THEMATIC REVIEW

Thyroid hormone signaling and consequences for cardiac development

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Abstract

The fetal heart undergoes its own growth and maturation stages all while supplying blood and nutrients to the growing fetus and its organs. Immature contractile cardiomyocytes proliferate to rapidly increase and establish cardiomyocyte endowment in the perinatal period. Maturation changes in cellular maturation, size and biochemical capabilities occur, and require, a changing hormonal environment as the fetus prepares itself for the transition to extrauterine life. Thyroid hormone has long been known to be important for neuronal development, but also for fetal size and survival. Fetal circulating 3,5,3'-triiodothyronine (T3) levels surge near term in mammals and are responsible for maturation of several organ systems, including the heart. Growth factors like insulin-like growth factor-1 stimulate proliferation of fetal cardiomyocytes, while thyroid hormone has been shown to inhibit proliferation and drive maturation of the cells. Several cell signaling pathways appear to be involved in this complicated and coordinated process. The aim of this review was to discuss the foundational studies of thyroid hormone physiology and the mechanisms responsible for its actions as we speculate on potential fetal programming effects for cardiovascular health.

Key Words

- ▶ fetal programming
- ▶ thyroid hormone
- ▶ cardiomyocyte maturation
- ▶ outcomes

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Introduction

Over the past 25 years, the many roles that thyroid hormone (TH) plays in regulating fetal development have come to light. Because maternal plasma thyroid hormone levels influence fetal levels, abnormal maternal thyroid hormone levels can lead to abnormal development of the brain, heart, lung and pancreas in the fetus (Oppenheimer & Dillmann 1978, Lorijn *et al.* 1980, Polk *et al.* 1995, Chan *et al.* 1998, Zimmerman 1999, Abalovich *et al.* 2002, Calvo *et al.* 2002, van Tuyl *et al.* 2004, Casey *et al.* 2007, Harris *et al.* 2017). The purpose of this review is to summarize the role of thyroid hormone in regulating the growth and maturation of the fetal myocardium and to highlight

features of thyroid hormone function that likely impart elevated susceptibility for adult-onset disease.

David Barker, his colleagues, and now many others have shown that low term birthweight is a significant risk factor for cardiovascular disease (CVD) in adulthood (Barker *et al.* 1989, Barker 1995). We now know the importance of the prenatal environment in determining risk for CVD throughout life via a process known as fetal programming (Barker & Osmond 1988, Gluckman *et al.* 2008). There is increasing evidence that fetal stressors, including poor nutrition and hypoxia, lead to detrimental changes in the heart and its blood vessels and elevated disease risk for life.

The capacity of cardiomyocytes to proliferate over the life course has been controversial, in part because of fraudulent or misleading research (Beltrami *et al.* 2003, Anversa *et al.* 2006), which reported that stem cells were able to regenerate cardiomyocytes in the adult heart of humans and rodents and repair regions of damage. In sheep, cardiomyocytes go through a maturation phase during the perinatal period whereupon cell division is slowed significantly. Jonker *et al.* showed myocyte number is reduced in both ventricles immediately before term, but proliferation increases myocyte number in the neonatal right ventricle (Jonker *et al.* 2015). There is evidence that human cardiomyocytes are able to divide very slowly over most of the adult lifespan in order to replace low levels of cellular loss (Bergmann *et al.* 2009). Bergmann's study reports that fewer than 50% of all cardiomyocytes are slowly exchanged over a normal life span with a gradual decrease from 1% turning over annually at the age of 25 to 0.45% at the age of 75 years. Studies of proliferation dynamics show that the number of heart cells is constant from early postnatal life into old age, when cell dropout begins to occur. Thus, cardiomyocyte proliferation during the prenatal period is important in determining the lifelong endowment of the myocardium. If the generative capacity of the myocardium is lost before it acquires an optimum number of cardiomyocytes, the heart could be vulnerable for coronary artery disease or heart failure in later life (Thornburg *et al.* 2011). A low cardiomyocyte endowment is especially important because cardiomyocyte loss is a hallmark of heart failure. CVD is the number 1 cause of death worldwide with a projected medical cost of \$1.1 trillion by 2035 in the recent American Heart Association report and heart failure represents a significant portion of that cost (American Heart Association 2017).

Low birth weight is associated with an increased risk for coronary artery disease (Barker *et al.* 1989, Rich-Edwards *et al.* 2005) as well as type 2 diabetes (Barker 1999a,b), hypertension (Barker & Osmond 1988), hypercholesterolemia (Barker *et al.* 1993) and hypercoagulopathies (Fall *et al.* 1995, Martyn *et al.* 1995). Babies that experienced intrauterine growth restriction (IUGR) are prone to have hearts that suffer detrimental changes over the lifespan. Hearts of 5-year olds who experienced IUGR had changes in cardiac shape, reduced stroke volume and increased heart rate (Crispi *et al.* 2010). The hearts were also characterized by abnormal systolic and diastolic functions. The cardiomyocyte population is clearly affected not only through changes in cardiac loading conditions but also by the chemical environment. Fetuses that grow slowly suppress the proliferation of

cardiomyocytes and their rate of maturation. In such cases, the myocardium suffers a cardiomyocyte deficit and is metabolically compromised at birth (Louey *et al.* 2007, Morrison *et al.* 2007).

