Published in final edited form as:
*Prog Biophys Mol Biol*. 2011 July ; 106(1): 289–299. doi:10.1016/j.pbiomolbio.2010.11.010.

# Regulation of the Cardiomyocyte Population in the Developing Heart

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## Abstract

During fetal life the myocardium expands through replication of cardiomyocytes. In sheep, cardiomyocytes begin the process of becoming terminally differentiated at about 100 gestation days out of 145 days term. In this final step of development, cardiomyocytes become binucleated and stop dividing. The number of cells at birth is important in determining the number of cardiomyocytes for life. Therefore, the regulation of cardiomyocyte growth in the womb is critical to long term disease outcome. Growth factors that stimulate proliferation of fetal cardiomyocytes include angiotensin II, cortisol and insulin-like growth factor-1. Increased ventricular wall stress leads to short term increases in proliferation but longer term loss of cardiomyocyte generative capacity. Two normally circulating hormones have been identified that suppress proliferation: atrial natriuretic peptide (ANP) and tri-iodo-L-thyronine ($T_3$). Atrial natriuretic peptide signals through the NPRA receptor that serves as a guanylate cyclase and signals through cGMP. ANP powerfully suppresses mitotic activity in cardiomyocytes in the presence of angiotensin II in culture. Addition of a cGMP analogue has the same effect as ANP. ANP suppresses both the extracellular receptor kinases and the phosphoinositol 3 kinase pathways. $T_3$ also suppresses increased mitotic activity of stimulated cardiomyocytes but does so by increasing the cell cycle suppressant, p21, and decreasing the cell cycle activator, cyclin D1.

### Keywords

Cardiomyocyte; Fetus; Programming; Terminal Differentiation; Atrial; Natriuretic Peptide; Tri-iodo-L-thryonine

## Common Pathways to Lethal Cardiac Pathology

There are 3 main causes of cardiac death in humans: 1) cessation of coronary arterial flow which leads to myocardial ischemia, 2) electrical abnormalities in the myocardium that lead to asynchronous contraction or ventricular fibrillation, and 3) failure of the heart to maintain



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its stroke volume or to fill properly. In short, cardiac death comes from myocardial infarction, ventricular fibrillation or heart failure. There are now well described links between early prenatal growth patterns and risks for all of these causes of death (Barker et al. 2010;Eriksson et al. 2010;Conen et al. 2010).

Heart failure is on the increase among western countries and is the most common reason for hospitalization in the USA. At present some 6 million people in the USA have heart failure with just under 700,000 new patients diagnosed every year (www.cdc.gov). Heart failure and associated hospitalization costs the United States some $40 billion annually. Contributing factors include an increasing number of people who are surviving ischemic cardiac events and increasing number of people living into old age. Another possible reason is increasing numbers of people who are vulnerable because of poor growth before birth, including inadequate numbers of cardiomyocytes.

## Cardiomyocyte endowment at birth

If the postnatal myocardium is poorly endowed with myocytes, the individual could suffer from mechanical deterioration and myocyte loss over decades of time, especially if new myocytes cannot be generated at a rate that matches cellular losses. Normally, growth of heart muscle is limited by the permanent suppression of cardiomyocyte mitotic activity that begins before birth. Thus, beginning in infancy, the developing heart must grow in size to match the body's oxygen demands, without the ability to make many new cells. Bergmann and colleagues (2009) investigated cardiac cell replacement rates in humans using the temporary "bubble" in C-14 content of myocardial DNA acquired during the Cold War nuclear testing era. They estimated that human hearts enjoy a 1% turnover per year in early adulthood and 0.5% per year in old age. Even with these new data, it is not yet possible to determine the mechanisms that underlie the cardiomyocyte number set point at birth nor the degree to which cardiomyocyte numbers at birth increase or decrease over a lifetime. It is known, however, that stressors *in utero* can depress cardiomyocyte numbers that will persist for life in rats (Li et al. 2003;Corstius et al. 2005). In cases where myocyte numbers are reduced, the remaining myocytes are required to bear a higher than usual share of contractile force generation which leads to myocyte enlargement, and perhaps, myocyte dropout. Thus, there is a need to understand biological pathways that lead to under-endowment of the myocardium and the consequences that must be borne by the heart if inadequately endowed.

Experiments in Lubo Zhang's laboratory (Li et al. 2003;Li et al. 2004) suggest that adult cardiomyocyte numbers are set during prenatal life. He exposed maternal rats to a hypoxic environment over the last week of gestation. Later he studied the once hypoxic offspring hearts in a Langendorf chamber. After 10 minutes of ischemia and 3 hours of reperfusion, the hearts from fetuses that were hypoxic in the womb suffered infarctions whereas the control hearts, that were never hypoxic, did not. In addition, the hearts that were exposed to hypoxia in the womb had depressed levels of the cardioprotectant heat shock protein HSP70. Perhaps the most exciting finding from this work is that the intrauterine hypoxia led to a reduction in cardiomyocyte numbers (Li et al. 2004). It should be noted that in rats, cardiomyocytes continue to proliferate after birth and do not become terminally differentiated until some 1–2 weeks after birth (Clubb, Jr. and Bishop 1984). In Zhang's studies hearts that were hypoxic during the prenatal period were not able to recover to attain their "normal" complement of cardiomyocytes during postnatal life (Li et al, 2004).

It is important to note that the once hypoxic hearts had no functional deficits under control conditions but following an ischemic injury they suffered larger deficits in function compared to controls and, unlike the control hearts, they never regained full function during the reperfusion period. Intrauterine events that suppress myocyte proliferation rates may

therefore diminish the cardiomyocyte endowment at birth and compromise the future cardiac health of the offspring. Thus it is important to determine the mechanisms that regulate cardiomyocyte proliferation before final numbers are set.

