the adult myoblast differentiation via a regulation of humoral environment. The role of larval cells was thus found to be very important, however, we would not be able to fully understand the regulatory mechanism for the trunk-specific adult muscle differentiation only by analysis of the cell-cell interactions between larval and adult myoblasts. This is because, there are two types of myoblasts (adult and larval) not only in the trunk but also in the tail muscles. Then, is there any regulation from cells (or tissues) other than myoblasts (or muscles) for the trunk-specific adult myogenesis? From this point of view, Yamane et al. (2011) reported the importance of interaction between myogenic cells and non-myogenic cells (i.e. notochord and spinal cord cells) for the trunk specific adult muscle differentiation during *X. laevis* metamorphosis. This study is described in detail in the next section (section 6).

6. Interaction between adult myogenic precursor cells and axial cells

The adult muscle differentiation occurs in the trunk (but not the tail) during *Xenopus laevis* metamorphosis. Then, Yanane et al. (2011) focused on the two major tissues, the notochord and the spinal cord, which adjacent to the axial muscles (i.e. dorsal and tail muscles) in order to assess the involvement from the neighboring cells in the region-specific adult muscle differentiation. Because, both two tissues (notochord and spinal cord) are known to regulate early axial myogenesis in vitro (Munsterberg and Lassar, 1995; Stern and Hauschka, 1995) and in vivo (Blagden et al., 1997). At first, Yamane et al. (2011) examined the difference in cross-sectional areas between trunk and tail regions using histological sections of *Xenopus* tadpoles and noticed that the cross-sectional ratio of spinal cord to notochord area (SC/Nc ratio) is nearly 1:1 in the trunk but about 1:15 in the tail (Fig.11). This big difference is due to the situation that notochord area in the tail region is about 1.5 times larger than that in the trunk region and, on the contrary, spinal cord area in the tail is extremely smaller (around 1/10) than that in the trunk part. From this observation, it is conceivable that the influence of the spinal cord on myogenesis is greater in the trunk than in the tail and, in contrast, the influence of the notochord is stronger in the tail than in the trunk.

Therefore, it is reasonable to hypothesize for the trunk-specific adult muscle differentiation that adult myoblast differentiation is promoted by the spinal cord but suppressed by the notochord. So, according to this hypothesis, each of adult (from hindlimb) and larval (from tail) myoblasts was co-cultured with the spinal cord (or notochord) cells so as to compare their responses to two axial cells (i.e. spinal cord and notochord cells)(Fig.12). The result

clarified the expected opposite roles of the two axial cells: The spinal cord cells increased twice the myotubes-forming activity of adult myoblasts but did not increase that of larval cells. On the other hands, the notochord cells strongly suppressed the myotube-formation by adult myoblasts but did not suppress that by larval cells (Fig.12 G-L). Thus, there is a high possibility that two contrasting mechanisms, i.e. the "spinal cord (SC)-promotion" and the "notochord (Nc)-suppression" on adult myogenesis, are involved in the trunk-specific adult muscle conversion (Fig.13).
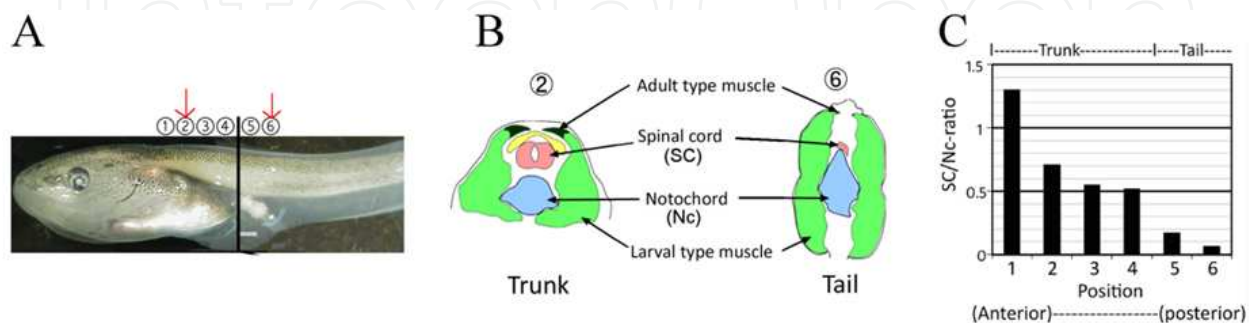