It is known that maternal thyroid function can influence the growth and maturation of fetal organs (Alemu *et al.* 2016). We have shown that in the ovine fetus, T3 is an important driver of fetal myocardial maturation (Chattergoon *et al.* 2012a). This finding may be important for human fetal development. Up to 10% of all pregnancies are afflicted by some form of thyroid dysfunction (Marx *et al.* 2008, Wang *et al.* 2011, Alemu *et al.* 2016). Treatment is recommended whenever maternal thyroid-stimulating hormone levels are high and free thyroxine (T4) is low (hypothyroidism) or when the opposite is found (hyperthyroidism) (Casey & Leveno 2006). The discovery that subclinical hypothyroidism also leads to intrauterine growth restriction (IUGR), preterm birth and permanent neurological deficits in offspring has given rise to a call by national organizations for screening of all pregnant women to determine plasma thyroid status (Haddow *et al.* 1999, Gharib *et al.* 2005, Casey & Leveno 2006, Casey *et al.* 2006) but such screening is to become routine (Casey 2014).

A few studies suggest the importance of thyroid hormone in fetal programming: (1) small-for-gestational age babies have elevated plasma free 3,5,3'-triiodothyronine (T3) levels postnatally (Radetti *et al.* 2004); (2) ethyl alcohol exposure in the fetal rat depresses thyroid hormone levels in the adult; (3) fetal T4 exposure in rats alters their thyroid hormone levels as adults (Wilcoxon & Redei 2004); (4) small body size at birth predicts hypothyroidism in offspring and adult women (Kajantie *et al.* 2006); (5) thyroid hormones in breast milk may alter the set point for other hormone levels in infants and as adults (Phillips *et al.* 1993) and (6) hypothyroidism in sheep stimulates increases in fetal pancreatic beta cell mass through proliferation (Harris *et al.* 2017). This group of facts suggests that early-life thyroid hormone levels are important for long-term health of offspring.

We should not be surprised that the thyroid system is important in regulating development in mammals. Thyroid hormone was the first morphogen to be discovered when in 1912, J F Gudematsch, a Cornell anatomist, showed that adult thyroid gland extract stimulated the rapid metamorphosis of tadpoles into frogs (Brown & Cai 2007). It is now known that the vast array of tissues that change dramatically as the aquatic tadpole transforms into a terrestrial frog are under the control of thyroid-sensitive genes. The muscles in the

tail of the tadpole are, for example, resorbed within mere hours, while muscles in the limb are stimulated to grow; both processes under the regulation of T3 (Brown & Cai 2007). Mammals do not, of course, develop by metamorphosis as found in anurans. However, many of the thyroid receptors and their binding partners are conserved among vertebrates from fish to mammals (Bertrand *et al.* 2004). Aquatic fetal mammals, like tadpoles, must prepare for postnatal terrestrial life. Thus, it is possible that many other T3-driven developmental processes among fetal mammals are important in the birth transition but remain undiscovered. In this review, we will focus on the role of thyroid hormones on cardiovascular development and potential long-term consequences for postnatal health.

Sheep as a model for cardiovascular developmental studies

The study of the mechanisms that regulate cardiomyocyte growth has required animal models because of the understandable rarity of viable cardiac tissue or blood samples from human fetuses and infants. The fetal sheep model allows for well-tolerated chronic surgical instrumentation, serial blood sampling and continuous measurement of hemodynamic factors. Experimental alterations in the fetal endocrine or hemodynamic environments can be utilized to determine the impact of cardiomyocyte growth and functional cardiac outcomes.

Nevertheless, the interspecies differences among sheep, humans and rodents must be taken into account when interpreting findings. To begin with, placental structure and function are widely variable among members across the mammalian phylogenetic scale. These differences may influence the fetal nutritional, hemodynamic and hormonal milieu in which the heart develops (Jonker *et al.* 2007a, Barry & Anthony 2008, Mu *et al.* 2008). Rodents have litters of up to ten pups or more and their location in the uterine horn influences nutrient delivery and their ultimate size (McLaurin & Mactutus 2015, Rennie *et al.* 2015). In contrast, sheep and humans have few offspring with each pregnancy. The longer gestation of sheep (145 vs 21 days in rats and mice) offers the opportunity to conduct experiments in a similar developmental time frame to compare to humans. Rodent pups are altricial, and thus, very immature at birth. They are hairless, have eyes that are sealed and the final steps of heart maturation occurs after birth, rather than prenatally as for humans and sheep (Soonpaa *et al.* 1996,

Jonker *et al.* 2007b). The fetal thyroid hormone system is similar between humans and sheep.

The ovine thyroid gland begins to secrete T4 around 50 days gestational age (dGA) (Hopkins & Thorburn 1972, Thorburn & Hopkins 1973). In humans, the fetus secretes T4 by 12 weeks gestation (Fisher & Polk 1989, Becks & Burrow 1991) and in rats it is detectable by 13–16 days of gestation (term is 21) (Perez-Castillo *et al.* 1985). Serum T3 concentrations are low in sheep and human fetuses over most of gestation. In the sheep, there is a slow increase in serum T3 concentrations during the last third of gestation. Serum T3 concentrations then increase rapidly during the week immediately preceding parturition (the prenatal T3 surge) as shown in Fig. 1A. The plateau phase coincides with the final developmental window for various organs before birth, including the heart (Fig. 1B). In the human fetus, serum T3 is essentially unmeasurable until about 30-week gestation, after which time serum T3 increases