## Dynamics of Cardiac Growth in the Fetus

There are two possible outcomes for a mononucleated cardiomyocyte (Figure 1a) that duplicates its DNA. It may undergo cytokinesis (Figure 1b) and completion (Figure 1c), yielding two mononucleated daughter cardiomyocytes or it may form a binucleated cell with approximately twice the volume of a mononucleated cardiomyocyte (Figure 1d). Such a cell rarely reenters the cell cycle.

The small size of immature cardiomyocytes allows them to function before an adequate t-tubular network is developed (Legato 1979). Mononucleated and binucleated cardiomyocytes each remain the same average length through the last third of gestation (Figure 2). However, binucleated cells are thirty percent longer than mononucleated cells. Cardiomyocyte diameter increases slowly over this period (Figure 3) with binucleated cells being about 15–20% wider than mononucleated cardiomyocytes at this stage. The result is that binucleated cells contain about twice the volume of mononucleated cells; both increase slowly in volume over the last third of gestation. Fetal RV cardiomyocytes are consistently larger in every dimension than LV cells, in contrast to the adult (Smolich et al. 1989;Jonker et al. 2007b) but demonstrate similar trends to the LV in terms of length and width throughout gestation.

Until about 100 days of gestation (term ~145 days), all cardiomyocytes of the sheep heart contain one nucleus (Figure 4). Over the next two weeks, binucleated cardiomyocytes gradually appear after which the proportion of cardiomyocytes that are mononucleated drops rapidly. At the end of gestation, about 70% of cardiomyocytes are binucleated (Burrell et al. 2003;Jonker et al. 2007b). In the last two weeks of gestation, a negligible number contain 4 nuclei. Because binucleated cardiomyocytes are rarely observed to enter the cell cycle and are phenotypically comparable to adult cardiomyocytes, they are considered to be post-proliferative and terminally differentiated. If true, their increasing proportion in the fetal heart represents the loss of proliferative potential.

Both proliferation and terminal differentiation require entry of mononucleated cardiomyocytes into the cell cycle. A notable characteristic of cell cycle activity in fetal sheep cardiomyocytes is the wide variability in this measure at any given age (Figure 5). An explanation for this variability is that the heart may undergo saltatory growth, that is, growing rapidly in spurts rather than slowly and constantly. Because measurement of cell cycle activity cannot be made longitudinally in the living heart, this hypothesis has not been tested. Despite the variability, cell cycle activity within the myocardium decreases over the last third of gestation due to the decreasing percentage of mononucleated cardiomyocytes in the heart. However, when expressed as cell cycle activity per mononucleate myocyte, activity is consistent across this period (Figure 5, **inset**).

At the early stages when the cell cycle activity is mostly proliferative, the number of mononucleated cardiomyocytes increases dramatically. This is the case throughout fetal development of sheep until about 115–125 days of gestation (Figure 6). At this time, the rate of terminal differentiation exceeds the rate of proliferation and the fraction of the myocardial cell population that is composed of mononucleated myocytes declines. If cell cycle kinetics were to remain constant following birth, the mononucleated population of cells would be depleted within weeks of birth and proliferative growth would cease.

Proliferative growth of cardiomyocytes is a relatively small component of overall cardiac growth at term (Bergmann et al. 2009). The gradual enlargement of both mononucleated and binucleated cardiomyocytes contribute to overall growth of the fetal heart. However, an increasingly important contributor to normal cardiac growth is terminal differentiation. Terminally differentiated cells undergo growth associated with doubling of the genetic material but do not divide. Although for a given cardiomyocyte this doubling in size occurs only once, it occurs for at least 70% of all cardiomyocytes by term and is a major contributor to the expansion of fetal heart mass at birth. In mice, binucleation and terminal differentiation occur in the first few days after birth (Soonpaa et al. 1996). In rats, it is complete by 14 postnatal days in most strains (Clubb, Jr. and Bishop 1984;Li et al. 1996).

It is now clear that during fetal life, both hemodynamic forces and circulating factors regulate cardiomyocyte proliferation. Among the chemical and mechanical factors that stimulate proliferation are arterial pressure load (Giraud et al. 2005), angiotensin II (**Ang II**) (Sundgren et al. 2003b), cortisol (Giraud et al. 2006) and insulin-like growth factor-1 (**IGF-1**) (Sundgren et al. 2003a). Among those that suppress cardiac cell proliferation are tri-iodo-L-thyronine ($T_3$) (Chattergoon et al. 2007), atrial natriuretic peptide (O'Tierney et al. 2010b), and reduced cardiac systolic load (O'Tierney et al. 2010a).

## Wall Stress and Myocyte Proliferation

It has been known for decades that cardiomyocytes in the living heart are sensitive to wall stresses. They enlarge if systolic wall stress is increased with elevated systolic pressure. Such hearts may undergo so called physiologic hypertrophy where the chamber wall thickens to the degree needed to normalize wall stress. In simple terms, wall stress can be estimated by the Laplace relationship

$$S_w=(P_{tr}/2) \times (r/h)$$

where wall stress ($\mathbf{S_w}$) is determined by ½ the transmural wall pressure ($\mathbf{P_{tr}}$) times the radius (**r**) to wall thickness (**h**) ratio. This relationship demonstrates that increasing wall thickness can normalize wall stress to accommodate increases in transmural pressure. The loading conditions of the fetal heart can be altered by constricting the arterial vessels through which the heart must pump its blood, by increasing protein concentrations of plasma or more commonly by increasing the vascular resistance of the placental bed due to poor vascularization.

