# Molecular Mechanism of Muscle Cell Death by Thyroid Hormone in Amphibian Tadpole Tail

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First slide (1) please. Our research has focused on elucidating the molecular mechanism by which the tails of tadpoles regress during metamorphosis, and applying the results of the study to development of an advanced thyroid hormone detection system.

Next slide (2) please. Almost all organs of anurans undergo transformation during metamorphosis, such as development of four limbs and regression of the tail and gills. The tails of *Xenopus* tadpoles that grow more than two times their body size almost disappear in one-week period from stage 61 to stage 65.

Next slide (3) please. These metamorphous transformations are primarily caused by an increase in concentration of thyroid hormone in the blood, but little was known about the molecular mechanism by which cell death is triggered by thyroid hormone during metamorphosis. It has been surmised that thyroid hormone regulates expression of genes that cause cell death in the regressing tail. In organ culture of fins and gills of tadpoles, an increase in collagenase activity and decrease in organ size due to thyroid hormone have been observed. It is therefore thought that synthesis of collagenase by thyroid hormone has got something to do with reconstitution of collagen.

Next slide (4) please. In the research of Dr. Don Brown et al, many genes that are up- and down-regulated by thyroid hormone in the regressing tail have been isolated by PCR-based subtractive hybridization and characterized by *in situ* hybridization. Extracellular matrix (ECM)-degrading enzymes such as stromelysin-3 and collagenase-3 are highly expressed in the subepidermal fibroblasts encircling the entire muscle flank, but not expressed in the tail muscle. These enzymes are also up-regulated in the myotendinous junctions to which the muscle fibers are attached. These observations lead to the idea that the increase of secreted matrix metalloproteinases induced by thyroid hormone results in the degeneration of the myotendinous junctions, which detaches muscle cells from ECM and causes their death. This is called the “murder model”.

Next slide (5) please. With this model, thyroid hormone induces secretory protein such as matrix metalloproteinase, which breaks down the extracellular matrix. This protein kills surrounding cells and itself.

Next slide (6) please. On the other hand, we have established a myoblastic cell line XLT-15 derived from the tadpole tail. The cells at the top are treated by the control and the lower cells are treated by 10 nM of active thyroid hormone T3 for three days. In the lower panel nuclei of T3-treated cells were stained strongly with Hoechst. These were found to be positive by TUNEL reaction, showing cell death by apoptosis. Because this suggests that cells of the tail die cell-autonomously, it supports the suicide model.

Next slide (7) please. This simply shows that only cells reacting to thyroid hormone die directly. However, it is possible that this is an *in vitro* artifact and does not reflect the physiological death in matrix-interacting cells *in vivo*. Furthermore, it does not exclude a possibility that thyroid hormone-treated myoblastic cells kill each other by secreting soluble factors.

Next slide (8) please. Because dominant-negative thyroid hormone receptor (DNTR) binds to thyroid hormone response elements (base sequence) but not to thyroid hormone, it prevents transcription of thyroid hormone-responsive genes by normal thyroid hormone receptors. In other words, it blocks the thyroid hormone signal. I will refer to this as DNTR from now on. We came up with the following experiment using DNTR to differentiate the suicide model and murder model from each other. We introduced DNTR and reporter genes into some of the cells and treated with thyroid hormone. With the

murder model, since a cell death inducing factor is secreted from cells where DNTR is not expressed, this factor should kill all cells in spite of the expression of DNTR. With the suicide model, since the cells are differentiated from each other, DNTR-untransfected cells should die and DNTR-expressing cells should survive. We conducted this experiment with cultured cells and with living tadpoles.

Next slide (9) please. First, we introduced the DNTR gene and GFP gene as a reporter gene to a tail-derived myoblastic cell line XLT-15 by transfection and treated with thyroid hormone for three days. C was untreated and $T_3$ was treated. The DNTR gene was transfected to the right side and vector to the left. The vertical axis shows the percentage of apoptotic cells in GFP-positive cells. The graph clearly shows that cell death is controlled by DNTR. In other words, it strongly supports the suicide model.

