REVIEW

Thyroid hormone-dependent regulation of metabolism and heart regeneration

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Abstract

While adult zebrafish and newborn mice possess a robust capacity to regenerate their hearts, this ability is generally lost in adult mammals. The logic behind the diversity of cardiac regenerative capacity across the animal kingdom is not well understood. We have recently reported that animal metabolism is inversely correlated to the abundance of mononucleated diploid cardiomyocytes in the heart, which retain proliferative and regenerative potential. Thyroid hormones are classical regulators of animal metabolism, mitochondrial function, and thermogenesis, and a growing body of scientific evidence demonstrates that these hormonal regulators also have direct effects on cardiomyocyte proliferation and maturation. We propose that thyroid hormones dually control animal metabolism and cardiac regenerative potential through distinct mechanisms, which may represent an evolutionary tradeoff for the acquisition of endothermy and loss of heart regenerative capacity. In this review, we describe the effects of thyroid hormones on animal metabolism and cardiomyocyte regeneration and highlight recent reports linking the loss of mammalian cardiac regenerative capacity to metabolic shifts occurring after birth.

Key Words

- thyroid hormone
- metabolism
- heart
- regeneration
- polyploid

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Heart attacks remain a leading cause of death worldwide due to the inability of adult humans to regenerate lost cardiac tissue after ischemic damage (, Nowbar *et al.* 2019). Myocardial infarction occurs when blood flow to the heart is blocked resulting in the massive loss of cardiomyocytes followed by the deposition of non-contractile fibrotic scar tissue at the sites of injury (Price *et al.* 2019). This compromises ventricular function leading to pathological remodeling, heart failure, and death (Riching & Song 2021). While adult mammals generally lack the capacity for heart regeneration, other vertebrate animals including urodele amphibians and teleost fish are capable of robust cardiac regeneration after injury throughout their lives (Oberpriller & Oberpriller 1974, Poss *et al.* 2002, Cutie & Huang 2021, Khyeam *et al.* 2021). Importantly, the seminal discovery that newborn rodents possess a transient ability to regenerate their hearts has stimulated much interest in the field to determine the physiological mechanisms driving the loss of mammalian heart regenerative potential after birth (Porrello *et al.* 2011, Wang *et al.* 2020).

We have recently reported that increasing levels of thyroid hormones after birth are key drivers that promote mammalian cardiomyocyte cell cycle arrest and loss of heart regenerative potential (Hirose *et al.* 2019). Interestingly, recent studies describe an emerging link between metabolic regulation, cell cycle control, and mammalian heart regenerative potential (Cao *et al.* 2019, Cardoso *et al.* 2020, Amram *et al.* 2021, Graham & Huang

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2021). Since thyroid hormones are traditionally recognized for their classical roles in regulating animal metabolism and body temperature (Maillet *et al.* 2013), we propose that metabolic control and heart regenerative potential in mammals are dually regulated by this hormonal pathway (Fig. 1). In this review, we highlight the roles of thyroid hormone in animal metabolism, its impact on cardiac regenerative capacity, and the growing evidence linking heart regeneration to metabolic control.

Thyroid hormones and metabolism

Thyroid hormones are synthesized as the prohormone thyroxine (T4) and the actions of deiodinases convert this prohormone to its active form triiodothyronine (T3). Binding to nuclear thyroid hormone receptors recruits transcriptional activators to induce the expression of target genes. Thyroid hormones are well-established modulators of processes that are essential for growth and development including metabolism in mammals (Brent 2012, Little & Seebacher 2014) and metamorphosis in amphibians (Furlow & Neff 2006). Its activity increases basal metabolic rate, which in turn leads to body heat production, oxygen consumption, and ATP hydrolysis (Shahid *et al.* 2021). For example, Gerdes *et al.* uncovered that T4-treated animals exhibited decreased body weights accompanied by increased heart rate and heart weight compared to untreated controls (Gerdes *et al.* 1983), phenotypes consistent with increased metabolism. Furthermore, thyroidectomy experiments showed that removal of the thyroid gland in echidna, a monotreme that may be regarded as a hypothyroid animal due to its low uptake and slow release of I^{125}, resulted in little change in standard metabolism. In contrast, rabbits and bandicoots – representing eutherians and marsupials, respectively – body temperature and standard metabolism were significantly reduced after radiothyroidectomy (Hulbert & Augee 1982). These results may suggest the possibility that thyroid hormone plays an increasingly significant role in metabolic control during the evolution toward placental mammals. Thyroid hormone may exert these effects by the regulation of brown fat-mediated thermogenesis, mitochondria biogenesis, and mitochondrial function.

Thermoregulation

A key evolutionary innovation in mammals is endothermy, the ability to generate heat to increase body temperature and metabolic rate. This adaptation allows mammalian species to maintain constant body temperatures regardless of environmental temperature. Mammalian thermoregulation is predominantly regulated in brown adipose tissue (BAT), where substrate oxidation is uncoupled from ATP synthesis and releases energy in the form of heat. This process is dependent on uncoupling protein-1, which allows the passage of electrons into the mitochondrial matrix without ATP synthesis, decreasing the proton motive force (PMF) required by the enzyme ATP synthase. As a result, energy substrate oxidation increases to restore the PMF leading to heat production rather than ATP synthesis in brown fat cells. Thyroid hormone has

