RAPID COMMUNICATION

Thyroid hormone inhibits proliferation of fetal cardiac myocytes *in vitro*N N Chattergoon¹, G D Giraud^{1,2,3,4} and K L Thornburg^{1,2,3}¹Heart Research Center, ²Departments of Physiology and Pharmacology and ³Department of Medicine (Cardiovascular Medicine), Oregon Health and Science University, L464, 3181 SW Sam Jackson Park Rd, Portland, Oregon 97239-3098, USA⁴Portland Veterans Affairs Medical Center, Portland, Oregon 97201, USA

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Abstract

Thyroid hormone (T₃) is a key regulator of fetal organ maturation. Premature elevations of thyroid hormone may lead to a 'mature' cardio-phenotype. Thyroid hormone will stimulate maturation of ovine fetal cardiomyocytes in culture by decreasing their proliferative capacity. Group 1 fetal cardiomyocytes (~135 days gestation) were incubated with T₃ (1.5, 3, 10, and 100 nM) and bromodeoxyuridine (BrdU; 10 µM) for 24 and 48 h. Group 2 cardiomyocytes were cultured with T₃ alone for later protein analysis of cell cycle regulators. At all concentrations, T₃ decreased BrdU uptake fourfold in serum media ($P < 0.001$ versus serum, $n = 5$).

Following serum-free (SF) T₃ treatment, BrdU uptake was inhibited when compared with serum ($P < 0.001$ versus serum, $n = 5$). p21 expression increased threefold ($P < 0.05$ versus serum free, $n = 4$) and cyclin D1 expression decreased twofold ($P < 0.05$ versus serum, $n = 4$) in T₃-treated cardiomyocytes. (1) T₃ inhibits fetal cardiomyocyte proliferation, while (2) p21 protein levels increase, and (3) cyclin D1 levels decrease. Thus, T₃ may be a potent regulator of cardiomyocyte proliferation and maturation in the late gestation fetus.

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Introduction

Thyroid hormones (3,5,3'-triiodo-L-thyronine: T₃; thyroxine: T₄) are key regulators of development in all vertebrates (White *et al.* 2001, Mai *et al.* 2006). In mammals, including humans, the circulating levels of T₃ are maintained within a precisely regulated range during fetal and neonatal life to support the appropriate cell proliferation and differentiation of organ-specific cells (Hernandez *et al.* 2006). While it is well known that T₃ causes cellular hypertrophy in the adult heart (Ledda-Columbano *et al.* 2006), its role in regulating the growth and maturation of the immature myocardium has been little studied.

The concentration of circulating T₃ in the fetus rises in late gestation as the fetal hypothalamic–pituitary–thyroid axis matures (White *et al.* 2001, van Tuyl *et al.* 2004). Most studies on the role of T₃ in development have been conducted in altricial species like the mouse and rat where maturation of the thyroid hormone system occurs after birth. In precocial species, such as the human, sheep, and pig, the thyroid gland becomes functional before birth and affects the metabolic activity and maturation of fetal organs. In sheep, plasma cortisol levels begin to rise some 10–15 days before birth and rise

rapidly in the last 3–5 days of gestation (Li *et al.* 1993, Forhead *et al.* 2000). Increasing levels of cortisol stimulate increases in 5'-monodeiodination activity (5'-MD) that deiodinates T₄ to yield T₃ and is responsible for the prepartum rise in plasma T₃ (Polk *et al.* 1986, Forhead *et al.* 2000). In studies, where plasma T₃ was measured in fetal sheep, it was undetectable through days 130–132 of gestation (dGA; Forhead *et al.* 2002). The plasma concentration of T₃ rises to 0.7–0.8 ng/ml at ~142 dGA (Breall *et al.* 1984, Forhead *et al.* 1998, 2002) and there is a further surge in T₃ in the first 6 h after birth. If the normal increase in T₃ is prevented, the resulting hypothyroidism suppresses growth and leads to low circulating insulin-like growth factor (IGF) concentrations in the fetus (Latimer *et al.* 1993, Forhead *et al.* 1998) and suppressed maturation of a number of fetal tissues (Fowden 1995, Fowden & Silver 1995).

