Cell cycle regulation during development and dormancy in embryos of the annual killifish

Austrofundulus limnaeus

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Embryos of the annual killifish Austrofundulus limnaeus can enter into a state of metabolic dormancy, termed diapause, as a normal part of their development. In addition, these embryos can also survive for prolonged sojourns in the complete absence of oxygen. Dormant embryos support their metabolism using anaerobic metabolic pathways, regardless of oxygen availability. Dormancy in diapause is associated with high ATP and a positive cellular energy status, while anoxia causes a severe reduction in ATP content and large reductions in adenylate energy charge and ATP/ADP ratios. Most cells are arrested in the G1/G0 phase of the cell cycle during diapause and in response to oxygen deprivation. In this paper, we review what is known about the physiological and biochemical mechanisms that support metabolic dormancy in this species. We also highlight the great potential that this model holds for identifying novel therapies for human diseases such as heart attack, stroke and cancer.

Introduction

Entrance into a state of reversible dormancy requires organismal, cellular and molecular alterations that support cellular structure and metabolism, often at severely reduced energy flows.1-3 The energetic constraints associated with hypometabolism are generally inconsistent with sustained progress through the cell cycle, and thus cell cycle arrest is likely a common occurrence in organisms entering hypometabolic states.4 However, very little work has been focused on evaluating where in the cell cycle cells may arrest when entering various stages of hypometabolism. Yet, where the arrest occurs will have a profound influence on the cost of maintenance, environmental stress tolerance and the probability of re-entering the cell cycle successfully when the organism exits a state of dormancy. In this paper, we review what is known about the cellular physiology of dormancy in embryos of Austrofundulus limnaeus and explore the role of cell cycle arrest as they enter into two distinct types of hypometabolic states, embryonic diapause and anoxia-induced quiescence. The environmental and cellular conditions that the cells of these embryos can endure provides a unique opportunity to investigate naturally evolved mechanisms to deal with cellular stresses associated with a number of human diseases and disorders, including cardiovascular disease, cancer and teratogenesis during development in vertebrates.

Life History of Annual Killifish

Annual killifish (Cyprinodontiformes) inhabit ephemeral aquatic habitats in regions of Africa and South America that experience distinct dry and rainy seasons. Many of these ponds are isolated from permanent waters by long distances and are subject to extremes in temperature and oxygen availability.5 A pond may be inundated for several weeks to several months, during which time the fish must complete their entire lifecycle (Fig. 1). Thus, annual killifish have extremely fast growth rates, as some larvae may complete their entire life cycle, from hatching to senescence and death, in as little as 6 weeks.6 When the ponds dry, the adult and juvenile fish perish, and the population survives the dry season as drought-tolerant embryos7 that can enter a state of metabolic and developmental arrest termed diapause.8-10

Embryonic Diapause

Diapause is defined as a state of developmental arrest that precedes the onset of unfavorable conditions.11 In annual killifish, diapause may occur at three distinct developmental stages termed diapause I, II and III9 (Fig. 1). Each of these stages of diapause appears to respond to different environmental cues for induction and breakage of dormancy.10,12-15 However, it is important to note that embryos may enter diapause under conditions that are conducive to normal development, which indicates an active cessation of development dependent on internal signals and not removal of some external resource, such as oxygen or water.

Diapause I. Diapause I may occur early in development at 4 days post-fertilization (dpf), just after the completion of epiboly.9 Diapause I is obligate in some species of annual killifish and facultative in others.16 In Austrofundulus limnaeus, this stage of diapause is rarely observed but may be induced by low incubation temperatures in some populations.9 Diapause I is associated with a unique feature of early development in all annual killifishes, the complete dispersion and subsequent reaggregation of the embryonic...
Diapause II. Diapause II may occur following the major events of neurulation and somitogenesis, but prior to initiation of the major phases of organogenesis, in a stage referred to as the long-somite embryo. In A. limnaeus, diapause II embryos have 38–42 pairs of somites (precursors to axial muscle and bone), a functional tubular heart and associated vasculature and the foundations of the central nervous system (fore-, mid- and hind-brain) and major sensory organs (olfactory placodes, otic vesicles and optic cups). Diapause II may be obligate or facultative, depending on the species. In A. limnaeus, diapause II is facultative. For incubation temperatures at or below 25°C, the vast majority of embryos enter diapause II at 24 dpf, while temperatures of 30°C or higher cause embryos to skip this diapause and follow an alternate developmental trajectory.