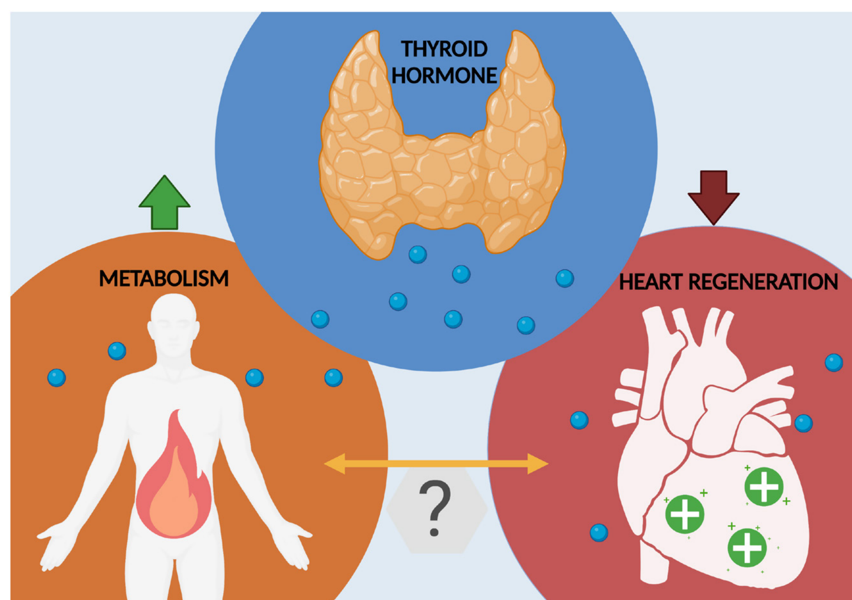

Figure 1
Thyroid hormone dually regulates metabolism and heart regenerative potential. Thyroid hormones (blue spheres) are secreted by the thyroid gland and drive mammalian metabolism and inhibit heart regenerative capacity. The interrelationship between metabolism and heart regeneration has been a subject of recent interest.

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been found to increase oxidation uncoupling in the BAT (Hulbert 2000). Proton leakage was found to be lower in mitochondria from hypothyroid rats and higher in those from hyperthyroid rats compared to euthyroid control animals (Harper & Seifert 2008).

Mitochondrial biogenesis and function

Mitochondria are key organelles regulating cellular metabolism and can participate in cell signaling by the release of reactive oxygen species (ROS). Thyroid hormones may modulate metabolism through the stimulation of mitochondrial biogenesis. In primary cultures of neonatal cardiomyocytes, T3 treatment upregulated the expression of genes involved in mitochondrial biogenesis as determined by RT-qPCR (Tan *et al.* 2019). *In vivo*, T3 was shown to increase mitochondrial numbers in the hearts of hyperthyroid rats (Tanaka *et al.* 1985). Mitochondrial processes, more specifically the electron transport chain, produce ROS as a byproduct of energy metabolism. Increased mitochondrial respiration leads to electron leakage and the production of superoxide radicals (O_2^-) from O_2 reduction. This is followed by dismutation to hydrogen peroxide (H_2O_2), which is among the major endogenous ROS species (Sedensky & Morgan 2006, Chen *et al.* 2009).

Peroxisome proliferator-activated receptor-alpha (PPARA) is a ligand-activated transcription factor that belongs to a family of nuclear receptors (van Raalte *et al.* 2004). Its coactivator is the peroxisome proliferator-activated receptor-coactivator-1 (PGC-1), and both have been associated with the regulation of genes involved in mitochondrial biogenesis, oxidative phosphorylation, and energy metabolism (McClure *et al.* 2005). Northern blot analysis demonstrates that PGC-1 expression is upregulated in the heart at birth, which may increase mitochondrial biogenesis and the metabolic switch from glycolysis to fatty acid oxidation (Lehman *et al.* 2000). PGC-1a has been found to mediate mitochondrial maturation with the action of the coactivator nuclear respiratory factor-1. Together, they increase the transcription of mitochondrial transcription factor A (Tfam), which induces mitochondrial biogenesis (Scarpulla 2008). T4-treated rats exhibit increased transcript levels of Tfam, as shown by quantitative RT-PCR (Goldenthal *et al.* 2004). Wulf *et al.* identified T3 as a rapid regulator of PGC-1 expression, both *in vivo* and in cell culture (Wulf *et al.* 2008). Cardiac overexpression of PGC-1 resulted in rampant mitochondrial biogenesis that led to cardiomyopathy (Lehman *et al.* 2000). T4 administration in rats resulted in a significant increase in PPARA and PGC-1 levels, which was accompanied by ~50% increase in myocardial mitochondrial DNA levels and oxygen consumption (Goldenthal *et al.* 2004, Liang & Ward 2006). These results identify PGC-1 and PPARA as critical regulators controlling mitochondrial number and function in response to energetic demands.

We have recently profiled differential gene expression in 2-week-old mouse hearts with cardiomyocyte-specific overexpression of dominant-negative thyroid hormone receptor alpha (Thra) inhibiting thyroid hormone-induced gene activation in these cells (Hirose *et al.* 2019). We additionally identified the direct target genes regulated by Thra by chromatin immunoprecipitation-sequencing (Hirose *et al.* 2019). Our studies revealed that many mitochondrial genes were downregulated and were direct targets of Thra. Among these genes is *Cpt2*, which encodes carnitine palmitoyltransferase 2, the rate-limiting enzyme for fatty acid transport into mitochondria for oxidation (Ceccarelli *et al.* 2011, Hirose *et al.* 2019). Consistent with these findings, Tanaka *et al.* determined that hyperthyroid treatment increased carnitine palmitoyltransferase enzyme activity in the hearts of rats (Tanaka *et al.* 1985). These findings suggest a role for T3 in the metabolic shift toward fatty acid oxidation that occurs shortly after birth and is associated with the increase of circulating thyroid hormone levels during the perinatal period.