The generation of appropriate numbers of cardiac cells during development is the key to the construction of a heart that will be appropriate for the work load it must bear during transition to postnatal life. The heart is composed of various cell types, including endothelial cells, fibroblasts, smooth muscle cells, and cardiomyocytes. Cardiomyocytes comprise 70–80% of the heart mass, but represent only 20–30% of total cardiac cells by number (Schaub *et al.* 1997) and contain almost two-thirds of

the cardiac protein mass (Dillmann 2002). In humans and large mammals, the heart grows entirely by cardiomyocyte proliferation during the first half of gestation (Smolich *et al.* 1989, Austin *et al.* 1995, Mayhew *et al.* 1997). Thereafter, cardiomyocytes undergo terminal differentiation and binucleation at an increasing rate (Barbera *et al.* 2000, Burrell *et al.* 2003), so that some 80% of cardiomyocytes are binucleated at the time of birth. Evidence suggests that binucleated cardiomyocytes cannot divide (Clubb & Bishop 1984), these cells contribute to increased heart weight only by cellular hypertrophy (increased cell size), but not by hyperplasia. Mouse and rat cardiomyocytes continue to proliferate well into the postnatal period (Clubb & Bishop 1984, Cluzeaut & Maurer-Schultze 1986, Li *et al.* 1996, Soonpaa *et al.* 1996) and terminally differentiate during the first 2 weeks of life.

Thyroid hormone has been well studied in the adult and it is known that a number of cardiac-related genes contain thyroid response elements. Studies in adult animals demonstrate that increased plasma T_3 levels lead to cardiac hypertrophy (Tang *et al.* 2005, Thomas *et al.* 2005). However, there is a significant gap in our knowledge of hormonal regulation in the fetal heart – specifically, the role of T_3 as a regulator of myocardial development. Our understanding of the mechanisms underlying the maturation of fetal cardiomyocytes and regulation the cell cycle is minimal. The decision for a cell to progress through the cell cycle often lingers at the G_1 checkpoint. For this reason, we chose to investigate two proteins important in G_1 . The cyclin-dependent kinase (cdk) inhibitor, p21, of the KIP/CIP family, is associated with cell cycle arrest in the G_1 phase. Cyclin D1 is a well-accepted marker of the G_1 to S transition (Soonpaa *et al.* 1997, Ledda-Columbano *et al.* 2006). Increases in cyclin D1 expression would be expected to correlate to increased proliferation of cardiomyocytes.

The present study was designed to determine the role of thyroid hormone in regulating maturation and proliferation of cardiomyocytes *in vitro*. The fetal period is a critical time of maturation when the number of working heart cells that will support heart function during the birth transition is set. Because T_3 induces rat skeletal muscle myoblasts to exit the cell cycle (Burton *et al.* 1999), differentiate, and express a muscle-specific phenotype (White *et al.* 2001), we hypothesized that T_3 would stimulate the maturation of fetal sheep cardiomyocytes in culture by decreasing their proliferative capacity. We also proposed that cell cycle proteins, p21 and cyclin D1, would play a key role in regulating the effects of T_3 in the heart. To test these hypotheses, we determined the role of T_3 in regulating the proliferation of immature cardiomyocytes in culture.

Materials and Methods

Animals

Hearts were taken from uninstrumented fetal sheep of time-mated ewes (*Ovis aries*; mixed western breed) at 135 ± 2 days

gestational age (dGA) where term is ~ 145 dGA. The sheep fetus is a good model for study because it has a long gestation and generally has only singleton or twin pregnancies (like the human).

Treatment groups

In order to determine the effects of T_3 on cardiomyocyte proliferation, we measured bromodeoxyuridine (BrdU) incorporation as an index of cell proliferation in three groups of cultured fetal cardiomyocytes: (1) 48 h in serum-free (SF) media with T_3 in different doses; (2) 48 h of fetal bovine serum containing media with T_3 ; (3) 24 h of T_3 pre-treatment followed by 24 h exposure to serum medium. Doses of T_3 included physiological and pharmacological concentrations. Other *in vitro* studies have investigated pharmacological concentrations of T_3 ranging from 10 to 100 nM (Burton *et al.* 1999, Kuzman *et al.* 2005). We chose to study this dose range to determine the degree to which physiological and higher than physiological concentrations affected cardiomyocyte growth in culture.

Among proteins that suppress the cell cycle are p21 and p27, which inhibit the activity of cyclin-cdk complexes and mediate exit from or arrest of the cell cycle (Tamamori-Adachi *et al.* 2004, Chattergoon *et al.* 2005). We sought to determine whether the cell cycle progressor, cyclin D1, or cell cycle suppressor, p21, were integral to the regulation of the cell cycle by T_3 by western blot analysis of these proteins following a 24-h treatment with T_3 .

