RAPID COMMUNICATION

Thyroid hormone inhibits proliferation of fetal cardiac myocytes in vitro

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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, Chazet & 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 T3 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 T3 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 G1 checkpoint. For this reason, we chose to investigate two proteins important in G1. The cyclin-dependent kinase (cdk) inhibitor, p21, of the KIP/CIP family, is associated with cell cycle arrest in the G1 phase. Cyclin D1 is a well-accepted marker of the G1 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 T3 induces rat skeletal muscle myoblasts to exit the cell cycle (Tamamori-Soonpaa 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 T3 by western blot analysis of these proteins following a 24-h treatment with T3.

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 T3 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 T3 in different doses; (2) 48 h of fetal bovine serum containing media with T3; (3) 24 h of T3 pre-treatment followed by 24 h exposure to serum medium. Doses of T3 included physiological and pharmacological concentrations. Other in vitro studies have investigated pharmacological concentrations of T3 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 T3 by western blot analysis of these proteins following a 24-h treatment with T3.

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 (Graud et al. 2006). Briefly, hearts were retrogradely perfused with gassed solutions, 95% O2 and 5% CO2 at 39 °C. First, with Tyrodes buffer for 5–10 min (no calcium added; 140 mM NaCl, 5 mM KCl, 1 mM MgCl2·6H2O, 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 KH2PO4, 20 mM taurine, 3 mM MgSO4, 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.

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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, before centrifugation and resuspension in sterile serum media slurry of myocytes rested for 30–60 min at room temperature (39°C). 6-well plates (for later protein analysis). Coverslips and plates (for later measurement of myocyte size or proliferation) or in PBS, and serum media containing 10 mM creatine, 5 mM taurine, 2 mM L-carnitine, pH 7.6 with 10 ng/ml 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, 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–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...
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, T₃ given at any dose in serum media dramatically inhibited BrdU incorporation ($P<0.001$, $n=5$) when compared with serum-stimulated cells. Thus, T₃ negatively affects the normal proliferation response to serum.

**BrdU incorporation after T₃ pre-treatment**

T₃ pre-treatment in SF media for 24 h, followed by 24 h in serum rich media (containing BrdU 10 µM) 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 T₃ 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 T₃ (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 T₃. Rather, in the thyroid hormone-treated cells, cyclin D1 levels were

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**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.

**Figure 2** BrdU (10 µM) incorporation in (A) LV and (B) RV fetal sheep cardiomyocytes. T₃ has no effect on BrdU incorporation of cells treated in SF media.
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 3** BrdU (10 μM) 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.

**Figure 4** BrdU (10 μM) 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.
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, TRα and TRβ, encoded by the genes, THRA (NR1A1) and THRB (NR1A2) respectively. Both TRs have alternative sliced variants: TRα1, TRα2, TRβ1, TRβ2, and TRβ3. TRβ1, α1, and α2 are widely expressed; TRβ2 is present mainly in pituitary cells. TRα is the predominant isoform in the heart and is thought to play a major role in determining heart rate; TRβ 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).

**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.
In a study using three different cancer cell lines, cyclin D1 was shown to repress the T3-dependent transactivation activity of TRβ1 (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 that IGF-I and angiotensin II stimulates cardiomyocytes.

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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