Next slide (10) please. We know that thyroid hormone treatment causes this cell line to secrete 92kDa gelatinase. The gelatinase activity was examined in the previously mentioned *in vitro* supernatant using zymography, and a similar activity after thyroid hormone treatment was observed whether either vector or DNTR gene was transfected. This means, if the murder model is correct, secretory cell death inducing factor should exist at the same concentration regardless of transfection with DNTR. Also it should mean that cell death is not suppressed by transfection of DNTR. This inconsistency with the experimental results negates the murder model.

Next slide (11) please. We conducted an experiment to ascertain whether cell death of tail muscle cells in living tadpoles by thyroid hormone is by murder or suicide. We used the method of injecting DNA into muscle tissue developed by Dr. Demeneix who is here with us today. The basic way is to inject DNTR and reporter genes into a tadpole tail, and determine the quantity of muscle cells expressing reporter genes in stage 64 when a tail almost disappears.

Next slide (12) please. Prior to starting this experiment, we conducted several pilot studies. After we injected the beta-galactosidase gene as a reporter gene, beta-galactosidase activity was visualized in muscle cells as shown here. When the cross sections of samples were prepared and subjected to the immunohistochemistry using anti muscle-specific protein tropomyosin antibodies, all cells with beta-galactosidase activity were identified as muscle cells.

Next slide (13) please. When the beta-galactosidase gene-injected tail was regressing, muscle cells have been fragmented and killed irrespective of the overexpression of beta-galactosidase. From this, it has been interpreted that the overexpression of a reporter gene has not affected cell death.

Next slide (14) please. In order to know the morphology of apoptosis-inhibited muscle cells in the regressing tail, we injected a cell death-suppresser bcl-XL gene, and beta-galactosidase gene. A big tail at stage 59 has shrunk this much at stage 64. This is a spinal cord and this is a notochord. A notochord has disappeared at stage 63-64. Only cells with the beta-galactosidase activity have survived at stage 64 in an almost cell-free structure. To see what this is, tails were stained at the various stages with antibodies against the type IV collagen.

Next slide (15) please. Type IV collagen is observed not only in the basement membrane within the skin and between muscle cells, but also around the spinal cord and in the notochord lamella. As the tail regresses after stage 64, it looks like the collagen takes up all space other than the spinal cord. In other words, it appears that only the bcl-XL-overexpressing cells are present and floating up in the extracellular matrix containing the type IV collagen.

Next slide (16) please. Here we analyzed a tail injected with DNTR and beta-galactosidase genes. This time we got the same results as bcl-XL gene-injected tails. When the part stained in blue is magnified, we observe the cross-striated structure particular to the muscle tissue.

Next slide (17) please. The results of conducting this experiment with many tails are as follows. “Surviving” indicates the number of tails which contain cells with beta-galactosidase activity at stage 64 as shown in the previous, and “Dead” indicates the number of tails which lost blue muscle cells. Blue cells

did not survive with negative control vector and luciferase expression construct, but cells injected with bcl-XL, particularly with DNTR, tended to survive. The data supports the suicide model.

Next slide (18) please. When a beta-galactosidase reporter gene was coinjected with both DNTR and TR expression constructs, no muscle cell with beta-galactosidase activity was found at stage 64. The result demonstrated that the inhibition of cell death via DNTR overexpression is mediated specifically by blocking the thyroid hormone-signaling, since a injection of this amount of DNTR expression construct inhibited death of muscle cells with beta-galactosidase activity. These data indicated that the suicide mechanism by thyroid hormone plays an important role in muscle cell death during stage 58-64.