Cardiac myocyte isolation

Ewes were euthanized by i.v. injection of a commercial solution of sodium pentobarbital (Euthasol (~ 65 mg/kg), Virbac, Fort Worth, TX, USA). Fetuses received a bolus dose of heparin (10 000 U), followed by 10 ml saturated KCl into the umbilical vein to arrest the heart in diastole. Fetuses were weighed and the heart excised, trimmed in a standard way, blotted dry and weighed. Hearts were enzymatically dissociated as previously described by our laboratory (Giraud *et al.* 2006). Briefly, hearts were retrogradely perfused with gassed solutions, 95% O_2 and 5% CO_2 at 39 °C. First, with Tyrodes buffer for 5–10 min (no calcium added; 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 10 mM glucose, 10 mM HEPES; pH adjusted to 7.35 with NaOH) until the vessels are clear of blood; 5–10 min with 160 U/ml Type II collagenase (Worthington Biochemicals, Lakewood, NJ, USA) and 0.78 U/ml Type XIV protease (Sigma) in Tyrodes buffer to digest the tissue; 5–10 min with a high potassium (KB) solution (74 mM glutamic acid, 30 mM KCl, 30 mM KH_2PO_4 , 20 mM taurine, 3 mM $MgSO_4$, 0.5 mM EGTA, 10 mM HEPES, 10 mM glucose; pH adjusted to 7.37 using KOH). The left and right ventricular (LV and RV) free walls were dissected from the heart and placed into separate tubes containing KB solution. The tissue was gently agitated to release the myocytes.

Cardiomyocyte cultures

Cardiomyocytes were cultured as previously described by our laboratory (Sundgren *et al.* 2003a,b). The freshly isolated slurry of myocytes rested for 30–60 min at room temperature before centrifugation and resuspension in sterile serum media (14.9 g/l; Sigma MCDB 105, 2 mM KCl, 0.2 mM glycine, 5 mM creatine, 5 mM taurine, 2 mM L-carnitine, pH 7.6 with 10 mg/l insulin–transferrin–sodium selenite supplement (Sigma), 1 ml/l antibiotic–antimycotic solution (Sigma), and final concentration 10% fetal bovine serum (FBS; Invitrogen). SF media are the same composition as serum (S) media without FBS. Of note, FBS contains 127 ng/ml T₄ and the S media contains 12.7 ng/ml T₄. We do not know what contribution T₄ may have in our culture system but our S- and SF-treated cells serve as proper controls for treatment groups under these serum conditions. Cells were pre-plated twice to remove non-myocyte cells (2 h each time, 39 °C; 95% air and 5% CO₂) and plated on 22×22 mm coverslips (for later measurement of myocyte size or proliferation) or in 6-well plates (for later protein analysis). Coverslips and plates were coated with laminin (3–5 mg/ml) at least 4 h prior (39 °C; 95% air and 5% CO₂) and then aspirated just before final cell seeding. Cells were incubated in S media at 39 °C (95% air and 5% CO₂) for 24 h. Cells were then incubated in SF for 48 h, and the media changed again to fresh SF media for another 24 h. Twenty-four hours after the last media change, cells were treated with the experimental drug (day 5).

BrdU immunofluorescence

Two sets of cells were cultured with 10 µM BrdU to determine proliferation rates in response to T₃ (1.5, 3, 10, and 100 nM) in both S and SF using BrdU uptake techniques that we have previously reported (Sundgren *et al.* 2003b). Both of these groups of cells were treated for 48 h before fixation in acidified ethanol. A third group of cultures were exposed to SF (control) and T₃ (1.5, 3, 10, and 100 nM) in SF media for 24 h. T₃-containing media were aspirated; cells washed in sterile 1× PBS, and serum media containing 10 µM BrdU was added to all coverslips for another 24 h. Cells were fixed as above and stained for myosin and BrdU. Doses were chosen from reports in the literature (Burton *et al.* 1999, Kuzman *et al.* 2005).