ROS production

Previous studies determined that experimental hyperthyroidism led to accelerated ROS production as indicated by lipid peroxidation products (Petrulea *et al.* 2009). More recently, a study showed that the administration of exogenous T3 increased H_2O_2 production by three-fold in primary cultures of neonatal mouse cardiomyocytes (Tan *et al.* 2019). In a study of thyroidectomized sheep, cytochrome c oxidase (Cyt aa3) and cytochrome c (Cyt c) contents were decreased, as indicated by quantitative spectrophotometry. These data suggest that low levels of thyroid hormone inhibit mitochondrial maturation (McClure *et al.* 2005). In contrast, hyperthyroidism resulted in increased cytochrome c oxidase-specific activity (Tanaka *et al.* 1985). Moreover, rats treated with T4 for 15 days showed increased mitochondrial proteins including cytochrome c and cytochrome c oxidase contents (Goldenthal *et al.* 2004). These results further substantiate the role of thyroid hormones in mitochondrial biogenesis and energy metabolism.

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Thyroid hormones and heart regeneration

Soon after birth, cardiomyocytes stop undergoing cell cycle and become terminally differentiated. In order to keep up with the increased circulatory demands, individual cells undergo physiological hypertrophy. This is characterized by multinucleation and an increase in size that results from the addition of new sarcomeres (Yang *et al.* 2016). Thyroid hormones are essential for these changes and stimulate hypertrophy via activation of nuclear receptors that upregulate transcription of genes including α-myosin heavy chain (Myh6) and sarcoplasmic reticulum Ca2+ ATPase and downregulate transcription of β-myosin heavy chain (Myh7) and phospholamban (Belakavadi *et al.* 2010, England & Loughna 2013). The changes in expression of these genes result in physiological heart growth that can accommodate the increasing demands for cardiac function post-birth (Maillet *et al.* 2013). Due to its role in contraction and calcium regulation, thyroid hormones are essential for cardiac function, and inappropriate levels of this hormone can lead to cardiovascular disease (Yamakawa *et al.* 2021).

Adult zebrafish are capable of fully regenerating their hearts after 20% resection of cardiac mass (Poss *et al.* 2002). The cardiac tissue of adult newts after injury regenerates through the division of pre-existing cardiomyocytes that dedifferentiate and undergo mitosis, as evidenced by the disappearance of Z-band patterning (Oberpriller & Oberpriller 1974). Consistent with these findings, elegant lineage tracing studies using the Cre-recombinase system demonstrated that the source of new cardiomyocytes in the regenerating zebrafish heart is pre-existing cardiomyocytes themselves (Jopling *et al.* 2010, Kikuchi *et al.* 2010). A similar lineage tracing study confirmed this mechanism in regenerating neonatal mouse hearts (Porrello *et al.* 2011). Taken together, the results of these studies suggest that the dedifferentiation and proliferation of pre-existing cardiomyocytes may be a fundamentally conserved mechanism for cardiac regeneration across phylogeny.

Lessons from adult zebrafish and neonatal mice teach us that heart regeneration occurs primarily through the proliferation of pre-existing cardiomyocytes rather than expansion and differentiation of stem cell populations (Jopling *et al.* 2010, Kikuchi *et al.* 2010, Porrello *et al.* 2011, Xin *et al.* 2013*b*). Unfortunately, mammalian cardiomyocytes withdraw from the cell cycle shortly after birth (Xin *et al.* 2013*b*). In newborn mice, cardiomyocytes undergo a final round of DNA duplication and division during the first weeks of life (Senyo *et al.* 2013). However, due to cytokinesis failure, approximately 90% of murine cardiomyocytes binucleate rather than completely divide into individual cells. The increase in heart size from neonatal to adult stages is predominantly a result of cardiac hypertrophy with cardiomyocyte renewal rates at less than 1% per year (Xin *et al.* 2013*b*). In contrast, greater than 95% of adult zebrafish and newt cardiomyocytes are mononucleated and retain proliferative activity (Bettencourt-Dias *et al.* 2003, Wills *et al.* 2008). Because of this, it is hypothesized that mammalian cardiomyocyte cell cycle exit and binucleation during neonatal stages restrict cardiac regenerative potential in adulthood. Consistent with this model, induction of cardiomyocyte polyploidization inhibits adult zebrafish heart regeneration (González-Rosa *et al.* 2018) and mouse strains retaining higher abundances of diploid cardiomyocytes exhibit higher regenerative potential (Patterson *et al.* 2017). Growing evidence from our lab and others has demonstrated a role for thyroid hormones in regulating cardiomyocyte cell cycle during this developmental transition (Fig. 2).