A: Side view of stage-55 tadpole.. The numbers ①-⑥ show the position of cross-sections analyzed. B: A schematic illustration of the cross-sections at positions ② and ⑥ (red arrows). C: The ratio of the spinal cord area to the notochord area (SC/Nc ratio) was shown in the ordinate and the abscissa showed the position along the anterior-posterior axis. The figures were reformed from the original papers (Yamane et al., 2011).

Figure 11. Cross-sectional proportion of spinal cord to notochord in *Xenopus laevis* tadpoles.
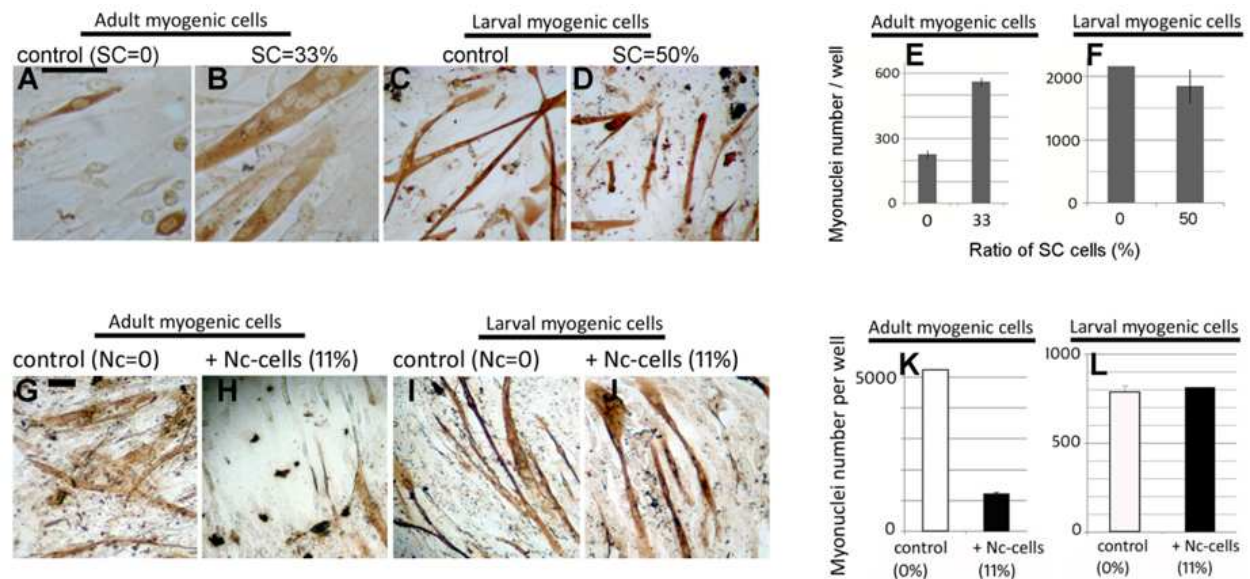

A-F: Adult (A, B and E) and larval (C, D and F) myogenic cells (1.6 x 10^4 cells) were co-cultured with (B and D) or without (A and C) SC cells (33%=8 x 103 cells or 50%=1.6 x 103 cells, respectively) for 7 days. Fixed cells were immunostained with antibodies for myogenic markers (brown color). The myo-nuclei number/well was measured and shown in E and F. G-L: Adult (G, H and K) and larval (I, J and L) myogenic cells (1.6 x 10^4 cells) were co-cultured with (H and J) or without (G and I) Nc cells (11%=2 x 10^3 cells) for 7 days. Fixed cells were immunostained with antibodies for myogenic markers (brown color). The myo-nuclei number/well was measured and shown in K and L. The bars in A and G show 50 μm. The figures were reformed from the original papers (Yamane et al., 2011).