Diapause III. Diapause III can occur near the completion of development just prior to hatching. Diapause III embryos have consumed the bulk of their yolk resources and are ready to feed almost immediately upon hatching. This stage of diapause appears to be obligate in most species that have been studied, including A. limnaeus.

Diapause and stress tolerance. Tolerance or resistance to stress is acquired during early embryonic development in A. limnaeus and peaks after about a week into diapause II (32 dpf at 25°C). These embryos can tolerate extremes in environmental salt concentration, water availability and oxygen content. However, it is worth noting that early embryos of A. limnaeus are very hardy and can survive environmental stresses that would be lethal to most other species of vertebrates. In addition, the extreme tolerance of environmental stress exhibited by diapause II embryos is retained for several days of post-diapause II development. Thus, although tolerance of environmental stress is greatest in diapause II, it is not unique to diapausing embryos.

Cellular physiology of diapause II. Most work on the physiology of diapause in A. limnaeus has been focused on early development and diapause II. The pace of early development in A. limnaeus is slow compared with other species of Cyprinodont fish with similar sized eggs. For instance, embryos of Fundulus heteroclitus complete development in about 12–14 dpf at 20°C, while embryos of A. limnaeus reach diapause II (roughly halfway through development) at about 24 dpf at 25°C. Thus, metabolic rate is low, and developmental progression is slow for all of early development in A. limnaeus. Entry into diapause II is defined by a lack of developmental progression, a severe decline or complete cessation of cardiac activity and the absence of cell division as evidenced by a lack of increase in the total DNA content. Embryos that enter diapause II have an extremely low metabolic rate.
rate, as estimated by both oxygen consumption and heat dissipation, that is depressed by over 90% compared with developing early embryos. While morphological development ceases when embryos enter diapause II, cellular physiology continues to change during the first week of diapause, resulting in even lower rates of oxygen consumption and a greater contribution of anaerobic pathways to overall heat dissipation by 32 dpf. This low metabolic rate is in part supported by a 90% decrease in the rate of protein synthesis, and low levels of ATPase activity are supported by embryonic membranes with exceptionally low permeability to salts and water.

Calorimetric:respirometric ratios indicate that diapause II embryos support a large proportion of their metabolism using anaerobic pathways despite being incubated in completely aerobic conditions. This conclusion is supported by the recent observation that diapause II embryos do not exhibit a decline in total heat dissipation when exposed to anoxia (see below). In addition, mitochondria isolated from diapausing embryos of *A. limnaeus* have severely reduced activities for the major respiratory complexes, and an almost complete absence of ATP synthase activity (Fig. 2). The metabolic capacity of diapause II embryos is highly biased toward anaerobic metabolic pathways as indicated by a 50-fold greater capacity for lactate dehydrogenase activity compared with citrate synthase activity. Despite low mitochondrial activity and a low metabolic rate, cellular ATP levels remain high, and ATP/ADP ratios and adenylate energy charge indicate a very positive cellular energetic status with respect to phosphorylated adenylates.