Myosin and BrdU staining

Myocytes were stained with anti-myosin (primary antibody: mouse anti-myosin, 1:5000, Abcam, Cambridge, MA, USA) and anti-BrdU (primary antibody: rat anti-myosin, 1:500, Abcam) overnight (O/N) at 4 °C. After washes in 1× PBS, cells were incubated in anti-mouse rhodamine red (myosin secondary antibody, 1:200, Jackson ImmunoResearch, West Grove, PA, USA) and BrdU secondary antibody (anti-rat FITC, 1:200, Jackson ImmunoResearch) for 2 h at room temperature (RT). Slides were mounted using Vectashield Hardset mounting medium with DAPI (Vector Laboratories,

Burlingame, CA, USA) and stored in the dark O/N at 4 °C to allow the mount to dry (Sundgren *et al.* 2003b). The proportion of BrdU positive myocytes was determined in a minimum sample of 300 myocytes using fluorescence microscopy and counting BrdU positive cardiomyocytes as those that stain for myosin (red) with punctuate nuclei (green) indicating BrdU (Fig. 1).

Cell cycle protein analysis (p21, cyclin D1)

Following incubation with T₃, cardiomyocytes were lysed (5 mM Tris–HCl, 5 mM EGTA, 5 mM EDTA, 0.06% SDS, Protease inhibitor Mini-complete tablet (Roche), and Phosphatase Inhibitor Cocktail 1 and 2 (Sigma), and collected into pre-chilled tubes. Protein concentration was quantified by BCA assay (Pierce, Rockford, IL, USA; Chattergoon *et al.* 2005). Equal amounts of total protein/sample (10 µg) were separated by SDS–PAGE on a 10% gel and transferred to Optitran BA–S 83 nitrocellulose membrane (Fisher, NH, USA). Membranes were blocked with 5% milk TBS–T (TBS + 0.01% Tween) buffer for 1 h. Membranes were incubated with primary antibodies to p21 (1:400, Santa Cruz Biotechnologies, Santa Cruz, CA, USA) and cyclin D1 (1:1000; Santa Cruz Biotechnology) O/N at 4 °C. Membranes were washed in large volumes of TBS–T before exposure to the secondary antibody in the ratio of 1:4000 (anti-mouse, GE Healthcare, San Diego, CA, USA) for 1 h RT. Antibody binding was detected using a chemiluminescence system (SuperSignal, Pierce); protein expression was quantitated from a digitized image of the blot.

Statistical analysis

A minimum of 300 cells per treatment group was assessed for BrdU incorporation and reported as a percentage of the total number of cardiomyocytes counted. Densitometry of western blots was determined using Image J software (NIH, Bethesda, MD, USA). One-way repeated measures ANOVA was used to analyze BrdU uptake among the treatment groups. Significant differences were further analyzed by Tukey's multiple comparison *post hoc* test for differences between treatment groups. Significance was determined $P < 0.05$. Graphs represent mean and S.E.M.

Results

BrdU Incorporation

BrdU incorporation was used as an index of proliferation in ventricular cardiomyocytes from both left and right ventricles (LV and RV) of 135 dGA fetal sheep hearts (Fig. 1). Figure 2 shows that the presence of T₃ in SF media did not increase or decrease BrdU incorporation of cardiomyocytes, regardless of ventricle, when compared with the SF control group which was already low at 1–2%. The average of BrdU uptake did not exceed 1.5% in all T₃ groups. However, cardiac myocytes

Figure 1 Immunohistochemical staining of fetal sheep cardiomyocytes in culture for BrdU analysis. BrdU positive cells are marked by positive FITC (green) staining in the nucleus of cells that are positive for myosin (red). This image represents a BrdU-positive mononucleated cell and a non-positive binucleated cell.

showed a very robust surge in proliferation in the presence of serum. Of the cells in the serum media control group, there was an 8–12% incorporation rate BrdU (Fig. 3). However, T_3 given at any dose in serum media dramatically inhibited BrdU incorporation ($P < 0.001$, $n = 5$) when compared with serum-stimulated cells. Thus, T_3 negatively affects the normal proliferation response to serum.

BrdU incorporation after T_3 pre-treatment

T_3 pre-treatment in SF media for 24 h, followed by 24 h in serum rich media (containing BrdU 10 μ M) significantly decreased uptake of BrdU when compared with treatment with serum alone (Fig. 4; $P < 0.001$, $n = 5$). There was not a

dose-dependent effect; rather, BrdU uptake was inhibited to similar degrees across all concentrations of T_3 treatment in this time period.