Cardiomyocyte cell cycle and ploidy

Polyploidization and multinucleation as an index of cardiomyocyte maturation and differentiation have been used by researchers to examine the role of thyroid hormone in heart regeneration. Adult mammals with little heart regenerative capacity have mostly polyploid cardiomyocytes (Alkass *et al.* 2015). In contrast, animals with heart regenerative and proliferative capacities were found to have mainly mononucleated and diploid cardiomyocytes (Hirose *et al.* 2019). Postnatal day 2 (P2) mice possess ~93.2% of mononucleated diploid cardiomyocytes compared to those identified at P10, when only ~5% of cardiomyocytes were mononucleated and ~95% binucleated (Soonpaa *et al.* 1996). Thyroidectomy experiments in ovine fetuses revealed a reduction in cardiomyocyte binucleation, suggesting that thyroid hormones promote cardiomyocyte polyploidization. Although the number of cardiomyocytes positive for cell cycle marker expression was reduced in thyroidectomized fetal sheep, cardiomyocytes from thyroidectomized animals were ~21.4% binucleated compared to ~56.8% observed in T3-infused animals (Chattergoon *et al.* 2012). This suggests that low or complete absence of thyroid hormone hindered cardiomyocyte binucleation.

Naqvi *et al.* reported that increasing circulating levels of thyroid hormone after birth lead to a second wave of cardiomyocyte proliferation during preadolescent development. This burst of cardiomyocyte proliferation was shown to contribute to ~40% increase in total cardiomyocyte numbers in adult rats, as determined by an

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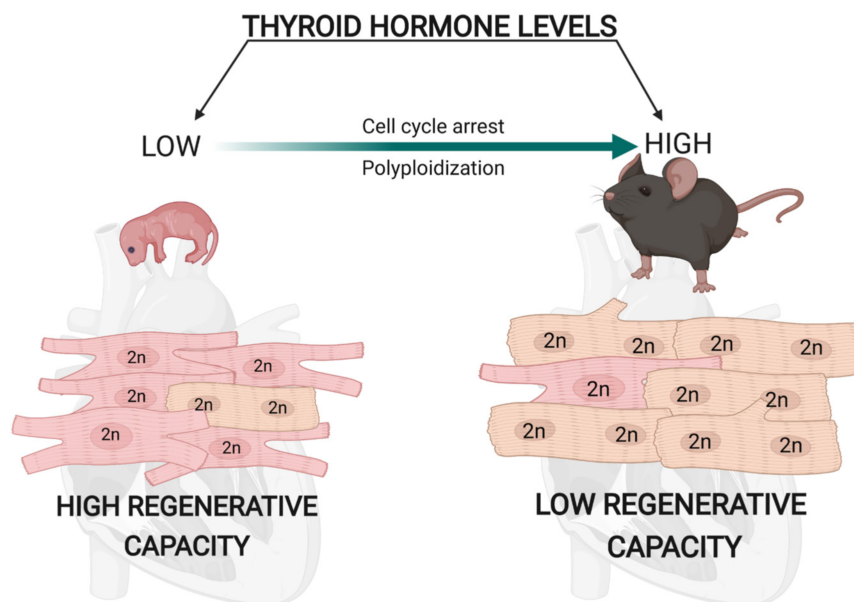

Figure 2
Thyroid hormone drives cardiomyocyte cell cycle arrest, polyploidization, and loss of heart regenerative capacity. During perinatal stages, the majority of cardiomyocytes are mononucleated and diploid (2n), which retain proliferative potential to support cardiac regeneration. Increasing levels of circulating thyroid hormones after birth drive cardiomyocyte maturation, cell cycle arrest, polyploidization, and loss of heart regenerative capacity.

enzymatic tissue digestion approach (Naqvi *et al.* 2014). In contrast to these findings, Alkass *et al.* employing design-based stereology to estimate cardiomyocyte numbers did not identify a preadolescence cardiomyocyte surge. Instead, they observed that the postnatal period was characterized by two waves of non-replicating DNA synthesis, when cardiomyocyte nuclei become polyploid. Mouse cardiomyocyte volume increased ~two-fold from P11 to P21 as determined by three-dimensional volume measurements of dissociated cells. This volume increase correlated to the ~2.0-fold volume increase in the left ventricle, providing little evidence of cardiomyocyte proliferation during this time period (Alkass *et al.* 2015). Thymidine analogs are commonly used to identify cycling cells that have undergone DNA synthesis or S-phase. By analyzing bromodeoxyuridine (BrDU) incorporation in genetically labeled cardiomyocytes, Soonpaa *et al.* observed low levels of cardiomyocyte DNA synthesis between P12 and P19 (Soonpaa *et al.* 2015), corroborating the findings of Alkass *et al.* These conflicting studies regarding thyroid hormone action and its role in cardiomyocyte cell cycle withdrawal after birth stress the need for further refinement in the approaches used to identify cardiomyocytes with high fidelity and quantify total cardiomyocyte numbers in the mammalian heart.

The direct effects of thyroid hormone on cardiomyocyte cell cycle and division have been controversial. BrdU incorporation was significantly decreased in primary cultured fetal ovine cardiomyocytes treated with T3 (pre-treatment or concurrent). T3-treated cells exhibited a 1.5% BrdU incorporation compared to 8–12% incorporation in serum-treated controls (Chattergoon *et al.* 2007). Thyroid hormone was also demonstrated to exert antiproliferative effects on mid-gestation ovine cardiomyocytes, with BrdU incorporation decreasing in a T3 dose-dependent manner (Chattergoon *et al.* 2012). Neonatal rats treated with T4 for 12 days exhibited increased heart weight-to-body weight ratios, cardiomyocyte hypertrophy, and inhibited cardiomyocyte proliferation (Gerdes *et al.* 1983). Taken together, these results suggest that exogenous administration of thyroid hormone negatively impacts cardiomyocyte proliferation.