**Cell cycle arrest during diapause II.** DNA content does not increase during long bouts in diapause II, suggesting withdrawal of cells from the cell cycle. Flow cytometry analysis indicates that over 90% of the cells possess a diploid complement of DNA during most of early development, including diapause II (Fig. 3). Thus, the great majority of cells in diapause II embryos arrest in the G1/G0 phase of the cell cycle. Arrest in G1 is controlled by a number of factors associated with regulating the presence and activity of the D and E cyclins. While nothing is currently known about cyclin E expression in this species, diapause II embryos have low levels of cyclin D1 compared with embryos that have broken diapause II and resumed normal development for 4 d (Fig. 4). High levels of D cyclins are associated with progression through early G1 toward the restriction point, and thus low levels of cyclin D1 are consistent with early G1 cell cycle arrest in diapause II. However, in embryos that are 12 d...
post-diapause II (dpd), cyclin D1 levels are also low despite high levels of morphogenesis and growth, suggesting a possible shift from cyclin D1 to other isoforms of D or perhaps E cyclins during late development to support cell cycle progression. Increased expression of p53 protein can also lead to cell cycle arrest in the G1 phase of the cell cycle in response to a number of cellular stressors. Arrest of the cell cycle during diapause in A. limnaeus is likely not dependent on induction of p53, as levels of this protein remain extremely low in diapause II embryos despite their withdrawal from the cell cycle (Fig. 5). This result is consistent with what is known about the role of p53 in the induction of diapause in insects. Somewhat unexpectedly, the amount of phosphorylated active AKT (pAKT, phosphorylated at serine 473) is relatively high in diapause II embryos despite their withdrawal from the cell cycle (Fig. 6). While elevated amounts of the phosphorylated active form of this kinase are often associated with development of cancer, AKT also plays a pivotal role in supporting cell survival by blocking positive regulators of apoptotic programmed cell death. Thus, without additional information we currently interpret increased abundance of pAKT protein to be a pro-survival signal in diapause II embryos.

**Figure 4.** Cyclin D1 protein levels in diapause II (DII) and embryos 4 d post-diapause II (4 dpd) under normoxia, after long-term anoxia equal to one-half of the LT50 for the developmental stage, and after 24 h of recovery from long-term anoxia. Diapausing embryos have low levels of cyclin D1 compared with post-diapause II embryos that are actively developing. Levels of this protein do not change in response to anoxia or recovery from 24 h of anoxia. Data from Meller et al.

**Figure 5.** Expression levels of p53 protein in diapause II (DII) and embryos 4 d post diapause II (4 dpd) under normoxia, after long-term anoxia equal to one-half of the LT50 for the developmental stage, and after 24 h of recovery from long-term anoxia. Diapausing embryos enter into diapause with extremely low levels of p53 compared with embryos that have exited diapause II and resumed active development. Levels of p53 are responsive to anoxia in diapause II (ANOVA, p < 0.05), but not in post-diapause II embryos.

**Anoxia-Induced Quiescence**

When faced with oxygen deprivation, diapausing and developing embryos of A. limnaeus experience a profound decrease in heat dissipation, while diapause II embryos are relatively unaffected (Fig. 8). Anaerobic metabolism is largely supported by lactate production in embryos of A. limnaeus. Embryos can accumulate over 30 mM lactate after extended periods of anoxia. In addition to lactate, smaller amounts of alanine and succinate are also accumulated, as are large quantities (5–10 mM) of gamma-aminobutyrate (GABA). Changes in free amino acid levels are consistent with a lack of significant protein degradation, even during long-term exposures to anoxia, but rather support a preferential consumption of amino acids such as glutamate, glutamine, aspartate and asparagine and production of alanine. Surprisingly, levels of ATP plummet during the initial exposure to anoxia in both diapausing and developing...
Figure 6. The amount of phosphorylated AKT (pAKT, serine 473) protein increases during the course of early embryonic development. pAKT protein levels are highest after 8 d in diapause II (32 dpf). This is consistent with blockage of apoptosis, but is surprising considering the lack of cell proliferation observed in diapausing embryos. Data are from Meller et al.25

Cell cycle arrest in response to anoxia. The vast majority of cells isolated from anoxic embryos of A. limnaeus contain diploid levels of DNA, suggesting a G1/G0 arrest of the cell cycle (Fig. 10).25 However, the overall distribution of cells in G1 and

Figure 7. Anoxia tolerance in embryos of A. limnaeus is two orders of magnitude greater than that of any other vertebrate, even considering the increased tolerance of anoxia exhibited by vertebrate embryos. Data for all species have been normalized to a temperature of 25°C assuming a Q10 of 2. 100% of development was set at birth or hatching for turtles, birds and placental mammals, emergence from the pouch for marsupial mammals, and at completion of larval development for fish and amphibians. Development past 100% is expressed as a percentage of the time to reach 100% development. The regression line was calculated based on all species except for the obvious outliers, embryos of A. limnaeus and turtle hatchlings. Additional details of this figure are outlined in Podrabsky et al.35

embryos, falling to levels that are only 20% of aerobic values within a few hours in embryos that are 4 dpd (Fig. 9).36 AMP accumulates as a consequence of the drop in ATP, while ADP values remain relatively constant. While anoxic, ATP/ADP ratios approach 1, and adenylate energy charge drops to near 0.3, indicating severe energy limitation during exposure to anoxia.36 The vast majority of vertebrate cells, especially cardiac myocytes and neurons, would be irreversibly damaged within minutes of experiencing this severe of a depletion of ATP. The fact that cells of A. limnaeus can tolerate this level of ATP depletion for months without obvious cell damage is exceptional.