Cyclin D1 and p21

Protein levels of p21 and cyclin D1 were determined by western blot analysis on lysates of cells incubated for 24 h in serum media, SF media, and T_3 (3, 10, and 100 nM) in SF media. Figure 5 shows representative images of protein analysis for cyclin D1 (A) and p21 (B) respectively. Cyclin D1 was increased in serum-treated cells, but not in cardiomyocytes treated in SF media alone or with T_3 . Rather, in the thyroid hormone-treated cells, cyclin D1 levels were

Figure 2 BrdU (10 μ M) incorporation in (A) LV and (B) RV fetal sheep cardiomyocytes. T_3 has no effect on BrdU incorporation of cells treated in SF media.

Figure 3 BrdU (10 μ M) incorporation in (A) LV and (B) RV fetal sheep cardiomyocytes with 10% FBS-serum media (S) challenge. T₃ at all doses in serum media significantly inhibited BrdU uptake. * $P < 0.001$ versus serum; $n = 5$.

significantly decreased in comparison with cardiomyocyte serum control. In contrast, p21 expression increased in the T₃-treated groups over the serum-treated group. p21 was not significantly increased by S or SF treatment in comparison with the T₃ groups.

Discussion

This study has demonstrated for the first time that T₃, at both physiological and pharmacological doses, inhibits fetal cardiomyocyte proliferation as indicated by BrdU uptake. T₃ exposure did not stimulate BrdU uptake in the presence of SF media. This is in contrast to our previous findings for IGF-I and angiotensin II, both of which are stimulants of fetal cardiomyocyte proliferation *in vitro* (Sundgren *et al.* 2003a,b). T₃ significantly decreased proliferation of cardiomyocytes in the presence of serum-rich media for 48 h. When the cells were pre-treated with T₃ for 24 h, the stimulatory effect of serum on proliferation was completely inhibited over the next 24 h, even though T₃ was no longer present. Thus, the degree of suppression of BrdU uptake by pre-treatment with T₃ was similar to that with concurrent T₃ treatment. This suggests

that 24-h incubation is adequate to modulate the transcriptional activity of genes that suppress cell cycle activity during the subsequent 24-h period. Western blot analysis of p21 and cyclin D1 suggests that well-recognized pathways of cell-cycle regulation were affected by the T₃ exposure. p21, a protein known to be associated with the inhibition of proliferation, was increased in cells treated with T₃ but not in cells exposed to either SF media or S media alone. Cyclin D1, a marker of proliferation, was increased in serum-treated cells, but decreased in SF- and T₃-treated cells with significance only in the T₃-treated cells.

The relationship between the numbers of working cardiomyocytes at birth and the numbers that are present throughout life is not known. However, the number of cardiomyocytes present in the heart at birth may be related to the number of cardiomyocytes in that individual for life. While there is evidence that the adult myocardium is able to maintain its working cell numbers through a 'stem cell' replication process (Anversa *et al.* 2006), the regulation of cardiomyocyte number for any individual has not been determined. It is clear that the last third of ovine gestation is a critical window for the developing myocardium because during that period the heart must gain the complement of

Figure 4 BrdU (10 μ M) incorporation in serum media in (A) LV and (B) RV following 24 h T₃ pre-treatment. All doses of T₃ significantly inhibited BrdU uptake in the 24-h period compared with serum control. ANOVA with Tukey post-test, * $P < 0.001$ versus serum; $n = 5$.

Figure 5 Cyclin D1 and p21 protein levels (A and B respectively) in cardiomyocytes following T₃ treatment. The same trend is seen in LV and RV cardiomyocytes. (A) Cyclin D1 levels significantly decrease in T₃-treated cells compared with serum. * $P < 0.05$ versus serum, $n = 4$. (B) p21 levels were significantly increased in all T₃-treated cardiomyocytes compared with serum or serum-free controls. ANOVA with Tukey post-test, * $P < 0.05$ versus serum, $n = 4$.

cardiomyocytes that will carry it through the stress-laden birth transition. During this period, both mono- and bi-nucleated myocyte populations are sensitive to nutritional, hormonal, and hemodynamic modifications (Barbera *et al.* 2000, Sundgren *et al.* 2003a, Giraud *et al.* 2006).