Figure 1

Circulating triiodothyronine (T3) and reverse T3 (rT3) levels in normal fetal sheep (Polk 1995) (A). The prepartum surge is driven by the prepartum cortisol surge stimulating deiodinase expression and conversion from T4. Cortisol stimulates D1 activity and suppresses D3 activity. Representation of the normal timing and changes in binucleation of fetal sheep left ventricular cardiomyocytes (B). Binucleation is an index of terminal differentiation, after which cells exit the cell cycle and no longer divide. In the fetal sheep heart this process begins just after 100 days gestational age and is ~75% binucleated at the time of birth. The dashed line at 125 days gestational age represents the timing of studies discussed in this review (data from Polk 1995, Jonker *et al.* 2007a,b, Chattergoon *et al.* 2012a,b).

to a mean concentration of about 50ng/dL at term (Fisher 1977). During the first 4–6 h after birth, serum T3 concentrations increase another three- to six-fold (Abuid *et al.* 1973, Fisher *et al.* 1973). Rodents appear to have a T3 surge which occurs after birth (Chanoine *et al.* 1993). The fetal sheep model is a good representation of human development with many organs sharing similar growth trajectories, including the heart (Wu *et al.* 2006). In this review, we primarily focus on data from sheep.

Thyroid hormone in development and its metabolism

There has been a significant body of work pointing to the importance of thyroid hormones in development and postnatal organ function. Prematurity is associated with low circulating thyroid hormone levels (T3 and T4) (Williams *et al.* 2004). The fetus is dependent on maternal transfer of T4 and T3 prior to the fetal gland secreting them, which is particularly important in early development and embryogenesis during the early phases of central nervous system development (Fisher 2008). In most mammals, extrauterine adaptation is supported by a cortisol surge near term mediated by increased cortisol production by the fetal adrenal gland and reduced conversion to cortisone. Cortisol increases deiodinase expression and activity in several fetal tissues (Wu *et al.* 1978). Three deiodinase enzymes, types I, II and III (D1, D2 and D3, respectively), are found in both fetal and adult tissues (Bianco *et al.* 2002, Bianco & Kim 2006) (Fig. 2). Their expression is tissue specific (Brent 2012) and is affected by glucocorticoids (Forhead *et al.* 2006). The activity of thyroid hormone is partially regulated by the removal of iodine moieties from precursor molecules by the iodothyronine deiodinases.

The enzymes are members of dimeric integral membrane thioredoxin family, containing proteins that activate or inactivate thyroid hormone by acting upon the phenolic or the tyrosil rings of the iodothyronines; each deiodinase prefers the ring from which it removes the iodine group (Bianco & Kim 2006, van der Spek *et al.* 2017). Figure 2 illustrates the conversion of the T4 and T3 molecules by the specific deiodinases.

D1 is a 5'-monodeiodinase which activates or inactivates T4 to T3 or reverse T3 (rT3), respectively. It is present in the fetal liver, kidney, thyroid and pituitary glands; the production of T3 by liver D1 is thought to be the main source of circulating T3 (Polk 1995). D2 is also a 5'-deiodinase that generates T3 from T4, though it is primarily found in the brain, pituitary gland, placenta and brown adipose tissue (Polk 1995). D2 conversion to T3 tends to be for that specific organ use rather than contributing to circulating levels. D3 is a 5'-monodeiodinase that inactivates T3 by conversion to T2 and metabolizes T4 to reverse T3 (rT3). D3 is found in liver, kidney, skin, uterus and placenta. It plays an important role in placental clearance of maternal thyroid hormones and protects the fetus by limiting its exposure to maternal thyroid hormones. Fetal thyroid hormone levels and metabolism are characterized by a predominance of D3 and rT3 (Polk *et al.* 1988, Polk 1995). While it is the primary circulating T4 metabolite in development, it has little activity. D1 activity is low through most of gestation therefore circulating T3 levels in the fetus are relatively low. Elevated glucocorticoids reduce D3 and increase D1 activity. In fetal sheep, kidney and liver D1 activities increase and placental D3 activity decreases in the 2 weeks prior to birth (Forhead *et al.* 2006). Forhead *et al.* has shown that those changes were induced by prepartum cortisol surge and that premature maternal administration

Figure 2

Basic deiodinase reactions regulating thyroid hormone metabolism. The reactions catalyzed by the deiodinases remove iodine moieties from the phenolic (outer rings) or tyrosil (inner rings) rings of the iodothyronines. These pathways can activate T4 by transforming it into T3 via D1 or D2 or prevent it from being activated by converting it to the metabolically inactive form, reverse T3 (rT3) via D1 or D3. T2 is an inactive product common to deiodination of both T3 and rT3 and is rapidly metabolized by further deiodination (data from Bianco & Kim 2006, van der Spek *et al.* 2017).

of the synthetic glucocorticoid dexamethasone yielded the same effect (Forhead *et al.* 2006, 2007). This results in overall preferential deiodination of T4 to T3 and reduced clearance of T3 leading to a rise in fetal plasma T3 levels.

Studies by Breall *et al.* in the 1980s showed that, as expected, fetal sheep that were thyroidectomized 2–3 weeks prior to birth or term delivery did not undergo the T3 surge (Breall *et al.* 1984). Forhead *et al.* showed that thyroidectomy of the sheep fetus abolished the prepartum rise in fetal plasma T3 but not the cortisol surge (Forhead *et al.* 2000). They also identified a role for thyroid hormone in controlling growth hormone receptor, insulin-like growth factor 1 and 2 (IGF1 and IGF2) expression in the fetal liver, which is important for the overall somatic growth (Forhead *et al.* 1998, 2000). When thyroidectomized, fetuses also showed reduced heart rate, cardiac output, oxygen consumption and lower blood flow to the peripheral circulation in the first 6 h after delivery (Breall *et al.* 1984). These foundational studies indicate that plasma thyroid hormone levels in the 2–3 weeks prior to delivery, and not just the prepartum and postnatal increase, are important for cardiovascular and metabolic adjustments required to prepare the fetus for extrauterine life.