Figure 12. Effect of spinal cord (SC) cells and notochord (Nc) cells on adult and larval myogenic cells in vitro.
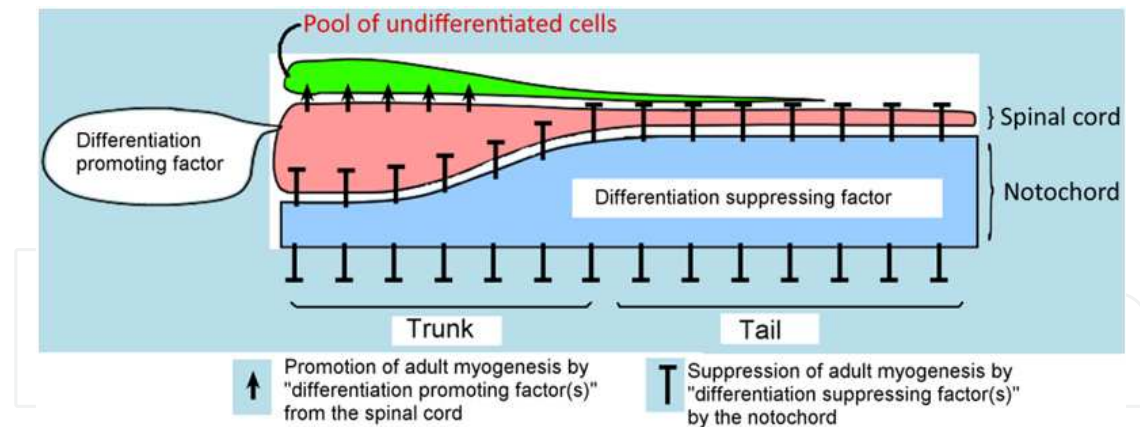
In trunk region, adult myogenesis is promoted by a "differentiation promoting factor(s)" from the spinal cord while, in the tail region, adult myogenesis is suppressed by a "differentiation suppressing factor(s)" from the notochord.

Figure 13. A model for the trunk-specific adult muscle differentiation during *Xenopus laevis* metamorphosis.
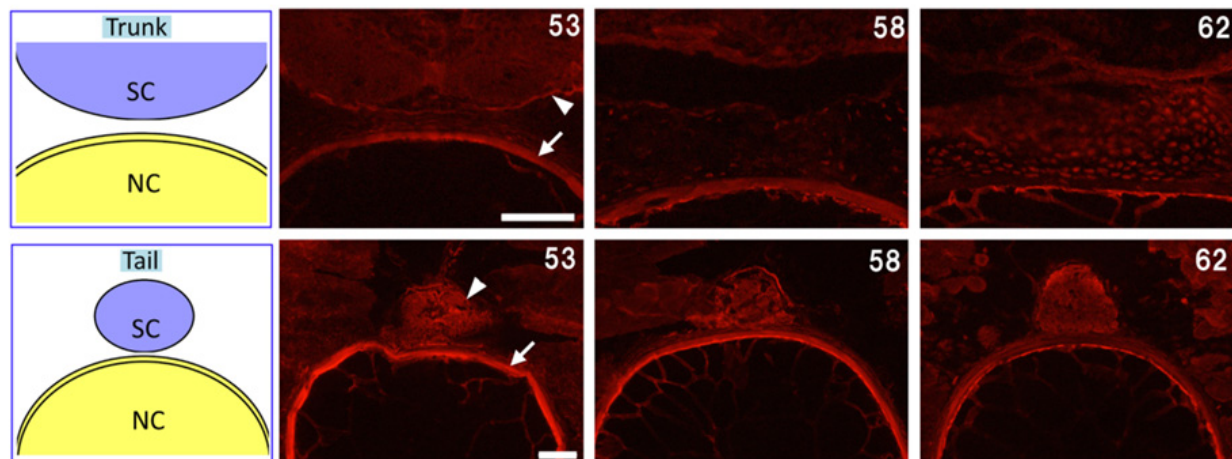