However, studies have also reported contrasting effects. Ledda-Columbano *et al.* suggested that cardiomyocyte proliferation increases as a result of T3 treatment in adult rats (Ledda-Columbano *et al.* 2006). It was proposed that the accumulation of cyclin D1 and its translocation to the nucleus driven by thyroid hormone-stimulated cell cycle re-entry in adult cardiomyocytes. BrdU labeling index was ~30.2% in T3-treated cardiomyocytes compared to ~2.2% in controls, results that were validated using phosphorylated histone-3 (PH3), a mitosis marker. However, while these findings indicate DNA synthesis and cyclin D1 accumulation after thyroid hormone treatment, changes in cardiomyocyte number were not directly examined after treatment. Increased S-phase or mitotic entry may still result in polyploidization and binucleation rather than true cell division. For these reasons, the use of molecular markers may not be sufficient to examine heart regeneration. In a recent study, exogenous T3 administration in mice at P2 and P3 was observed to increase 5-ethynyl-2'-deoxyuridine (EdU) incorporation, expression of mitotic markers,

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and cardiomyocyte cell numbers (Tan *et al.* 2019), which oppose the observations by Gerdes *et al.* (1983). It is possible that exogenous administration of T4 compared to T3 may exert different effects on gene expression. Thyroid hormone effects on cardiomyocyte proliferation may be concentration-dependent, which is further complicated by the local conversion of T4 to T3 in downstream tissues.

As noted above, the effects of exogenous thyroid hormone administration on cardiomyocyte proliferation are unclear. In an effort to clarify the role of endogenous thyroid hormone signaling during mammalian cardiomyocyte postnatal development, our lab has analyzed cardiomyocyte cell cycle activity in mice expressing cardiomyocyte-specific dominant-negative thyroid hormone receptor alpha (Thra). In this genetic model, other functions of Thra including gene repression and non-genomic actions are not inhibited in this mutant. Genetic inhibition of thyroid hormone signaling increased cardiomyocyte cell cycle activity by five to six-fold and increased the abundance of diploid cardiomyocytes by three-fold at P14 (Hirose *et al.* 2019). In these animals, we quantified cardiomyocyte numbers by design-based stereology and observed a two-fold increase in cardiomyocyte numbers. Together, these results are consistent with an endogenous role of thyroid hormones to promote cardiomyocyte cell cycle arrest, polyploidization, and loss of cardiac regenerative capacity.

Molecular regulators of cardiomyocyte proliferation

The decrease in proliferation associated with thyroid hormone may be partially explained by the modulation of cyclin expression. It was previously reported that nuclear localization of the cyclin D1-CDK4 complex was associated with rat neonatal cardiomyocyte proliferation. However, proliferation ceased after one or two cell cycles, associated with the accumulation of the CDK inhibitor p27 in the nuclei (Tamamori-Adachi *et al.* 2004). Burton *et al.* suggested that cardiomyocyte cell cycle exit was associated with increasing levels of p27 and p18 in the adult heart and determined T3 necessary for p18 expression *in vitro* (Burton *et al.* 1999). Following proliferation analysis after T3 treatment in cultured cardiomyocytes, Western blot analysis of ovine cardiomyocyte lysates showed that cyclin D1, which had been previously associated with cell cycle progression, was decreased two-fold in T3-treated cells, while p21 levels, involved in cell cycle arrest, were increased three-fold in T3-treated cells (Chattergoon *et al.* 2012, 2007, Pasumarthi & Field 2002). In contrast, in accordance to the effect of thyroid hormone on cardiomyocyte proliferation suggested by Ledda-Columbano *et al.,* cyclin D1 mRNA was increased in T3-treated cells and protein expression was present both in cytosol and nuclei, whereas in control cells, cyclin D1 protein was present only in the cytosol. This suggests that cyclin D1 accumulation in the nucleus might be responsible for increased proliferation. However, due to these opposing findings, further research is necessary to define the role of thyroid hormone in cardiomyocyte proliferation through the modulation of cyclins.

During adolescence, mammalian heart size increases as a result of cell hypertrophy. Cardiomyocyte size and protein synthesis are used as indexes of terminal differentiation and permanent cell cycle exit, both associated with the loss of cardiac regeneration. Elevated T3 levels increase the maturation rate of fetal ovine cardiomyocytes, which is accompanied by 14% increase in the width of binucleated cells compared to control (Chattergoon *et al.* 2012). Synthesis of proteins including PI3K, Akt, and mTOR, known to be involved in cardiomyocyte hypertrophy both *in vivo* and *in vitro,* was also increased (Kuzman *et al.* 2005). A study by Kinugawa *et al.* showed that both thyroid hormone and overexpression of THRA without thyroid hormone treatment promoted cardiomyocyte hypertrophy, indicated by ~75% increase in protein synthesis (Kinugawa *et al.* 2005). Induced cardiomyocyte hypertrophy either by thyroid hormone treatment or thyroid hormone receptor overexpression was inhibited with preincubation with SB201290, a p38 inhibitor. p38 is a subfamily of mitogen-activated protein (MAP) kinases, shown to play a role in mammalian cell signaling induced by stress (Liao *et al.* 2001). p38α is the best characterized of the four members of this subfamily of kinases (Thornton & Rincon 2009). In mammalian cardiomyocytes, p38α MAPK activity is low during the cardiac hyperplastic fetal period. Its activity increases during the neonatal period and is maintained during the adult stage, when heart growth is mainly due to cardiomyocyte hypertrophy. In embryonic zebrafish, p38α activation resulted in decreased cardiogenesis due to cardiomyocyte proliferation inhibition. In adult zebrafish, the active expression of p38α was shown to inhibit cardiomyocyte proliferation after amputation (Jopling *et al.* 2010). In neonatal rats, p38α MAPK inhibition with SB203580 promoted cell cycle re-entry after apex resection shown by BrdU incorporation (Hertig *et al.* 2019). In fetal rat cardiomyocytes, activation of p38α by MKK3bE reduced proliferation, indicated by a 17.6% decrease in BrdU incorporation. Moreover, cardiomyocyte-specific p38 knockout resulted in increased neonatal cell mitosis by ~92.3% (Engel 2005). Inhibition of p38 resulted in increased expression of cell cycle