Cardiomyocyte proliferation and maturation

The question of what determines the numbers of cells in a mature organ has not been answered. Some organs have regenerative powers, like liver, but most have little. However, in spite of reports on the presence of stem-like cells that can be recruited or that are resident in the myocardium, the heart cannot adequately replace cardiomyocytes when it really counts, following an infarction or in heart failure when cardiomyocytes become ineffective and are dying.

The developing heart is continuously growing to meet the hemodynamic and metabolic needs of the growing fetus, while cardiomyocytes continue through their own growth and maturation processes. During the first

two-thirds of gestation the ovine heart grows by cardiomyocyte division before the majority of them leave the cell cycle permanently in late gestation. During this high proliferative stage cardiomyocytes are mononucleated. Figure 1B shows that an increasing number of fetal ovine cardiomyocytes transition into terminal differentiation in the last third of gestation. This process begins around 110 days gestational age (dGA) and continues to birth (~145 dGA) (Burrell *et al.* 2003, Jonker *et al.* 2007b). This process is marked by karyokinesis, the formation of a second nucleus, without cell division; the resulting binucleated cardiomyocyte permanently exits the cell cycle (G_0). At birth, some 75% of ovine cardiomyocytes have become binucleated and can no longer divide (Jonker *et al.* 2007b). The mechanisms that underlie the normal transition of fetal cardiomyocyte growth from a predominantly hyperplastic to a hypertrophic mode are not known. The maturation process results in the gradual replacement of the mononucleated population by a binucleated cell population that can grow only by hypertrophy (increase in cell size). Terminal differentiation is about 75% complete at term (Fig. 3) in the sheep (Jonker *et al.* 2015, Jonker & Louey 2016). In mice and rats, the terminal differentiation process occurs in the newborn and is complete by 2 weeks of life (Clubb & Bishop 1984, Cluzeaut & Maurer-Schultze 1986, Li *et al.* 1996). Less is known about the maturation process in the human. The human heart has a higher population of mononucleated cardiomyocytes but most of them are polyploid and appear to be terminally differentiated. In addition, the human myocardium also has binucleated cardiomyocytes (Schmid & Pfitzer 1985, Ahuja *et al.* 2007), but only to ~8–14% at birth (Rakusan 1984), which increases up to 33% in early childhood. However, because humans are born in a more mature state than are altricial rats and mice, the human heart growth patterns are more closely related to the relative growth and maturational timing of the sheep compared to rodents.

Several stressors *in utero* can reduce cardiomyocyte numbers for life (Li *et al.* 2003, Corstius *et al.* 2005). As mentioned earlier, having a reduced cardiomyocyte

Figure 3

Cardiomyocyte growth in the fetal heart. Mononucleated cardiomyocytes are capable of proliferation but can also become binucleated. Binucleated cardiomyocytes are unable to undergo further cytokinesis. Both mononucleated and binucleated cardiomyocytes can undergo cellular enlargement (length or width changes) during hypertrophic growth.

endowment puts an individual at greater risk for CVD and impaired recovery from an event like myocardial infarction. The terminal differentiation process, as evidenced by binucleation rates begins almost 30 days prior to the prepartum T3 surge. T3 becomes detectable at about 110 dGA (Fraser & Liggins 1988), suggesting that T3 is an important contributor to the maturation process. The effect of T3 on proliferation of isolated cells from fetal sheep hearts has been studied at two developmental ages that represent 100% mononucleated cardiomyocytes (100 dGA) and ~50% mononucleated (135 dGA; ~10 days prior to birth). The proliferation rates are different between the two ages with the younger cells having a 10–12% rate over 24 h of 5-bromo-2'-deoxyuridine (BrdU) incorporation compared to 1–2% in 135 dGA cells. We found that T3 inhibits cardiomyocyte proliferation when in the presence of a physiological growth stimulus like IGF1. IGF1 is an important stimulant of cardiomyocyte growth throughout gestation.

We reasoned that immature cardiomyocytes that are highly proliferative, as at 100 dGA and earlier, would be necessarily immune to the inhibitory effects of T3. We hypothesized that the viability of the fetus might be at stake if heart cells could not divide rapidly at a time when the cardiomyocyte endowment is being established for life. However, we proved our hypothesis wrong. We found that T3 has a stronger inhibitory effect on 100-day cardiomyocytes than in near term cells (Chattergoon *et al.* 2012b). This was an unexpected finding. The data suggest that maternal, and therefore, fetal hyperthyroidism would leave the fetal heart with far fewer cardiomyocytes than required for optimal function over the life course.

The effect of T3 on fetal cardiomyocyte proliferation was tested *in vivo*. T3 (54 µg/day) was infused into the circulation of fetal sheep from 125 to 130 dGA when T3 levels are very low. This is about 15 days prior to the normal T3 surge that occurs near the end of gestation. Hearts from these fetuses were not bigger or smaller

than hearts from control fetuses or when normalized to body weight. However, cardiomyocyte proliferation was reduced, as indicated by lower Ki-67 expression, and the cell cycle inhibitor p21 was elevated (Fig. 4). Binucleation rates were increased under the influence of T3 and cells were enlarged, particularly with increased width (Fig. 5). In addition, several genes related to hypertrophy and maturation were increased. For example, mammalian target of rapamycin (mTOR), atrial natriuretic peptide and sarcoplasmic reticulum Ca²⁺-ATPase 2A (SERCA2A) were increased. This is consistent with our *in vitro* studies showing that p21 protein levels are elevated and the cell cycle promoter, cyclin D1 levels were decreased after 24 h of T3 treatment over a range of doses (Chattergoon *et al.* 2007, 2012b).