Shh expression was examined by immunostaining of tadpole sections. Left panels show the schematic illustrations of cross-sections of trunk and tail regions. SC: spinal cord. Nc: notochord. The other panels show the immunofluorescent photomicrographs with anti-Shh antibody (H160) of frozen section of tadpoles. The numbers show the stages of tadpoles. Upper panels: trunk region. Lower panels: tail region. The arrows and arrowheads show the position of notochord sheath and spinal cord, respectively. The bars show 100 μm. The figures were reformed from the original paper (Yamane et al., 2011)

Figure 14. Sonic hedgehog (Shh) distribution in notochord and spinal cord of the trunk and tail regions during *Xenopus laevis* metamorphosis.

As to the former mechanism, i.e. the "SC-promotion", the involvement of sonic hedgehog (Shh) signaling is expected, because the spinal cord and notochord expresses Shh and positively regulates the early embryonic myogenesis (Munsterberg and Lassar, 1995, Stern and Hauschka, 1995, Blagden et al., 1997). In fact, the analysis with antibody staining revealed that the N-terminal fragment (active form) of Shh proteins is present in the spinal cord and notochord regions throughout metamorphosis of *X. laevis* (Fig.14). And further analysis with a Shh inhibitor (cyclopamine) revealed that cyclopamine suppress the adult myoblast differentiation but not affect the larval one (Fig.15). In addition, the Shh protein

was found to be expressed also in the myotubes formed in the adult myoblast cultures, thus suggesting the involvement of Shh signaling in the auto-regulation of adult myoblast differentiation. Since both the two events, i.e. cyclopamine inhibition and the "SC promotion", occur in an adult type-specific manner, the "SC promotion" might involve a kind of Shh signaling released from the spinal cord.
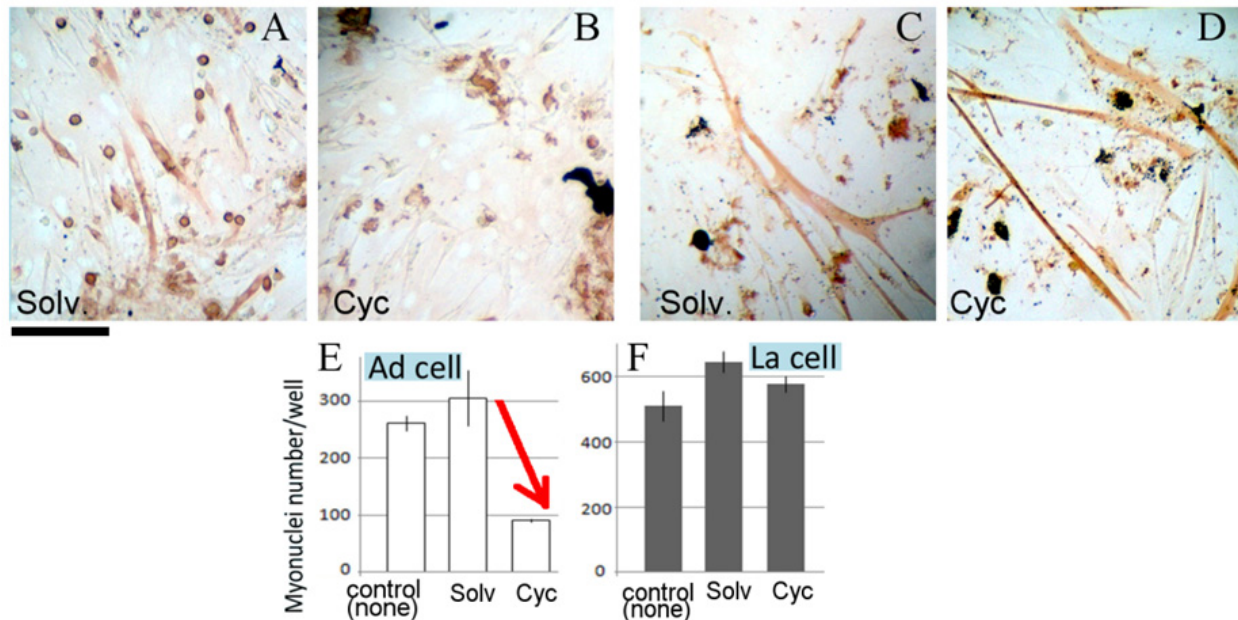
Adult (A, B and E) and larval (C, D and F) myoblasts were cultured for 6 days in the presence (B and D) or absence (A and C) of cyclopamine (1 μM). A-D: Photomicrographs of immunostained (brown color) cells at 6th day with an antibody mixture (anti-desmin and anti-myosin antibodies). The bar in A=100 μm. E and F: The numbers of nuclei in the antibody+ myotubes were counted and indicated in the ordinate. Mean values of two determinations (two wells) were shown and the bar indicate the range of the two. Solv: solvent without cyclopamone. Cyc: cyclopamine. The figures were reformed from the original paper (Yamane et al., 2011).