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regulators including cyclin A2, which is normally silenced in postnatal cardiomyocytes and had been previously associated with increased cardiomyocyte proliferation when expressed constitutively from the embryonic stages into adulthood (Chaudhry *et al.* 2004). Thyroid hormone may inhibit cardiac regeneration via the p38 MAPK pathway, as indicated by the demonstrated role of p38 in cardiomyocyte proliferation blockage, and the activation of this signaling pathway induced by thyroid hormone or overexpression of TR α_1 in primary rat cardiomyocyte cultures (Kinugawa *et al.* 2005).

In an effort to identify new pathways interacting with thyroid hormone signaling to regulate cardiomyocyte proliferation, we examined the roles of glucocorticoid and vitamin D receptor signaling. We observed that activation of these pathways can inhibit mammalian cardiomyocyte proliferation *in vitro*, while genetic inhibition of these pathways was not sufficient to significantly alter cardiomyocyte proliferation *in vivo*. Furthermore, combined inhibition of these pathways in a thyroid hormone mutant background did not affect cardiomyocyte proliferation beyond that observed with thyroid hormone signaling inhibition signaling alone (Cutie *et al.* 2020). These data suggest that thyroid hormones, glucocorticoids, and vitamin D likely regulate cardiomyocyte proliferation through distinct mechanisms.

Metabolism and heart regenerative potential

Post-birth mammalian cardiomyocytes undergo maturational processes that include metabolic changes. During early development, cardiomyocytes obtain energy primarily from glycolysis. Alongside maturation and terminal differentiation, there is a metabolic switch from glycolysis to fatty acid β-oxidation as well as an increase in mitochondrial oxidative capacity. Recent studies have revealed an association between glycolytic metabolism maintenance and the prolongation of the proliferative state in developing cardiomyocytes (Chung *et al.* 2007, Lopaschuk & Jaswal 2010). It has been suggested that the transcriptional cofactor Yes-associated protein (YAP), a key component in the Hippo signaling pathway, is involved in glucose metabolism during organ development (Cox *et al.* 2018, Zheng *et al.* 2017). YAP expression is higher in fetal and postnatal murine hearts compared to adults, which correlates with the loss of cardiomyocyte proliferation (von Gise *et al.* 2012). Overexpression of YAP has been found to increase cardiomyocyte proliferation and cardiac muscle contraction after injury in adult mice (Xin *et al.* 2013*a*). As this metabolic switch correlates with the loss of heart regenerative capacity during early development, the role of metabolism in heart regeneration received recent interest.

ROS and DNA damage

As described above, ROS are byproducts of various cellular processes including mitochondrial metabolism (Forrester *et al.* 2018). ROS have been demonstrated to induce DNA damage response (DDR) signaling pathways that can in turn cause cell cycle arrest (Puente *et al.* 2014, Tao *et al.* 2016, Nakada *et al.* 2017). The activation of the DDR signaling pathway as a result of increased ROS production was shown to promote the production of p53 and p21, both of which had been associated with inhibition of cell cycle progression (Lakin & Jackson 1999). Puente *et al.* demonstrated that ROS, oxidative DNA damage, and DDR pathways all increase in the heart during the first-week post-birth and promote hypertrophic growth. Inactivation of *Tfam*, a gene essential for mitochondrial function, leads to increased ROS production and activation of the DDR signaling pathway due to the impaired function of the electron transport chain (Kang *et al.* 2007, Vernochet *et al.* 2012). *Tfam* ablation in fetal mice resulted in cardiac hypoplasia and reduced cardiomyocyte proliferation (Zhang *et al.* 2018). In contrast, ROS scavenging with N-acetylcysteine (Halasi *et al.* 2013) and DDR inhibition enhanced postnatal cardiomyocyte proliferation and decreased binucleation (Puente *et al.* 2014). Mice deficient in the Pitx2 gene, which activates ROS scavengers (Tao *et al.* 2016), failed to regenerate after resection and exhibited increased scarring and reduced function, whereas Pitx2-gain-of-function mice demonstrated regenerative capacities.

Fetal cardiomyocytes are protected from ROS and oxidative damage due to the low oxygen level condition of the intrauterine environment, suggesting oxygen levels a regulator of cardiomyocyte proliferation (Puente *et al.* 2014). A 2-week exposure to hypoxic conditions (~7%) resulted in heart growth, cell proliferation, and decreased fibrotic tissue after induction of myocardial infarction (Nakada *et al.* 2017). Moreover, using a Cre–Lox-based fate-mapping system, Kimura *et al.* identified a population of hypoxic cardiomyocytes expressing Hif-1a (Semenza 2010), which exhibit characteristics of undifferentiated cardiomyocytes such as smaller size, mononucleation, and lower DNA damage. This cardiomyocyte population was found to contribute to cardiomyocyte proliferation in the adult heart (Kimura *et al.* 2015). Mitochondrial maturation may also play a role in the general inability

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of adult hearts to regenerate. Honkoop *et al.* discovered reduction in mitochondrial gene expression and activity in immature cardiomyocytes, which retain their proliferative capacities. In these cells, mitochondria appeared to be immature due to their morphology and reduced cristae (Honkoop *et al.* 2019).