To determine whether some basal level of T3 was required to support cardiomyocyte growth and maturation, we thyroidectomized (TX) a group of fetuses. Thyroidectomized fetuses showed lower rates of proliferation of cardiomyocytes and binucleation (Figs 4 and 5) as well as reduced cardiac mass when corrected for body weight (Chattergoon *et al.* 2012a). The characteristics are similar to a heart that has been grown under the condition of placental insufficiency where it is nutrient deprived (Louey *et al.* 2007). Interestingly, cardiomyocytes from TX fetuses were enlarged (Fig. 5). We speculate that under TX conditions, cardiomyocytes responded to wall thinning because of the cardiomyocyte deficit which led to elevated wall stress and a hypertrophic stimulus. Interestingly, we also noted a reduced estimated cardiomyocyte number in hearts from both TX and T3-infused (Fig. 6) (Thornburg 2015). The mechanism of this reduction is different between the two groups where T3 is required for normal growth as reflected by reduced cardiac mass in the TX group. These and findings of others showed that the loss of T3 support results in reduced glucose transport genes, contractile protein expression, hypertrophic genes and calcium handling genes

Figure 4

Cell cycle activity, measured by Ki-67, decreased in both T3-infused and TX fetuses (A). Mitotic activity of myocytes, measured by phospho-histone 3 expression, was decreased in both T3-infused and TX fetuses (B). T3 also elevated p21 protein levels, inhibiting the cell cycle (C). Bars that do not share letters are significantly different ($P < 0.05$); $n = 8$. Data are mean $\pm$ S.E.M. Reproduced, with permission, from Chattergoon NN, Giraud GD, Louey S, Stork P, Fowden AL & Thornburg KL; 2012; Thyroid hormone drives fetal cardiomyocyte maturation; *FASEB Journal*; volume 26, pages 397–408.

Figure 5

Increased circulating T3 promotes terminal differentiation of fetal sheep cardiomyocytes. Elevated T3 concentration increased the portion of LV myocytes that were binucleated (A) and caused myocytes to be wider (B; hypertrophy). Cardiomyocytes from TX fetuses were also wider to maintain wall stress and cardiac function. Bars that do not share letters are significantly different ($P < 0.05$); $n = 8$. Data are mean $\pm$ S.E.M. Reproduced, with permission, from Chattergoon NN, Giraud GD, Louey S, Stork P, Fowden AL & Thornburg KL; 2012; Thyroid hormone drives fetal cardiomyocyte maturation; *FASEB Journal*; volume 26, pages 397–408.

(Mai *et al.* 2004, van Tuyl *et al.* 2004, Chattergoon *et al.* 2012a, Segar *et al.* 2013), which contribute to normal cardiac growth and function. T3 stimulates pathways of terminal differentiation which inhibits cardiomyocyte proliferation. The evidence suggests that fetal thyroid hormone concentrations must be kept within a narrow window of concentration, not too high and not too low, to produce a normal heart.

Signaling pathways

Extracellular control of cell cycle progression is achieved in all cells by the activation of extracellular signal-regulated kinase (ERK) (Wilkinson & Millar 2000,

Figure 6

Elevated or loss of circulating T3 levels significantly reduced cardiomyocyte number and overall endowment, though the mechanism of inhibition of proliferation is different between the two. T3 is needed to support cellular growth and to stimulate the pathways of maturation and terminal differentiation. Bars that do not share letters are significantly different ($P < 0.05$); $n = 8$. Data are mean $\pm$ S.E.M. Reproduced, with permission, from Thornburg KL; 2015; The programming of cardiovascular disease; *Journal of Developmental Origins of Health and Disease*; volume 6, pages 366–376.

Chang & Karin 2001). In particular, ERK stimulates production of cyclin D1 (Graves *et al.* 2000) and facilitates the formation of a cyclin D1–Cdk4 complex to increase the transcription of growth-promoting genes to trigger the G₁/S transition of the cell cycle. T3 is known to stimulate the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and AKT/PKB pathways (Hu *et al.* 2003, Sundgren *et al.* 2003a,b, Kuzman *et al.* 2005b, Chattergoon *et al.* 2014). ERKs are one member of the MAPK family that are associated with cardiomyocyte proliferation in the fetal heart. When the ERK signaling cascade is inhibited, proliferation stimulators like IGF-1 for example are unable to promote cell division. Furthermore, AKT signaling is required for IGF-1 to stimulate proliferation in fetal sheep cardiomyocytes (Sundgren *et al.* 2003a, Chattergoon *et al.* 2014).