Anoxic preconditioning. It is well appreciated that short, non-lethal exposures to anoxia or ischemia (blockage of blood flow) can induce greater tolerance of subsequent bouts of oxygen deprivation in mammalian tissues.37,38 Such inducible protection has been termed “preconditioning,” the positive effects of which have a complex kinetics of induction but can last for several days following the preconditioning stimulus. Embryos of A. limnaeus that exhibit extreme tolerance of anoxia do not appear to respond to anoxic preconditioning, suggesting that they may be already pre-adapted for maximal survival of anoxia. As extreme tolerance of anoxia is lost during post-diapause II development, the ability to respond to preconditioning stimuli is gained, and in embryos that are 12 dpd, a significant increase in survival time is observed in response to a preconditioning treatment of 24 h of anoxia followed by 24 h of aerobic recovery.39 However, the extreme tolerance of anoxia exhibited by earlier developmental stages cannot be induced in these embryos through preconditioning. The underlying mechanisms that support anoxic preconditioning in these embryos have not yet been identified.
vertebrate that can enter into a profound state of metabolic depression and exhibits extreme tolerance of anoxia. The cells of these embryos can survive conditions, both extrinsic and intrinsic, that would be lethal to the vast majority of vertebrate cells. Mechanistic studies of this system have only just begun, but already a number of promising discoveries suggest this may be a fruitful model that could provide novel strategies for altering the stress tolerance or cell cycle progression of vertebrate cells. For instance, these cells survive a severe decline in cellular ATP concentrations without losing cellular and organellar integrity and the often-associated induction of cell death.

Regulation of the cell cycle is complex in this system, with some potentially interesting departures from other systems that may be adaptive for survival of exposure to anoxia and during metabolic dormancy associated with diapause. For instance, the vast majority of cells are found to be in G1 throughout development despite the obvious need for large amounts of cell division. Further, preferential arrest in G1 occurs regardless of whether the cue to halt cell cycle progression arose internally or externally. This pattern of cell cycle progression may be explained by a rapid progression through the S, G2, and M phases of the cell cycle, or a prolonged G1 phase, and may be adaptive, because the embryos are always prepared for a G1 arrest if faced with environmental stresses such as anoxia. Arrest in G1 may favor survival due to a

G1 does not change in response to anoxia, suggesting that cells can arrest in presumably any stage of the cell cycle. Detailed microscopy analysis would be needed to fully evaluate if cells are arresting in the G1 or M phases of the cell cycle, and if these cells are viable during recovery from anoxia. The protein concentration of cyclin D1, which drives G1/S progression, is not affected by anoxia in diapause II or 4 dpd embryos (Fig. 4).25 This lack of change following prolonged anoxia might suggest an underlying, yet persistent, production of cyclin D1 that is insensitive to cellular stressors and cell cycle arrest signaling through Cdk inhibitors. Interestingly, p53 protein levels increase in diapause II embryos exposed to anoxia but not in embryos that are 4 dpd (Fig. 5), despite the fact that both embryos share the same extreme tolerance of anoxia.20 An increase in p53 during exposure to oxygen deprivation has been associated with hypoxia-induced apoptosis in some systems.30 Thus, the induction of p53 in diapause II embryos exposed to anoxia is unexpected and warrants further investigation. In embryos that are 12 dpd, which have an LT50 in anoxia of around 6 d, levels of pAKT protein plummet after 48 h of anoxia and return to near control levels after 24 h of aerobic recovery (Fig. 11).25 A loss of pAKT is consistent with a role for this kinase in signaling for cell cycle arrest in response to anoxia. How this decline in pAKT will affect the regulation of programmed cell death has yet to be determined. It is interesting that pAKT is upregulated during aerobic dormancy in diapause II but severely downregulated during anoxia-induced quiescence in actively developing embryos. Obviously the role of AKT in the regulation of the cell cycle and apoptosis in this species is complex and will require additional studies to fully appreciate.