Next slide (19) please. This time we injected the cells with GFP gene as a reporter gene and observed change during the developmental process in the tails of living tadpoles. After injecting 50ng of vector, or 50ng or 300ng of DNTR with GFP gene, we traced the fluorescent light given off by GFP. The numbers between the panels indicate the number of days it took for development. The white lines show the outline of the tail other than fins. With vector, at stage 63 where the tail rapidly regresses, the muscle cells become round as the fluorescent light rapidly decreases. The light almost disappears at stage 64. The muscle cells of tails injected with DNTR likewise become round at stage 63 and the fluorescent light diminishes at stages 64 and 65. This decrease is the same when injected with either 50 or 300 ng of DNTR, so it appears that DNTR expression was saturated. Thus the delay by DNTR in fluorescent light reduction and the existence of GFP positive cells still at stage 66 altogether support the suicide model, and the decrease in fluorescent light at stages 64 and 65 indicates the validity of the murder model.

Next slide (20) please. This shows the quantification of fluorescent light. The horizontal axis shows the number of days after stage 61. The delay by DNTR in fluorescent light decrease and existence of GFP positive cells at stage 66 support the suicide model and the reduction in fluorescent light at stages 64 and 65 supports the murder model, again.

Next slide (21) please. GFP positive cells continue to exist in all tails injected with DNTR even three weeks after metamorphosis.

Next slide (22) please. When both GFP and DNTR genes were injected into every other myotome, they look like this at first, but as they develop, GFP negative myotomes disappear leaving GFP positive myotomes only. The cells become rounded regardless of location. The remaining cells subsequently begin to diminish. In other words, both the suicide model and murder model are correct for muscle cell death after stage 62 and it appears the muscle cells become rounded by lowered tension caused by regression of the tail.

Next slide (23) please. GFP positive cells are observed at stage 66 where tails injected with DNTR have disappeared. This could be the thyroid hormone resistant muscle tissue or cord suggested by Dr. Brown. The cord is TM228 and TM311 positive, but the muscle cells of the tail are TM311 positive and TM228 negative. As you see, GFP positive cells are not a cord but larval tail muscle cells. TM228 and TM311 positive muscle cells are the adult muscle cells around the legs and rectum.

Next slide (24) please. Muscle cells injected with GFP and vector begin to die rapidly at stage 62 and those injected with DNTR begin to die at stage 63. Because these cells are recognized as anti-active caspase-3 antibody, we know that both die by apoptosis.

Next slide (25) please. Next, in order to study when the muscle cells of the tail begin to die, we injected the tails of younger tadpoles with GFP and vector and observed the developmental process. As a result, we found that the cells began to die in stage 57, that is, prior to the climax of metamorphosis. We succeeded in perfectly suppressing cell death in stages 57 through 62 by injecting DNTR.

Next slide (26) please. As is evident from the quantification graph for the previous experiment, we found that reduction of fluorescent light is inhibited by DNTR, cell death in stages 57 through 62 is clearly

in keeping with the suicide model, and muscle cells themselves are extremely susceptible to thyroid hormone.

Next slide (27) please. We propose the hypothesis that muscle cells of larva begin to die at stage 57, thyroid hormone induces suicide (autonomous cell death) of muscle cells in the tail between stages 57 and 62, and cell death is executed through the mechanism of murder and suicide to quickly eradicate muscle during stages 62 to 64.

Next slide (28) please. We are in the process of developing an advanced method of detecting thyroid hormone activity in organisms that make use of this molecular mechanism. In other words, we inject one myotome with GFP and thyroid receptor genes and another with GFP and DNTR, treat them with thyroid hormone and observe the fluorescent light of GFP. This enables us to easily detect activity of $T_3$ in 0.3 nM. I hope our research can contribute to detection of endocrine disrupters. That brings my presentation to an end.

## Q&A

Yoshizato: Questions or comments, please.

Q: Very clear data. Thank you for your data. From stage 57-62, suicide model can be applied, but beyond 62 murder model factor is working. Another marker, 92kDa gelatinase activity, is low up to 62, but it becomes higher beyond 62. Is that true?

Yaoita: Yes. Beyond 62, the value is extremely high, at least for messenger RNA.

Q: I have a question, but because of the time limitation maybe we can discuss it later. Thank you very much.