Figure 15. Effect of a Shh inhibitor, cyclopamine, on the differentiation of *Xenopus* adult and larval myogenic cells.

On the other hands, as to the "Nc suppression", the causing factor(s) remains to be unknown. However, another interesting feature of the notochord cells was found by a "separated co-culture" experiment. In the "separated co-culture", when the notochord cells and adult myoblasts were placed each in separated two areas on the same culture dish in order to avoid direct cell-cell interaction between them, the notochord cells lost their ability to suppress adult myogenesis but rather promoted the adult myoblast differentiation (Fig.16). Interestingly, the same effect (promotion of adult myogenesis) was also observed in a "separated co-culture" with a whole notochord tissue instead of isolated notochord cells. Thus, it was found that notochord cells have a long-distance promotive effect for adult myogenesis (i.e. the "notochord promotion") other than the "notochord suppression" effect appearing at a short distance. Since the notochord also expresses Shh throughout metamorphosis (Fig.14) and Shh is also known to positively regulate early embryonic myogenesis (Munsterberg and Lassar, 1995), this molecule is possibly be a major candidate molecule for the "notochord promotion".
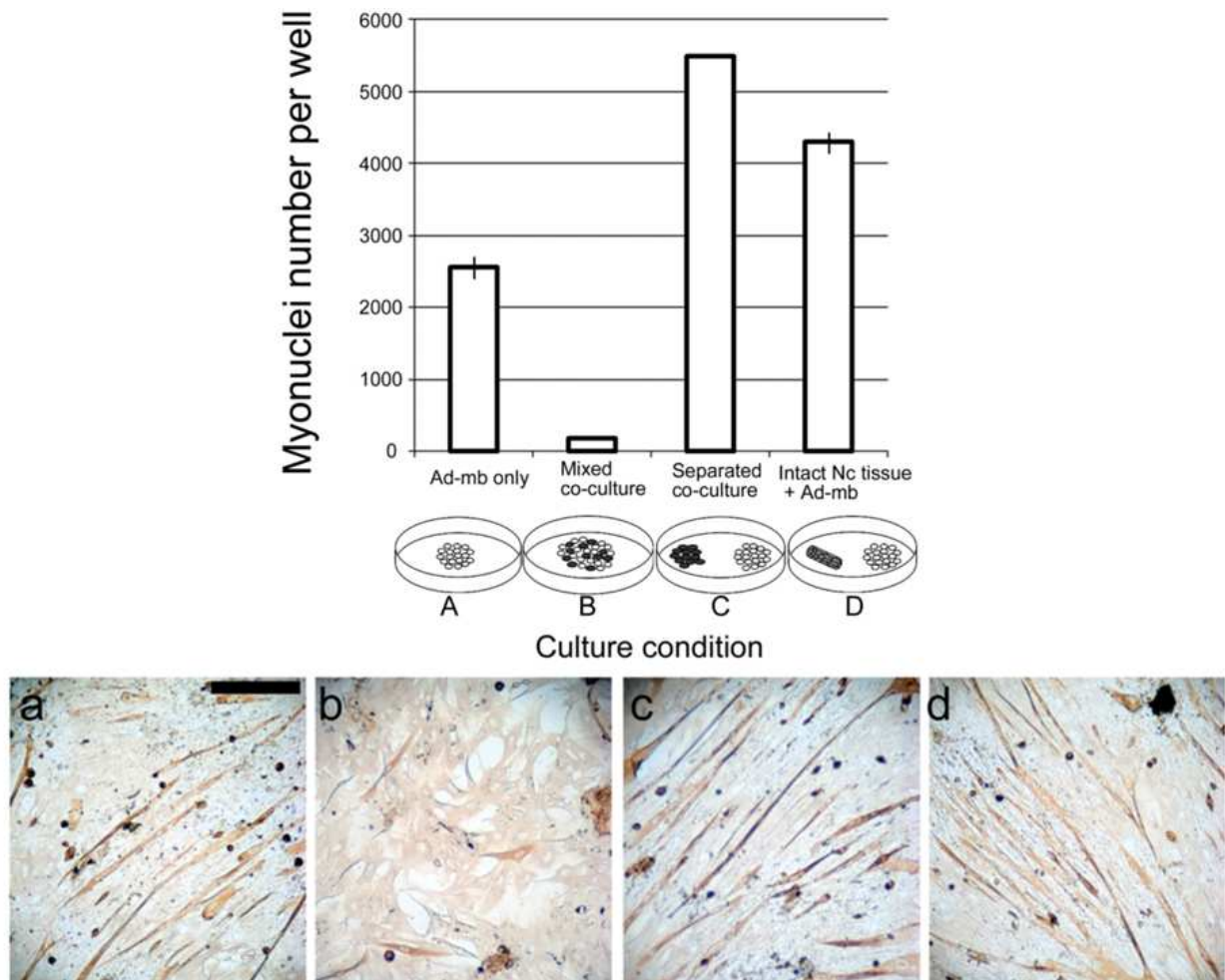