When the pulmonary artery of the near term sheep fetus is mildly constricted over a week's time (Barbera et al. 2000), systolic pressures can be readily increased in the upstream artery by some 10 mmHg above their usual mean of 40 mmHg. This rather pure systolic load leads to a number of changes in the right ventricle. The load stimulates proliferative and hypertrophic growth in the ventricular wall so that its thickness increases and the chamber volume becomes relatively smaller. This modification also increases the wall thickness relative to the radius of the chamber which provides an amazing mechanical benefit to the heart and ejection fraction is maintained or improved. There are important cellular changes within the right ventricular wall. Cardiomyocyte size increases above cells in control hearts and there is an elevated fraction of the population of myocytes that undergo terminal differentiation and become binucleated. Thus, mechanical load, by increasing stress in the wall of the heart, augments the rate at which the myocardium matures and accelerates the rate at which it loses mononucleated cells and thus its generative capacity.

## Decreasing systolic load

The above mentioned loading experiments do not inform the question of whether reduced systolic load slows the growth and maturation of the fetal heart. To address this question, arterial pressures were reduced in fetal sheep and their cardiomyocytes examined (O'Tierney et al. 2010a). Blockade of the angiotensin converting enzyme, which normally converts angiotensin I to angiotensin II, decreases fetal arterial pressure and thus systolic cardiac load.

When the angiotensin-converting enzyme inhibitor, enalaprilat, was infused (345 μg/day) into near term fetal sheep for 8 days, fetal arterial blood pressure decreased from a mean 42 mmHg to 24 mmHg (O'Tierney et al. 2010a). Mean right atrial pressure did not change. Fetal heart rate decreased as did arterial $PO_2$. Hematocrit did not change. Hearts from animals with decreased blood pressure were undergrown in that they weighed only 80% of control hearts. Cardiomyocytes isolated from these hearts did not differ in size compared to the normotensive fetuses. However, the percentage of cells actively in the cell cycle was dramatically reduced by 76% in the right ventricle and 88% in the left ventricle.

In these experiments, two factors changed. Systolic, but not diastolic, load was dramatically reduced. In addition circulating, Ang II levels were reduced. Thus, there may have been two changes that down-regulated mitotic activity and slowed growth of the heart. Circumstantial evidence suggests that the load had the major effect in these experiments. For example, in chronic fetal experiments, blockade of the Ang II AT1 receptor, does not inhibit myocardial remodeling when systolic load is increased (Segar et al. 1997). In addition, depression of the renin-angiotensin system does not prevent pressure induced hypertrophic and hyperplastic growth (Jonker et al. 2007a). These experiments suggest that an active renin-angiotensin system is not required for cardiac remodeling in response to decreased systolic load.

## Increasing Diastolic plus Systolic Load

In one series of experiments, sterile sheep plasma was infused into the vascular space of near term ovine fetuses over either 4 or 8 days (Jonker et al. 2007a). The protein infusion caused arterial and venous pressure increases of 20 and 3 mmHg, respectively. The animals in the 4 day experimental group received ~1,170 ml plasma containing ~60g plasma protein. The fetuses in the 8 day group received a total of ~2,500 ml plasma containing ~135g plasma protein. Hearts from hypertensive fetuses were heavier; the heart weight to body weight ratio increased by about 25% in both the 4 day and 8 day groups compared with age-matched, vehicle-infused controls. The portion of the cardiomyocyte population that was binucleated did not increase over the first 4 days of loading but by the 8th day, the portion had increased from a baseline of about 50% to nearly 70%. Compared with control cells, binucleated cardiomyocytes had increased in length by 4 days of increased pressure and both length and width of cardiomyocytes had increased by 8 days. The percentage of cardiomyocytes that were in the cell cycle (Ki-67) more than doubled in both groups in response to a pressure load. These experiments brought two new facts to light: 1) the myocardial growth response to elevations of both central arterial and venous pressures is similar to a systolic pressure load alone; 2) cardiomyocytes increased their length and volume several days before changes in terminal differentiation were detected. The delay is in part due to the fact that the time it takes a nucleus to complete its division is measured in days rather than hours.

## Atrial Natriuretic Peptide (ANP)

Ang II and IGF-1 both stimulate proliferation of fetal ovine cardiomyocytes through the mitogen activated protein kinase (MAPK) cascade down the extracellular signal-regulated

kinase (ERK) branch (Sundgren et al. 2003a;Sundgren et al. 2003b). While definitive experiments have not been performed for Ang II signaling, it is clear that IGF-1 signaling takes advantage of both the phosphoinositol-3 kinase (PI3K) and the ERK pathways (Figure 7). If either of these pathways is blocked in isolated fetal cardiomyocytes in culture, proliferation is also largely blocked (Sundgren et al. 2003a;Sundgren et al. 2003b).

The signaling pathways that regulate the actions of ANP are somewhat controversial. Silberbach *et al*. (Silberbach et al. 1999) found that the ERK pathway was stimulated by ANP in primary neonatal rat cardiomyocytes. In a more recent study, ANP was found to inhibit the MAPK-ERK pathway in immature rat cardiomyocytes (Hayashi et al. 2004). In both studies, ANP inhibited hypertrophic enlargement of the cardiomyocyte. These findings suggest that ANP restrains mitotic activity in fetal cardiomyocytes by altering the ERK pathway. Thus, to this point, studies had only found a piece or two of the ANP signaling puzzle for immature cardiomyocytes. To advance the field, ANP signaling was studied in isolated cardiomyocytes from fetal sheep (O'Tierney et al. 2010b).