Glycolysis and fatty acid β-oxidation

Zebrafish has been used as a model to study the role of the metabolic switch from glycolysis to fatty acid oxidation in heart regeneration. Neuregulins belong to a family of ligands that regulate glucose and lipid metabolism (Zhang *et al.* 2018). After injury in the zebrafish heart, neuregulin 1 (Nrg1) expression activates ErbB2, which promotes cardiomyocyte dedifferentiation and re-entry into the cell cycle. Researchers showed that activation of the Nrg1 signaling pathway by overexpression of a constitutively active ErbB2 receptor upregulated gene expression involved in glycolysis and shifted metabolism from oxidative phosphorylation to glycolysis. Aharonov *et al.* showed that ErbB2 influence on cardiomyocyte dedifferentiation and proliferation was mediated via the downstream activation of YAP, a component of the Hippo pathway, widely associated with cardiac regeneration (Xin *et al.* 2013*a*, Aharonov *et al.* 2020). Moreover, glycolysis inhibition in adult zebrafish with the glucose analog 2-deoxyglucose resulted in impaired proliferation, as indicated by quantification of double Mef2c/PCNA-positive cells (Honkoop *et al.* 2019). Cao *et al.* uncovered that inhibition of fatty acid β-oxidation with etomoxir (ETO), a carnitine palmitoyltransferase I (CPT1) (Lopaschuk & Jaswal 2010), increased proliferation in cultured neonatal mouse cardiomyocytes as demonstrated by Ki67 and Aurora B kinase (Cao *et al.* 2019). *In vivo*, ETO-treatment promoted cardiomyocyte cell cycle activity as shown by EdU incorporation and decreased binucleation. Additionally, GW7647-mediated PPARα activation to promote fatty acid β-oxidation resulted in a ~26% increase in binucleated cardiomyocytes, cell size, and decreased expression of fetal gene programs, all of which are indicative of enhanced cardiomyocyte terminal differentiation. In support of these results, we have observed that partial depletion of *Cpt2,* an enzyme essential for mitochondrial β-oxidation of long-chain fatty acids (Ceccarelli *et al.* 2011), increases cardiomyocyte cell cycle entry (Hirose *et al.* 2019). Taken together, these studies suggest that preserving glycolysis and delaying the metabolic switch to mitochondrial fatty acid β-oxidation increases cardiomyocyte proliferation.

Thermogenesis and heart regenerative potential

Using the abundance of mononucleated diploid cardiomyocytes as a proxy of heart regenerative capacity, we surveyed cardiomyocyte ploidy across phylogeny (Hirose *et al.* 2019). We observed that animal basal metabolic rate is inversely correlated with mononucleated diploid cardiomyocyte abundance. Furthermore, using a genetic loss-of-function mouse model to abrogate thyroid hormone signaling after birth, we showed that thyroid hormones play an autonomous role in driving cardiomyocyte cell cycle arrest, binucleation, and loss of heart regenerative potential (Hirose *et al.* 2019). Due to the established role of thyroid hormones as regulators of mammalian thermogenesis, we have recently investigated the interrelationship between postnatal endothermy and heart regenerative capacity.

Newborn mice are born poikilothermic and gradually develop the ability to self-regulate body temperatures after birth. Recent data from our lab demonstrate that pharmacological inhibition of thyroid hormone signaling with propylthiouracil prevents the acquisition of homeothermy. This is further repressed when combined with adrenergic receptor inhibition, suggesting pathway interactions between thyroid and adrenergic receptor signaling (Payumo *et al.* 2021). Importantly, combinatorial blockade of thyroid hormone and adrenergic signaling after birth dramatically increased cardiomyocyte cell cycle entry by 8-fold, abundance of diploid mononucleated cardiomyocytes by 11-fold and enabled a robust cardiac regenerative response to myocardial infarction in P14 mice.

Conclusions

While newborn mammals possess robust capacities to regenerate their hearts after injury, this ability is lost shortly after birth. A growing body of literature suggests that metabolic changes occurring during postnatal development likely impact the ability of the mammalian heart to regenerate after injury. Classical thyroidectomy experiments support a role of thyroid hormones in driving animal metabolism and thermogenesis, particularly in placental mammals, through brown fat-mediated mediated mechanisms and regulation of mitochondrial function. Furthermore, direct roles for thyroid hormones in controlling cardiomyocyte proliferation, ploidy, and heart regenerative capacity have been discovered. Therefore, thyroid hormones are a likely candidate linking animal metabolism and heart regenerative potential (Fig. 3).

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While inhibition of thyroid hormone signaling during perinatal development may preserve diploid mononucleated cardiomyocytes that retain regenerative potential, it remains unclear if manipulation of thyroid hormone signaling in adult mammals can improve cardiac regenerative capacity. The feasibility of this approach will depend on whether or not thyroid hormone-driven cardiomyocyte maturation, cell cycle exit, and polyploidization are reversible by acute thyroid hormone depletion at later stages in development. It may be possible that thyroid hormones initiate the maturation process and other mechanisms act to reinforce it. Further research is needed to determine a possible cross-talk between thyroid hormones and other signaling pathways involved in cell proliferation such as the Hippo-YAP pathway in cardiomyocytes. Regardless, understanding if distinct aspects of cardiomyocyte maturation represent barriers to cardiomyocyte proliferation will be an important area for future research.