Several studies have shown that thyroid hormone stimulates hypertrophic growth of the adult heart. Thomas *et al.* (2005) showed an increased heart weight to body weight (HW:BW) ratio in rats following 10 days of thyroid hormone treatment, which was exaggerated over the next 2 months. At the 2 month time point, cardiomyocytes were significantly longer than normal. LV chamber dimensions, determined by echocardiography were also increased. Hu *et al.* (2005) also found significantly increased HW:BW ratios in young thyroidectomized rats following i.p. treatment of T₄ over 7 days at two doses (Hu *et al.* 2005). Hypothyroid rats had decreased HW:BW ratio at the end of 7 days. A previous study by the same group reported significantly increased cross-sectional myocyte width without marked cardiac fibrosis after T₄ treatment (Hu *et al.* 2003). Tang *et al.* (2005) also showed that hypothyroidism in adult rats caused a rapid decrease in cardiac mass that they believed resulted from decreased myocyte cross-sectional area over 6 weeks of study. They also found myocyte lengthening from series addition of sarcomeres in cardiomyocytes after 1 year of hypothyroidism. Tang and Hu's data suggest that thyroid hormone can affect cardiac mass in the immature myocardium. Our data are compatible with the idea that increased T₃ levels promote maturation of fetal sheep cardiomyocytes in that we observed decreased proliferative capacity in T₃-treated fetal cardiomyocytes as would be expected during the maturation phase, although we do not yet know to what degree it affects heart maturation *in vivo*.

In the adult rat myocardium, T₃ was shown to increase DNA synthesis and cyclin D1 suggesting that T₃ induces

re-entry into the cell cycle (Ledda-Columbano *et al.* 2006). The results of our study differ dramatically in several ways from that study: (1) experiments were performed *in vitro*, (2) immature cardiomyocytes were used, and (3) protein was extracted from isolated cardiomyocytes, rather than whole heart tissue lysates.

As demonstrated in Fig. 2, T₃ did not alter the number of cardiomyocytes incorporating BrdU when grown in media lacking serum. These non-proliferating cells were characterized by increased p21 levels and a significant reduction in cyclin D1 when compared with the serum-treated cells (Fig. 5). While one might argue that the lack of proliferation found in cells grown in media without serum is obviously due to the lack of nutrients, we have previously reported significant increases in BrdU uptake by fetal cardiomyocytes following IGF-I and angiotensin II treatment in SF media (Sundgren *et al.* 2003a,b).

The major actions of T₃ are known to be transduced by nuclear thyroid hormone receptors (TRs). It is also known that T₃ can act in a non-genomic fashion (Falkenstein *et al.* 2000), but our experiments were designed to exclude this mechanism. In most vertebrates there are two isoforms, TR α and TR β , encoded by the genes, *THRA* (*NR1A1*) and *THRB* (*NR1A2*) respectively. Both TRs have alternative spliced variants: TR α_1 , TR α_2 , TR β_1 , TR β_2 , and TR β_3 . TR β_1 , α_1 , and α_2 are widely expressed; TR β_2 is present mainly in pituitary cells. TR α is the predominant isoform in the heart and is thought to play a major role in determining heart rate; TR β is found in lower levels in heart cells and is involved in the response to acute stimulation by T₃ (Mai *et al.* 2006). Cyclin D1 has been shown to affect the function of other nuclear receptors – it positively regulates estrogen receptor α -mediated transcription in breast cancers (Zwijsen *et al.* 1998, McMahon *et al.* 1999), while it selectively inhibits ligand-dependent androgen receptor function in breast cancer and bladder cancer (Knudsen *et al.* 1999, Reutens *et al.* 2001).

In a study using three different cancer cell lines, cyclin D1 was shown to repress the T₃-dependent transactivation activity of TR β ₁ (Lin *et al.* 2002). The role of cyclin D1 actions on TRs in the immature heart has not been well studied.

From our studies, we know that fetal heart weight and BrdU uptake increase in response to elevated IGF-I levels indicating that the proliferation of myocytes in the late-term fetus continues to be important in building heart cell numbers (Sundgren *et al.* 2003a). In another study, subpressor levels of cortisol infused into the circumflex coronary artery resulted in an increased HW:BW ratio without affecting LV or RV myocyte size or maturational state. It appears that heart weight was increased through increased proliferation because Ki-67, a marker of cell cycle activity, was increased (Giraud *et al.* 2006). Thus, it appears that cortisol can act as a mitogen for immature cardiomyocytes. These studies together with those showing that IGF-I and angiotensin II stimulates cardiomyocytes proliferation *in vitro*, offer insight to the complex roles of hormone regulated heart growth in the late-term fetus.

The present study has brought to light a potential role for thyroid hormone as an important regulator of heart development in the near term fetus. Nevertheless, the contribution of thyroid hormone in the regulation of heart growth in the fetus over the last weeks of gestation remains unanswered.

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