Atrial natriuretic peptide is a 28 amino acid peptide that is manufactured within atrial cardiomyocytes in adult mammals. It is released into the blood stream when the atrial wall is stretched, as with volume expansion. In the periphery, it binds to a guanylyl cyclase coupled receptor (natriuretic peptide receptor; NPRA or Npr1) on the surface of many cell types. When bound, NPRA stimulates natriuresis and diuresis through its actions on the kidney and vasodilation through relaxation of vascular smooth muscle (reviewed by McGrath et al. 2005). ANP also binds the C-type receptor, NPRC, widely known as the clearance receptor, which leads to the internalization and degradation of the bound peptide. However, there is increasing evidence that NPRC has physiological functions other than clearance, perhaps playing a role in cellular growth regulation (Rose and Giles 2008).

During fetal life, ANP is manufactured and released from all chambers of the heart. The fetal ventricles produce ANP in a constitutive manner over the course of gestation. The fetal atrial myocardium releases ANP with stretch, similar to adult atria. The actions of ANP at the cellular and organ level are similar to those in the adult (Brace and Cheung 1987;Brace et al. 1988;Jaekle et al. 1995) but the circulating concentrations of ANP in the sheep fetus are some 5 times higher than found in the adult (Cheung et al. 1987;Rosenfeld et al. 1992).

In the adult, ANP is not expressed in the ventricles of the normal myocardium. However, ANP is expressed in the ventricles under conditions that lead to increased wall stress and hypertrophic growth and thus is a marker for pathological hypertrophic growth of the myocardium (Gardner 2003). This association has led some investigators to suppose that ANP is pro-hypertrophic. However, the opposite is true. Instead, it is now clear that the hormone suppresses hypertrophic growth of adult cardiomyocytes in response Ang II or increased wall stress (Rosenkranz et al. 2003;Hayashi et al. 2004). That ANP can keep myocardial growth in check has been shown by Knowles et al. (2001) and Holtwick et al. (2002) who found that NPRA knockout mice have larger hearts at birth than do their wild-type counterparts.

We performed experiments using isolated 135 gestational day ovine cardiomyocytes (term ~145 days) from both the right and the left ventricles to determine the degree to which ANP affects cardiomyocyte proliferation. We used bromodeoxyuridine (BrdU) as an index of proliferation. We also characterized the signaling pathways that were stimulated when ANP binds its primary receptor, NPRA.

Ovine cardiomyocytes have low rates of proliferation in serum free culture conditions—in the 2.0–3.0 % range over 48 hours (Sundgren et al. 2003b). When ANP was added to the serum free media, even at pharmacological levels, the proliferation of cardiomyocytes was

not changed from control conditions. Thus, ANP does not alter the baseline rates of proliferation that are found in the absence of stimulation.

As mentioned above, it is well known that Ang II and IGF-1 are powerful stimulators of cardiomyocyte proliferation both *in vivo* and *in vitro*. To address the question whether ANP augments or reduces proliferation of cardiomyocytes under stimulated conditions, a series of experiments were performed on isolated fetal ovine cardiomyocytes. The role of ANP and its second messenger, cyclic guanosine monophosphate (cGMP), in regulating cardiomyocyte proliferation was determined.

Figure 8 shows the dose-dependent inhibition of Ang II-stimulated cardiomyocyte proliferation by ANP. It is clear that the suppression of Ang II-stimulated proliferation is increased with increasing doses of ANP well into the pharmacological range. What is also striking about the data is the difference between the right and left ventricles. Right ventricular cardiomyocytes are more sensitive to increases in ANP than are left ventricular myocytes. This may mean that pro-growth hormones affect left ventricular myocytes more potently than right ventricular myocytes at this stage of development.

It is clear that either ANP or its second messenger alone, cGMP, suppresses proliferation (Figure 9). This figure also suggests that it is the NPRA receptor, which is known to signal through cGMP, that mediates the suppressive signal. However, the involvement of downstream cell signaling pathways such as ERK or PI3K in ANP's actions remains unresolved. The finding that ANP suppresses the Ang II-induced increase in phospho-ERK and phospho-AKT in cardiomyocytes from both right and left ventricles (Figures 10 & 11) supports an inhibitory effect of ANP on the ERK and PI3K pathways in the immature heart. Thus, ANP acts as a "brake" on fetal heart growth in response to growth-promoting stimuli.

## Thyroid Hormone

In 1912, J.F. Gudematsch, a Cornell anatomist discovered that adult thyroid gland extract stimulated the rapid metamorphosis of tadpoles into frogs (Brown and Cai 2007). It is now known that the dramatic metamorphosis of the aquatic tadpole into a terrestrial frog is under the control of thyroid sensitive genes. Mammals do not, of course, develop by metamorphosis but many of the thyroid receptor forms and their binding partners are conserved from fish to mammals (Bertrand et al. 2004). Fetuses live in an aquatic environment before birth and must also prepare for postnatal terrestrial life. Thus, it is possible that there are $T_3$-driven developmental processes in fetal organs that remain undiscovered.

Thyroid releasing hormone (TRH) is secreted in the hypothalamus and stimulates the manufacture and release of thyroid stimulating hormone (TSH, or thyrotropin) by the anterior pituitary. The latter regulates TH production and its release by the thyroid gland. $T_3$ binds the thyroid receptors with an affinity about 20–30 times higher than that of $T_4$ (Samuels et al. 1979;Schueler et al. 1990) and is considered the "active form" of the thyroid hormone. The binding affinities of thyroid hormone receptors for $T_3$ and $T_4$ have not been studied in the fetal heart. It is known, however, that during the second half of gestation in fetal mammals, the hypothalamic-pituitary-adrenal-thyroid axis matures progressively. This results in an ever-changing hormone profile in fetal plasma from mid-gestation through late gestation until delivery. The result is an exponential increase pre- and post-natal $T_3$ concentration in plasma (Polk 1995). This surge coincides with the critical window of development during which the fetal heart undergoes terminal differentiation. The actions of thyroid hormone are largely determined by the availability of the biologically potent form, $T_3$. The iodothyronine deiodinases types I, II, and III (D1, D2, and D3, respectively) are responsible for removal of iodine moieties from precursor molecules to activate or degrade

the precursor form of the hormone, $T_4$. Their actions have been nicely reviewed by Bianco and Kim (2006). The enzymes are members of a group of dimeric integral membrane thioredoxin fold-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 an iodine group. In the fetus D2 generates the active form of thyroid hormone, $T_3$, via deiodination of $T_4$. D3 inactivates $T_3$ by conversion to $T_2$ and by metabolizing $T_4$ to reverse $T_3$ ($rT_3$). Finally, D1 activates or inactivates $T_4$ but is relatively ineffective on an equimolar basis and its importance in the fetus has yet to be clarified. Thyroid hormones can also undergo alternate routes of metabolism including glucuronidation, ether bond cleavage, oxidative deamination and reversible sulfation.