Ad-mbs were cultured for 7 days in various conditions (A-D). A: Ad-mbs only. B: Ad-mbs and Nc-cells were co-cultured within the same area of a culture dish. C: Ad-mbs and Nc-cells were separately placed in two different area of the same culture dish and cultured within the same medium. D: Separated co-culture with Ad-mbs and an intact Nc tissue. After 7 days, each culture was fixed, stained with an antibody mixture (anti-desmin and anti-myosin antibodies) and photographed (a-d for conditions A-D, respectively). The bar=200 μm. The nuclei numbers in antibody+ myogenic cells (i. e. myotubes and myoblasts) per dish were determined. The mean value for the two determinations was shown with the range of the two (vertical bar). The figures were reformed from the original paper (Yamane et al., 2011).

Figure 16. Notochord suppression on adult differentiation requires a direct cell-to-cell interaction between notochord (Nc) cells and adult myoblasts (Ad-mbs).

As described above, the multiple cell-to-cell interactions coordinately regulate in diverse ways the trunk-specific promotion and the tail-specific suppression of adult myogenesis during metamorphosis. The molecular features of such cell interactions have not fully characterized. Especially, it should be primarily emphasized as an important future work to get insight into the molecular mechanism for the "notochord suppression". Hebrok et al. (1998) reported with chick embryo that factors from notochord, such as fibroblast growth factors (FGF) and activin, suppress the prepancreatic dorsal endoderm Shh expression and thereby permit early pancreatic development. Such a FGF (or activin)-like molecule(s) might be a factor(s) responsible for the "notochord suppression". Further investigations are needed for clarifying this question.