AKT signaling and cardiomyocyte survival

Barreto-Chaves' group has shown that normal T3 status is important to maintain appropriate angiotensin receptor levels in the immature rodent heart (Diniz *et al.* 2009). They also showed that the angiotensin type 1 receptor (AT1R) mediates thyroid hormone-induced cardiomyocyte hypertrophy through the AKT/glycogen synthase kinase-3 β /mammalian target of rapamycin (AKT/GSK-3 β /mTOR) pathways in primary cultures of neonatal rat (1–3 days old) cardiomyocytes. Gerdes' research group has studied the role of T3 in neonatal rat cardiomyocyte signaling and survival (Kuzman *et al.* 2005a). Under serum-starved conditions, neonatal cardiomyocytes underwent loss of sarcomeric structure and apoptosis quantified by histology, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, DNA laddering and TUNEL assay (Kuzman *et al.* 2005a). The addition of T3 rescued this effect via the phosphatidylinositol-3 kinase/AKT/GSK-3 β (PI3K/AKT/ GSK-3 β) pathway. It was attenuated by LY294002 (a specific PI3K inhibitor). These studies were carried out in postnatal rodent cardiomyocytes that, as noted above, are in the different growth phase than late-gestation fetal sheep cardiomyocytes. However, they offer evidence that immature cardiomyocytes require T3 to signal through each of several pathways. However, it is not known whether cardiomyocytes from newborn rodents use the same pathways under the same conditions as do prenatal cells in large mammals like sheep and humans because T3 has different effects on fetal sheep cardiomyocytes.

Interaction with IGF1

As discussed earlier, T₃ exerts a strong inhibitory effect on proliferation of ovine cardiomyocytes at the two stages that we have studied most (100 and 135 dGA). We showed that IGF1 stimulated the proliferation rates of 135 dGA sheep cardiomyocytes by nearly four times *in vitro*, which caused a 30% increase in cardiac mass when administered *in vivo* (Sundgren *et al.* 2003a). When T₃ and IGF1 were given alone in culture, they each stimulated phosphorylation of both ERK and AKT (Fig. 7). In the younger mononucleated cells, the combination of the two (T₃+IGF1) led to the suppression of both BrdU uptake and phosphorylation of ERK and AKT (Chattergoon *et al.* 2014). In other words, while IGF1 would ordinarily stimulate proliferation via ERK and PI3K pathways, when T₃ levels are also elevated, proliferation stops. In older 135 dGA cardiomyocytes with a mixed cell population in terms of maturation, T₃+IGF1 led to suppression of BrdU uptake but a super stimulation of ERK and AKT well above the levels of T₃ or IGF1 alone (Chattergoon *et al.* 2014). This high level of stimulation was an unexpected finding. Upon further investigation, histology revealed that even

the binucleated cells, which can no longer divide, were highly positive for phosphorylated ERK and AKT and thus contributed to the overall signal. This suggests that cell signal patterning changes with maturation so that the signaling outcomes of IGF1 and T₃ stimulation lead to different patterns of phosphorylation and different outcomes than was found at earlier ages.

In the presence of IGF1, T₃ and LY29004 (PI3K inhibitor), phospho-p70 S6K (p-p70 S6K) levels in younger cardiomyocytes were higher than those found under the same conditions with the ERK inhibitor, U0126 ($P < 0.05$). These data are compatible with the hypothesis that p-p70S6K is more tightly linked to the ERK cascade than to the PI3K cascade at this stage of development. In older 135 dGA cardiomyocytes, both inhibitors resulted in a ~50% reduction in the signaling of the alternate pathway suggesting that when cells are treated with T₃ and IGF1 together, both the ERK and AKT pathways are important in bringing about a cellular response. Perhaps, these well-established pathways that were required for cell growth at younger ages may promote hypertrophy or metabolic functions in the more mature cells (Matsui *et al.* 2003, Kehat & Molkentin 2010, Chattergoon *et al.* 2012a).

Figure 7

MAPK and PI3K signaling analysis in 100 dGA (A, B and C) and 135 dGA cardiomyocytes (D, E and F). Western blot analysis shows that IGF1 (1 µg/mL) and T₃ (1.5 nM) each separately leads to the phosphorylation of ERK (A and D), AKT (B and E) and p70^{S6K} (C and F). IGF1 activates phosphorylation these proteins to a greater degree than T₃ in 100 dGA cells, but equally in 135 dGA cells. The combination of T₃ and IGF1 in 100 dGA cardiomyocytes reduces the phosphorylation of each protein to half that of IGF1 alone. The combination of T₃ and IGF1 in 135 dGA cardiomyocytes stimulates further activation of ERK and AKT in a 'super stimulatory way'. Bars that do not share letters are significantly different ($P < 0.05$); $n = 8$. Data are mean $\pm$ s.e.m. Reproduced, with permission, from Chattergoon NN, Louey S, Stork PJ, Giraud GD & Thornburg KL; 2014; Unexpected maturation of PI3K and MAPK-ERK signaling in fetal ovine cardiomyocytes; *American Journal of Physiology: Heart and Circulatory Physiology*; volume 307, pages H1216–H1225.

The data suggest that when physiological levels of T3 are normally low, cells are designed to proliferate under the influence of IGF1, which is predominant throughout development. As the older cardiomyocytes become exposed to both hormones when T3 levels rise, they may require stimulation of both pathways for normal maturation.

As mentioned above, fetal sheep given exogenous T3 for 5 days had increased the phosphorylation of ERK, AKT and mTOR in their cardiomyocytes (Chattergoon *et al.* 2012a). TX did not result in changes in the phosphorylation of these signaling molecules. In a study by the Segar group, a restrictive pulmonary artery band (PAB) was applied in fetuses at the time of thyroidectomy so that the fetuses grew into an enhanced constriction (Olson *et al.* 2008, Segar *et al.* 2013). They noted that markers of cellular proliferation but not apoptosis or expression of growth-related genes were lower in the TX and TX+PAB groups relative to thyroid-intact animals. They concluded that in the late-gestation fetal heart, thyroid hormone is required for adaptive fetal cardiac growth in response to pressure overload.