There is no controversy surrounding the fact that maternal TH levels influence fetal levels in humans. However, the sheep placenta appears to be less permeable to thyroid hormones. The actual mechanism by which the transplacental transport of thyroid hormone is regulated during human pregnancy has not yet been determined. There are at least six important pieces of the puzzle in humans: 1) $T_3$ is relatively lipophilic and crosses cell membranes (Elkins and Edwards 1988); 2) maternal blood levels of thyroid hormone strongly influence fetal levels (Burrow et al. 1994). 3) the $T_3$/$T_4$ transporter, monocarboxylate transporter 8 (MCT8), is expressed in the human trophoblast throughout gestation and is upregulated in placentas of intrauterine growth retarded (IUGR) babies (Chan et al. 2006); 4) the placenta contains the D2 and D3 deiodinases which can deactivate $T_3$ and $T_4$ (Chan et al. 2003); 5) thyroid hormone is important in placental development and function (Laoag-Fernandez et al. 2004;Oki et al. 2004;Barber et al. 2005); 6) IUGR is associated with low $T_3$ levels (Chan et al. 2006); and 7) the stimulatory maternal autoantibodies against TSH receptor that lead to Graves disease (Gupta 1992), are transported by the placenta with other IgG class antibodies into the fetal circulation where they have similar effects as those found in the adult.

Figure 12 shows that the *in vitro* proliferation of near term fetal cardiomyocytes in the presence of a growth stimulant (medium supplemented with 10% fetal bovine serum) is increasingly suppressed with increasing levels of $T_3$ over a 48 hour period. In addition, the cell cycle promoter, cyclin D1 and the cell cycle suppressor, p21, are down- and up-regulated respectively in a dose dependent fashion (Figure 13). Thus, there is evidence that thyroid hormone is instrumental in regulating the growth of the myocardium by mechanisms that ordinarily regulate the cell cycle. Because $T_3$ promotes the maturation of cardiomyocytes by slowing proliferation and because $T_3$ levels rise over the last few weeks of gestation, there is good reason to suggest that $T_3$ is the driving stimulus for the terminal differentiation of fetal cardiomyocytes.

## Placental Insufficiency and Heart Development

The sheep model of placental insufficiency has been used to investigate the role of placental function on fetal organ development. Two different animal models in sheep yield similar results. Sheep are different from humans and many animals in that the sheep placenta can be formed only at a hundred or so specialized sites called caruncles. In one model of placental insufficiency removal of the majority of maternal uterine implantation sites before pregnancy means there are so few sites available at which the placenta can be established that overall placental mass is dramatically reduced. In these circumstances, fetal growth becomes slower than normal when fetal size approaches the capability of the compromised placenta to provide oxygen and nutrients. Another well studied model of placental insufficiency is experimentally produced in fetal sheep that have reached at least mid-term in development. Small plastic or Sephadex inert microspheres are injected into the supply arteries of the fetal placenta to gradually obstruct micro-vessels within the placenta. Outcomes approximate those found in growth restricted human fetuses including

hypoxemia, hypoglycemia and hypercortisolemia (Murotsuki et al. 1996;Cock et al. 2001). Embolization leads to other fetal changes that are in common with human growth restriction syndromes including reduced, absent or reversed diastolic flow in the umbilical artery (Trudinger et al. 1985;Morrow et al. 1989). This flow pattern is found when placentas offer a high resistance to flow or, more precisely, high impedance against pulsatile flow. Because fetal cardiac myocytes are sensitive to sustained loading conditions (Barbera et al. 2000) one might argue that placental insufficiency would increase systolic load to the heart and cause myocardial hypertrophic growth and increased binucleation as discussed above.

However, that is not the case with models of placental insufficiency. In the two models discussed here, hearts responded similarly (Louey et al. 2007;Morrison et al. 2007). Both experimental methods led to a fetal growth restriction and reduced birth weight. Fetuses became hypoxemic but not hypertensive. The cardiomyocytes from hearts of undergrown fetuses had normal sized mononucleated and binucleated cardiomyocytes. In the placental embolization model, the percentage of cells within the cell cycle was greatly suppressed (Louey et al. 2007) and in both models, fewer cells were binucleated than their age-matched controls indicating that the suppression of fetal body growth was accompanied by a less mature myocardium (Figure 14).

There is evidence that placental insufficiency may predispose the fetal heart for heart failure in later life. In a birth cohort of 13,345 people born in Helsinki, Finland from 1934–44, those that developed heart failure had small placentas (Barker et al. 2010). The findings from fetal sheep studies suggest that reduced placental mass leads to hearts with immature myocytes with low cell cycle activity among mononucleated cells. If these hearts are unable to generate myocytes as rapidly as required to populate the myocardium to a normal level before cell proliferative activity ceases, they may have fewer cardiomyocytes than is optimal for life and be more vulnerable for heart failure in later life.