Secondary, it is also very important issue to clarify the signaling cascade for the "SC promotion" of adult myogenesis. The "SC promotion" shows adult-specific effect on the muscle differentiation and Shh is expected to be a responsible factor for this phenomenon. As detailed above (section 4), adult myoblasts proliferate many times as undifferentiated stem cells and then after few days stop cell divisions to transit to the differentiation steps (Shibota et al., 2000). Therefore, it is reasonable that the "SC promotion" is involved in the transition step from the undifferentiated stemness stage of the adult cells to the more committed differentiation stage. Borycki et al. (1999) reported that Shh, produced by the notochord and floor plate, control epaxial muscle determination through *myf-5* activation in the mouse embryo. Gustafsson et al. (2002) also reported that *myf-5* is a direct target of long-range Shh signaling through Gli transcription factors for the specification of mouse somitic epaxial muscle progenitor cells. Therefore, also in case of *X. laevis* "SC promotion", the factor(s) (possibly Shh-related molecules) may act on commitment stages (determination and/or specification steps) of undifferentiated stem cells. If the "SC promotion" factor is indeed Shh, *myf-5* upregulation may possibly be enhanced in the adult-cells co-cultured with SC cells. These points should be clarified in the near future.

The clarification of the cell-cell interaction mechanisms and their molecular cascades for the adult muscle differentiation during *Xenopus laevis* metamorphosis would finally contribute to the accumulation of technical knowledge about how to manipulate and maintain the mammalian embryonic stem (ES) cells (Martin, 1981; Evance and Kaufman, 1981) or induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006) for their induction toward complete (adult-type) differentiation.

7. Conclusion

Nishikawa and Hayashi firstly examined the larval-to adult-muscle isoform transition and put forward a new model, the "cell replacement model", that clearly explains the larval-to-adult myogenic conversion during frog metamorphosis. In this model, larval-to-adult conversion of tadpole dorsal muscles was achieved through the cell replacement by both death of larval-type myogenic cells and proliferation and differentiation of adult-type myogenic cells. The death of tadpole trunk dorsal muscles was found to occur through apoptotic processes including nucleosomal DNA-fragmentation, apoptotic body formation and phagocytosis by macrophages.

In subsequent research, larval- and adult-type myogenic precursor cells (myoblasts) were isolated each from *Xenopus laevis* tadpole tail or hindlimb leg muscles and cultured in vitro to see their basic nature, developmental fates, i.e., to die or not to die, and hormonal responses. It was thus clearly demonstrated that larval-type myoblasts are to die in the presence of T_3, while adult-type myoblasts can proliferate and differentiate into myotubes in the same hormonal condition. More interestingly, cell separation using different two methods showed that not only trunk muscles but also tail muscles contain both larval- and adult-type myoblasts. In order to see cell interaction between adult- and larval-type myogenic cells, heterokaryon analyses were conducted. The results revealed the possibility of the rescue of nuclei-death of larval type cells by adult cell nuclei in the syncytium and the promoting effect by a larval cell-releasing factor(s) for the adult myoblast differentiation.

Finally, co-culture system using myogenic cells (larval and adult myoblasts) and non-myogenic axial cells (notochord and spinal cord cells) was developed to examine how adult myogenesis is promoted in the trunk muscle region but suppressed in the tail muscle region through the interactions between myogenic and non-myogenic cell or environmental signals. The results revealed the suppression of adult myogenesis by notochord cells ("notochord suppression"), promotion of adult myogenesis by spinal cord cells ("spinal cord promotion") and upregulation of adult myogenesis by sonic hedgehog (Shh)-signaling. These results present a model for the region-specific regulatory mechanism of adult myogenesis by cell-cell interactions, i.e., "spinal cord promotion" and "notochord suppression", during *X. laevis* metamorphosis.

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