Potential of FOXO1–p21 interaction

We have identified a role for ERK and AKT signaling that results in the inhibition of the cell cycle and increased levels of p21 and reduced cyclin D1. This occurs when T3 levels are elevated. There is a wide array of possible effectors but we believe that the Forkhead box O (FOXO) protein is a key intermediate. The family includes 1, 3, 4 and 6; FOXO1 is an important factor in cardiac equilibrium

(Puthanveetil *et al.* 2013). It plays an important role in cell cycle arrest, oxidative stress resistance, cell survival, energy metabolism and cell death in the heart (Maiese *et al.* 2009, Puthanveetil *et al.* 2010, 2011). The transcription factor, FOXO1, is an effector of the phosphatidylinositol-3-OH kinase (PI3K)/AKT pathway which can link growth and metabolism in some cells (Vander Heiden *et al.* 2009, Ronnebaum & Patterson 2010, Ward & Thompson 2012) as in Fig. 8. PI3K signaling inhibits FOXO1 through AKT-mediated phosphorylation at Ser256 leading to its nuclear exclusion and inactivation (Salih & Brunet 2008). The nuclear export is trafficked by 14-3-3s, which only binds FOXO in this phosphorylated state (Tzivion *et al.* 2011). Stress signals including 5' AMP-activated protein kinase (AMPK) and ERK phosphorylate FOXO1 at sites different than AKT, disrupt binding to 14-3-3, leading to nuclear import and retention. FOXO1 can then induce the transcription of a variety of downstream target genes that collectively inhibit proliferation and induce cell cycle withdrawal (Huang & Tindall 2007). Direct downstream targets of FOXO1 include the cyclin kinase inhibitors, p21 and p27, which have also been implicated in cardiomyocyte cell cycle withdrawal after birth (Nakamura *et al.* 2000, Seoane *et al.* 2004, Bicknell *et al.* 2007). The role of FOXO1 and their regulation during cardiomyocyte cell cycle withdrawal is not well characterized. T3 stimulates not only the phosphorylation of AKT and ERK (Chattergoon *et al.* 2014) just like IGF1, but also the cell cycle inhibitor, p21. FOXO1 activity could be the discriminatory key to different actions of T3 and IGF1. Although T3 activates AMPK (Irrcher *et al.* 2008), we speculate AMPK phosphorylates FOXO1 at a site that

Figure 8

Proposed mechanism of T3-FOXO1 regulation of cardiomyocyte proliferation. T3 signals by nuclear TRs, p43 in the mitochondria and cytoplasmic TRs. T3 stimulates AKT and ERK just like IGF1, but further stimulates p21. The PI3K and MAPK pathways stimulate FOXO1 differently. FOXO1 activity could be the discriminatory key to different actions of T3 and IGF1. ERK or AMPK phosphorylates FOXO1 at a site that disrupts its nuclear export and results in nuclear retention, leading to p21 transcription. AKT-mediated phosphorylation of FOXO1 at Ser256 leads to its expulsion from the nucleus allowing proliferation to continue. The mechanism by which T3 prevents activated FOXO1 activity is unclear.

disrupts its nuclear export and allows T3 to arrest the cell cycle through p21. The specific mechanism by which T3 inhibits FOXO1 inactivation are not clear but one might be through sirtuins. Sirtuins are deacetylases and T3 has been shown to activate it through thyroid receptor β (TR β) binding; one of the complex's targets is FOXO1 (Singh *et al.* 2013).

Thyroid hormone receptors and actions

Research into the signaling biology of thyroid hormone is a dynamic area, at present, because of newly discovered signaling pathways through which TH molecules stimulate cellular actions. The so-called classical or 'genomic' pathways involve TH binding specific nuclear thyroid receptors (TR), which affect gene expression via thyroid hormone response elements (TRE). Thus, thyroid hormone receptors behave as ligand regulated transcription factors. In most species two thyroid receptors and their isoforms are expressed by separate genes that bind T3 and thyroxine (T4) (THRA (TR α) and THRB (TR β)) (Schueler *et al.* 1990, Yen 2001, Mai *et al.* 2004, Kahaly & Dillmann 2005). There is a general view that most of the cardiac effects of T3 are mediated via TR α , while most actions in liver and other tissues are via TR β in multiple species including humans and rodents (Yen 2001). The affinity of T4 for TR α 1 and TR β 1 in fetal myocardium and other organs (Mai *et al.* 2004, Kahaly & Dillmann 2005, Kinugawa *et al.* 2005), is some 10–15 times less than those for T3 (Chopra *et al.* 1978). The fetal sheep heart expresses both receptor types (Macchia *et al.* 2001, White *et al.* 2001) with TR α 1 being the predominant isoform which increases over gestation and more so in adulthood (Chattergoon *et al.* 2019). Cytoplasmic thyroid hormone receptors (rat) may also have signaling properties (Moriscot *et al.* 1997, Mai *et al.* 2004, Makino *et al.* 2009). Of the TH alpha isoforms, only TR α 1, the predominant isoform expressed in the adult heart, is known to bind T3. However, the other isoforms, α 2 and shortened cousins, $\Delta\alpha$ 1 and $\Delta\alpha$ 2 may have other physiological actions, mostly inhibitory. All of the beta TH isoforms, β 1, β 2 and β 3 (rat), bind T3; $\Delta\beta$ 3 is a potent TH repressor *in vitro* (Belke *et al.* 2007). It is clear that unliganded TH can bind DNA and suppress gene expression but regulation of such action is unknown.