## Programming Mechanisms

Two decades of research show that nutritional and/or hypoxic stress in the womb lead to chronic disease in the exposed offspring. The process by which embryonic or fetal responses to a stressor generate a pathway of disease vulnerability in the adult is known as programming. Programming is thought to underlie a significant portion of cases of chronic disease in adults worldwide. Low birthweight 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 1999), hypertension (Barker and Osmond 1988), hypercholesterolemia (Barker et al. 1993), and hypercoagulopathies (Fall et al. 1995;Martyn et al. 1995). Babies that were undergrown in the womb are prone to have hearts that suffer detrimental changes. Crispi et al. (2010) showed that the hearts of 5 year olds who grew slowly before birth had changes in cardiac shape, reduced stroke volume and increased heart rate. The hearts were also characterized by abnormal systolic and diastolic function. The children with fetal growth restriction also had elevated blood pressure and increased intimal thickness of major arteries.

The fetal response to an inadequate nutritional supply is complex. However, studies in the fetal sheep model show clearly that the cardiomyocyte population is affected not only through changes in cardiac loading conditions but also by the chemical environment. Undergrown fetuses are able to slow the growth and maturation of their hearts to the extent that one must suspect that long term cardiac health is at stake. The finding that fetal growth restriction induces primary cardiac and vascular changes is likely to explain the increased predisposition of small babies to cardiovascular disease in adult life.

The mechanisms by which cardiovascular diseases are determined in the womb are unclear. However, there is increasing evidence that two processes underlie the risk for chronic disease in later life. The first is the trading off of fetal organ structure. The reduction in the number of nephrons in the kidney with fetal undernutrition is an example. Low nephron number is associated with hypertension in the adult. The fact that people who were born small have coronary arteries with a reduced diameter is another example. In addition to structural tradeoffs, a second mechanism that has long term disease consequence is the epigenetic modification of genes during development. The epigenetic modification of gene promoter regions by the increased methylation of cytosines and/or modification of histone proteins in the nucleosome are examples of epigenetic modification that have been demonstrated in animal models of undernutrition and hypoxia (Pogribny et al. 1995;Thornburg et al. 2010). The role of microRNA species in the modification of DNA transcription is another possible mechanism for epigenetic control.

Recent evidence suggests that a week of intrauterine hypoxia leads to diminished protection of the rat myocardium against ischemia reperfusion injury in the once hypoxic adult through down regulation of the injury protecting phosphokinase C-epsilon (PKCξ) gene. This down regulation is apparently accomplished by excess methylation of specific SP-1 sites in the promoter region of the PKCξ gene during development (Patterson et al. 2010). In the coming years, it is likely that a host of epigenetic links between prenatal conditions and adult onset diseases will be discovered.

## Conclusions

The growing myocardium is populated by cardiomyocytes that proliferate rapidly over the first two thirds of gestation. During the last third of gestation, an increasing percentage of the myocyte population becomes binucleated and permanently exits the cell cycle. In response to increased wall stress, systolic pressure loading mildly increases cardiomyocyte proliferation but also increases the rate at which cardiomyocytes become terminally differentiated. That the immature myocardium senses wall stress is suggested by the loss of growth of the myocardium when systolic pressure is chronically reduced. A reduction in wall stress with decreased arterial pressure in the fetus leads to reduced cardiomyocyte proliferation and binucleation. This response is very similar to the effects of placental insufficiency where arterial pressures remain normal. By definition, placental insufficiency leads to reduced placental gas exchange and some degree of hypoxemia. However, placental nutrient transport is also affected in an environment of reduced levels of circulating IGF-1, increased cortisol and increased levels of ANP. The outcome of placental insufficiency in fetal sheep is a heart with fewer cardiomyocytes and less mature cardiomyocytes than found in age matched controls.

Thyroid hormone, in the form of $T_3$, and ANP have similar effects on isolated fetal cardiomyocytes. They both, through very different signaling pathways, prevent the proliferation of cardiomyocytes under the stimulatory actions of receptor-mediated growth factors. It is highly likely that the combination of structural changes in the myocardium and epigenetic down-regulation of protective genes that occur under conditions of low oxygen, high glucocorticoid levels and malnourishment is responsible for the vulnerability of the hearts of people who suffered insults before birth.

## Acknowledgments

**Funding**

This study was supported by funds from NICHD P01 HD 34430, NHLBI R21 HL093617, and R01 HL102763. Dr. Natasha Chattergoon was supported by NHLBI training grant T32HL094294. Dr. Sonnet Jonker was supported by

Office of Research on Women's Health and the National Institute of Child Health and Human Development, Oregon BIRCWH HD043488. Dr. Thornburg was supported by the M. Lowell Edwards Endowment.

The authors thank the M. Lowell Edwards Endowment, as well as Mr. Robert Webber and Ms. Loni Socha for excellent technical assistance.

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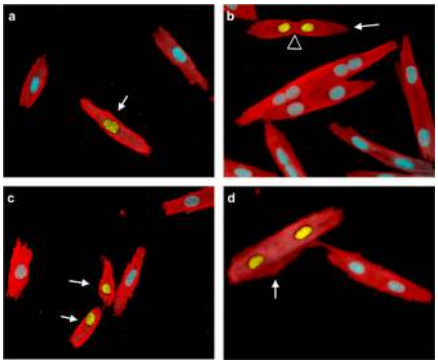


**Figure 1.**
Cardiac myocytes from the fetal sheep with immuno-fluorescence staining for myosin (red; primary antibody Abcam ab15) and a Hoechst nuclear marker (cyan). Myocytes were also probed with an antibody against Ki-67 (DAB staining shown in yellow for contrast). The types of myocytes observed include, (A) a mononucleated myocyte positive for Ki-67 (arrow). (B) a cleavage furrow is clearly visible (arrowhead); (C) a dividing myocyte positive for Ki-67 (arrows) (D) a myocyte containing two nuclei that is positive for Ki-67 (arrow). Jonker, S.S. *et al. J. Appl. Physiol.* 2007. 102: 1130–1142.