A. limnaeus as a Model for Cell Cycle Regulation in Health and Disease

Embryos of A. limnaeus present a unique opportunity to study global regulation of cell proliferation and metabolism in a vertebrate that can enter into a profound state of metabolic depression and exhibits extreme tolerance of anoxia. The cells of these embryos can survive conditions, both extrinsic and intrinsic, that would be lethal to the vast majority of vertebrate cells. Mechanistic studies of this system have only just begun, but already a number of promising discoveries suggest this may be a fruitful model that could provide novel strategies for altering the stress tolerance or cell cycle progression of vertebrate cells. For instance, these cells survive a severe decline in ATP and significant increase in AMP in response to anoxia, but show a nearly complete reversal within 12 h of aerobic recovery. Data are from Podrabsky et al.25

**Figure 8.** Heat dissipation of diapause II (green) and embryos 4 d post-diapause II (blue) exposed to normoxia (pre-dashed line) and anoxia. Post-diapause II embryos experience a profound decrease in heat dissipation in response to anoxia while diapausing embryos do not experience a statistically significant decrease. Data are from Podrabsky et al.16

**Figure 9.** ATP levels are not defended in embryos of A. limnaeus exposed to anoxia. Aerobic diapause II embryos have significantly lower levels of total adenylates and ATP compared with embryos 4 d post-diapause II that are actively developing. Both stages of development experience a severe decline in ATP and significant increase in AMP in response to anoxia, but show a nearly complete reversal within 12 h of aerobic recovery. Data are from Podrabsky et al.25
lower cost of genome maintenance, a higher probability of recovery from dormancy due to the molecular phenotype of G1 cells and the ability to harness existing molecular signaling cascades that have evolved with respect to withdrawal from the cell cycle prior to the restriction point. In addition, this may be the safest stage to arrest in terms of genome integrity, because disruption of DNA replication and mitotic chromosome rearrangements are not of concern. Future research in this area holds great promise for elucidating the possible significance of G1 arrest during metabolic dormancy.

Another interesting observation is the lack of p53 expression during diapause II and subsequent induction in response to anoxia. Because the cell cycle is already arrested during diapause, an increase in p53 protein may be important in blocking apoptosis, but future studies will be needed to evaluate the possible role of p53 in diapauing embryos. The fact that p53 is not induced in post-diapause II embryos is also interesting, although baseline levels are already high in these embryos compared with those in diapause II. The role of p53 in embryonic development is complex, and thus protein expression levels may indicate a critical role in maintaining healthy development, rather than a specific adaptation for tolerance of anoxia. Furthermore, it is important to note the high protein expression levels of pAKT in diapause II embryos. From a cell cycle perspective, this would be consistent with progression through the cell cycle via inactivation of Cdk inhibitors.33 However, the cell cycle is obviously arrested, and thus perhaps novel mechanisms exist in this system for inhibition of the cell cycle independent of the activity of AKT. This would allow for the expression of pAKT to support negative regulation of apoptosis without the possible loss of cell cycle control associated with reduced levels of Cdk inhibitors. The obvious importance of dysregulated AKT activity in many human cancers suggests that this could be a fruitful avenue for discovering new ways to control the cell cycle in cancer.

Embryos of the annual killifish A. limnaeus offer a unique model for studies of cell cycle control during embryonic development, during entry into a profound state of hypometabolism, and in response to cellular stress. A better understanding of the unique phenotype expressed by these cells could lead to novel avenues for treatments of diseases associated with altered progression through the cell cycle, and a number of metabolic syndromes...
associated with reliance on anaerobic pathways despite access to aerobic conditions. This relatively unknown model holds great potential for transforming our understanding of how to engineer vertebrate cells to survive relatively extreme environmental conditions relevant to very practical applications, such as extension of transplant organ shelf life, global control of cell proliferation independent of cell type, preservation of cellular integrity for long-term storage of gametes or fertilized embryos and increased survival of heart attack or stroke.

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