We recently presented an evolutionary model suggesting that the loss of mammalian cardiac regenerative potential may be a tradeoff for thyroid hormone-dependent thermogenesis and the development of endothermy (Hirose *et al.* 2019). Circulating thyroid hormone levels increase after birth to drive thermogenesis and the acquisition of endothermy, which is beneficial to animal survival. However, the levels of thyroid hormones necessary for this adaptation inhibit cardiomyocyte cell cycle activity and promote cell cycle arrest. Therefore, the lack of cardiac regenerative capacity in mammals could have co-evolved with other beneficial traits to ensure species success. Within this framework, it will be of key importance to identify other physiological pathways regulating the logic of regenerative diversity across the animal kingdom, understand why this capacity is limited in adult mammals, and reveal new mechanisms that may be therapeutically manipulated to regenerate the adult mammalian heart.

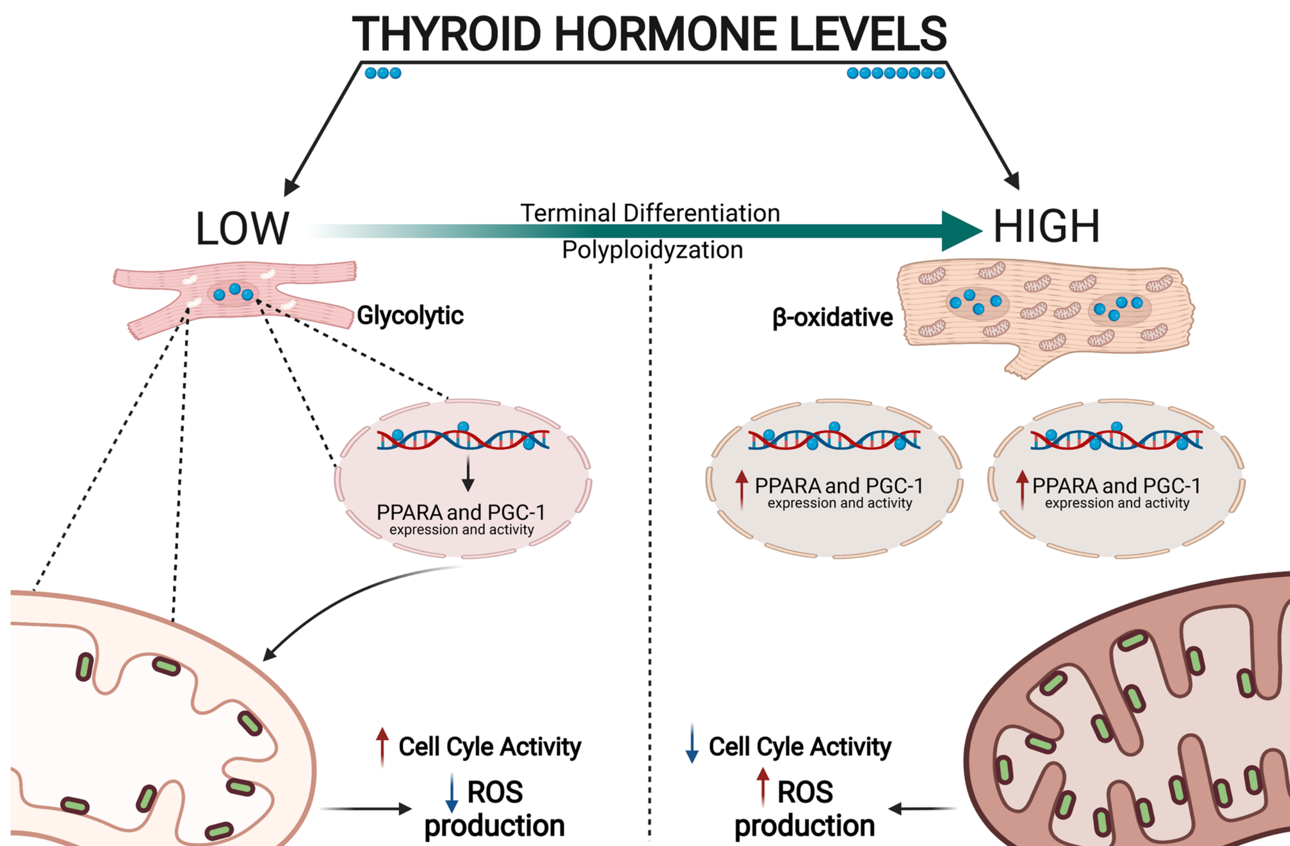

Figure 3
Thyroid hormone drives mitochondrial biogenesis and increases ROS generation, which may limit cardiomyocyte proliferation and regeneration. Thyroid hormone upregulates PPARA and PGC-1 resulting in mitochondrial biogenesis and increased levels of cellular ROS production, which may promote cardiomyocyte polyploidization, cell cycle arrest, terminal differentiation, and the loss of heart regenerative capacity.

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Declaration of interest

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

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Author contribution statement

I R and A Y P conceptualized the manuscript. I R, D B O, G N H, and A Y P wrote and edited the manuscript.

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