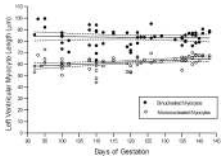

**Figure 2.**
Lengths of fetal sheep LV cardiac myocytes throughout the last third of gestation. The best-fit linear regression line and 95% confidence intervals of each data set are shown. Jonker, S *et al. J. Appl. Physiol.* 2007. 102: 1130–1142.

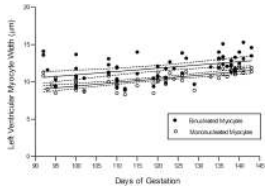


**Figure 3.**
Widths of fetal sheep LV cardiac myocytes throughout the last third of gestation. The best-fit linear regression line and 95% confidence intervals of each data set are shown. Jonker, S.S. *et al. J. Appl. Physiol.* 2007. 102:1130–1142.

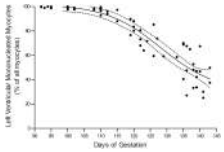

**Figure 4.**
The proportion of total LV cardiac myocytes that are mononucleated during the last third of gestation. The proportion of mononucleated myocytes begins to decrease after ~100dGA. Most of these myocytes become binucleated. Shown are Boltzmann's sigmoidal equations fit to each data set and the 95% confidence intervals. Jonker, S.S. *et al. J. Appl. Physiol.* 2007. 102: 1130–1142.

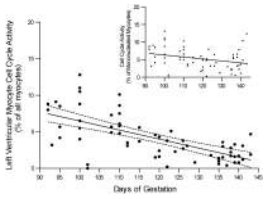


**Figure 5.**

The proportion of all cardiac myocytes that are in the cell cycle decreases towards term. *Inset panel:* Cell cycle activity (Ki-67) normalized to the proportion of myocytes that are mononucleated and thus capable of proliferating or becoming terminally differentiated. The best-fit linear regression line and 95% confidence intervals are shown. Jonker, S.S. *et al. J. Appl. Physiol.* 2007. 102: 1130–1142.

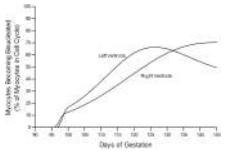

**Figure 6.**

The calculated total number of cardiac myocytes increased throughout the last third of gestation in the LV. The number of binucleated myocytes increased at the expense of mononucleated myocytes, which declined in number following ~115dGA. The proportion of myocytes becoming binucleated (rather than proliferating) increased rapidly after ~100dGA. Jonker, S.S. *et al. J. Appl. Physiol.* 2007. 102: 1130–1142.