The functional characteristics of the TR isoforms have been identified by phenotype analysis in mutant mice. Over a dozen mutant strains have been developed (Gloss *et al.* 2001, Flamant & Samarut 2003) and show that loss of TR α 1 and TR α 2 led to reduced heart rate and decreased pacemaker current gene expression,

while loss of TR β 1 led to increased heart rate. Mutation studies show that TR α 1 is necessary during pre-weaning postnatal life for survival. Mouse thyroid hormone receptors alpha- and beta-knockout experiments demonstrate receptor-mediated effects of T3 (Macchia *et al.* 2001). Cardiac contractile performance is reduced by direct action on the cardiomyocyte and electrical activity is suppressed via decreased ion channel activity. The sodium–calcium exchanger, the L/T type calcium channels, the ryanodine receptor and the sarcoplasmic reticulum calcium ATPase (SERCA2) are all targets of T3 action via the receptor system. In addition, the hormone influences the expression and contractile state of the contractile proteins in striated muscle (Sayen *et al.* 1992). Makino *et al.* showed that TR β has been shown to be required for angiogenesis during cardiac development and that administration of physiological concentrations of T3 upregulates RNA and protein expression of TR β and VEGFR2 in the mouse heart (Makino *et al.* 2009).

TRs partner with retinoid X receptors and perhaps other receptors to form heterodimers before binding DNA targets sites on promoters of target genes (Glass 1994). If they are 'unliganded', they adopt the aporeceptor conformation, or conversely, when the ligand-binding domain is occupied by T3, they adopt holoreceptor conformation (Chassande 2003). These two conformations are in equilibrium under physiological conditions. The unliganded receptors also bind T3 regulatory elements and appear to suppress gene action. Thus, unlike most other types of nuclear receptor systems, TR-sensitive gene expression ranges from stimulation to suppression depending on the ligand-binding state of the receptor (Chassande 2003).

Wrutniak-Cabello *et al.* (Wrutniak *et al.* 1995, Wrutniak-Cabello *et al.* 2001) identified and described a 43-kDa protein related to c-Erb A α 1 (p43) with TRE- and T3-binding activities in the mitochondrial matrix. Further studies show that p43 stimulates mitochondrial transcription and protein synthesis in the presence of T3 (Casas *et al.* 1999). p43 overexpression stimulates mitochondrial activity and potentiates terminal differentiation in myoblasts (Rochard *et al.* 2000, Seyer *et al.* 2006). More recently, it was reported that p43 overexpression in mouse skeletal muscle increased mitochondrial transcription, biogenesis and respiration and induced a shift in metabolic and contractile type toward a more oxidative and slow twitch fiber (Casas *et al.* 2008). This receptor has not been studied in cardiomyocytes.

If too much or too little T3 underlies programming changes in the myocardium, it is important to know

whether these changes are through binding the thyroid hormone receptors or by a non-receptor mechanism. Recent progress in signaling biology has enlightened our understanding of non-classical receptor signaling for thyroid hormone (40). There is evidence for two primary types of non-nuclear thyroid hormone signaling. One type is via a cell membrane receptor, an integrin perhaps, and the other is via a thyroid hormone receptor in the cytoplasm (Davis *et al.* 2005, 2008). Thus far, it appears that in each of these pathways, thyroid hormones stimulate well-known phosphorylation cascades within minutes. In addition, it has been reported that cellular calcium ion entry and regulation of protein kinase C are influenced by TH. As an example of the first, TH can activate the MAPK pathway when bound to the $\alpha V\beta 3$ integrin at the cell membrane; this can induce angiogenesis and promote cell growth in monkey CV-1 fibroblasts that do not express TRs (Bergh *et al.* 2005). For the second type, Cao *et al.* reported a thyroid hormone- β receptor-activated cascade, TH β -PI₃K-Akt/PKB-mTOR-p70^{s6k}, which was stimulated by T3 in human skin fibroblasts (Cao *et al.* 2005). This cascade leads to the induction of hypoxia-sensitive HIF1 α and its target genes (Moeller *et al.* 2006). Whether these nongenomic pathways operate similarly in immature ovine cardiac cells is unknown. Figure 8 illustrates how T3 is known to work in cardiomyocytes and other cell types and well as a proposed mechanism for incorporating FOXO1 signaling.

Conclusion

While there is little disagreement that stresses experienced by fetuses can predispose them for later disease, the biological mechanisms that link stressors to outcome have not been fully explained. There are four categories of stress that are well documented as causes of fetal programming: malnutrition, chronic maternal stress, intrauterine hypoxia and toxic environmental chemicals (Giraud *et al.* 2006, Louey *et al.* 2007, Jonker *et al.* 2010, Zohdi *et al.* 2014, Dubois *et al.* 2015, Elias *et al.* 2017). However, there are other potential causes of programming including the biochemical environment that includes abnormal levels of cytokines, growth factors and hormones through which a mother can influence her offspring. There is mounting evidence that concentrations of T3 at the extremes, high or low, alter the growth patterns of cardiomyocytes and vascular elements. In addition, there may be other programming-related mechanisms wrought by T3 in the immature myocardium. These have the potential for

elevating risk for various forms of heart disease including ischemic heart disease and heart failure. Now that it is clear that beta cell growth in the fetal sheep pancreas is also suppressed by elevated T3 levels (Harris *et al.* 2017), we can add even more evidence that dysregulation of the pituitary thyroid axis lays the foundation for programming the heart and pancreas for the life of the affected fetus.

David Barker was well aware that hormonal imbalances in pregnancy have the potential to lead to offspring disease. What he did not know and what we do not yet understand is the degree to which hormonal systems can change physiological and epigenetic regulatory systems during development that promote vulnerability for a disease that has its roots in the mother's thyroid gland.

Declaration of interest

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

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