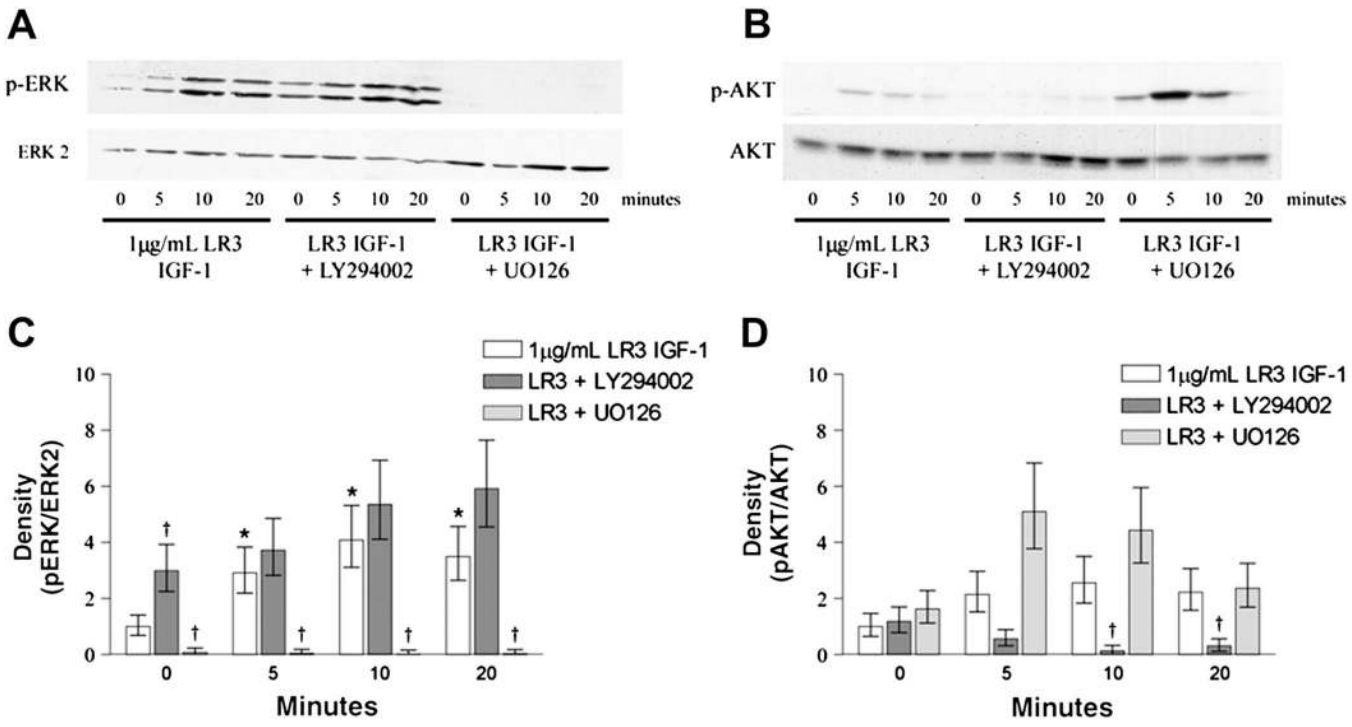


**Figure 7.**

IGF-1 receptor (IGF1R) signaling in cardiomyocytes. Isolated near-term fetal sheep cardiomyocytes were treated with the IGF-1 analog Long R3 IGF-1 (LR3 IGF-1, 1μg/ml), the PI3K inhibitor LY294002 (10 μM) and the MEK inhibitor UO126 (10 μM). Panels A and B show a representative Western blot for ERK (*A*) and AKT (*B*) stimulation. *C* and *D*: values are plotted as median fold change ± SE of blot density normalized to baseline LR3 IGF-1 stimulation (0 min, *n*=4). *C*: density is plotted in normalized units of phospho-ERK (p-ERK)/ERK2. *D*: density is plotted in normalized units of phospho-AKT (p-AKT)/ AKT. *P<0.05 compared with within-treatment baseline. †P<0.05 compared with LR3 IGF-1 treatment at same time point. Sundgren, N.C. *et al. Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2003. 285: R1481–R1489.

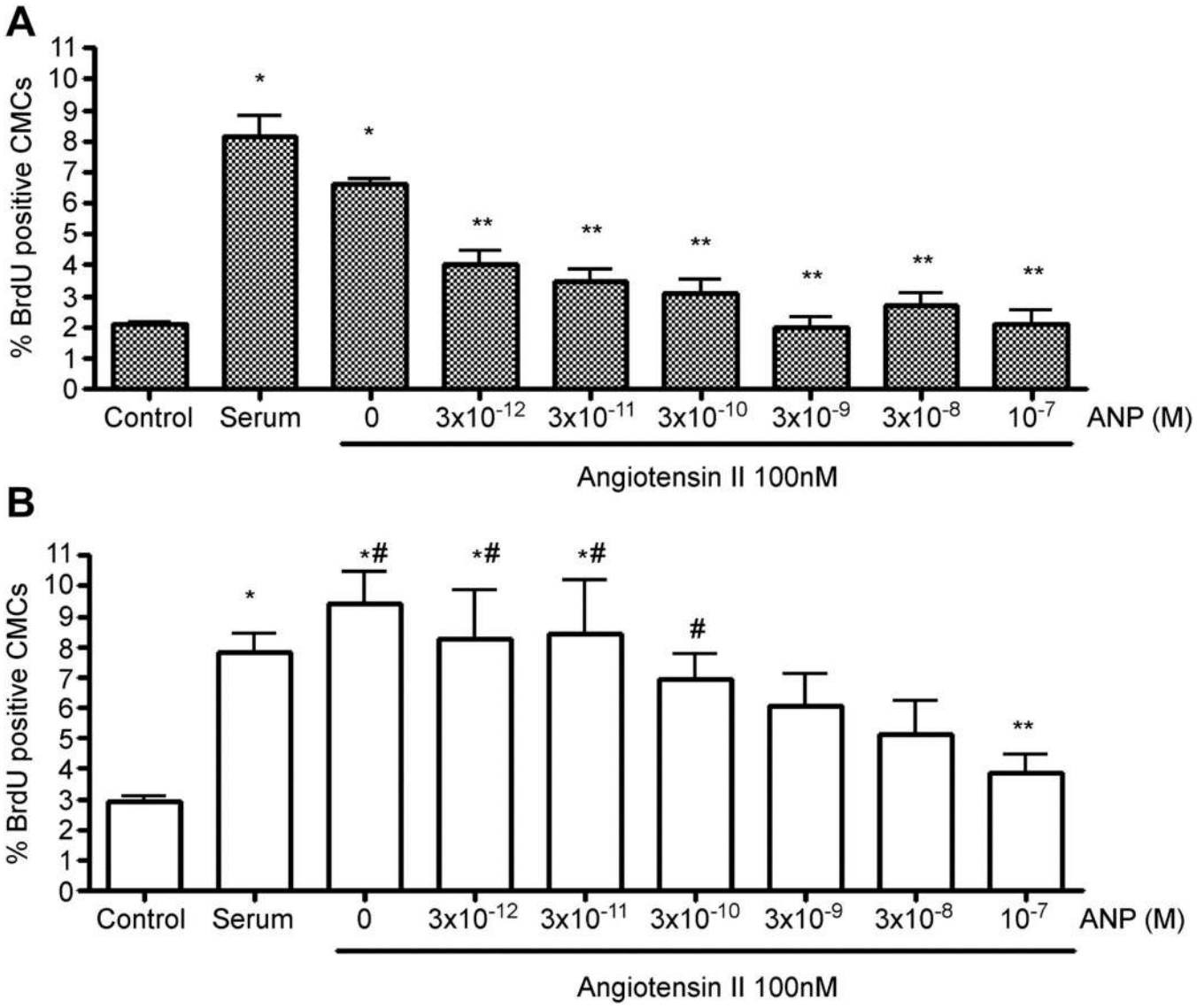


**Figure 8.**

Ang II-stimulated BrdU uptake in cardiomyocytes isolated from the right (A) and left (B) fetal ventricles was inhibited by ANP in a dose-dependent manner (48h treatment). Left ventricular cardiomyocytes were less sensitive to ANP than were right ventricular cardiomyocytes. Data are means ± SEM. *n*=6 fetuses per group. $^{\#}$P<0.05 vs. matching treatment in RV; *P<0.05 vs. serum-free control in same ventricle; $^{**}$P<0.05 vs. Ang II alone in same ventricle. O'Tierney, P.F. *et al. J*ournal of Physiology, 2010. *588*, 2879–2889.

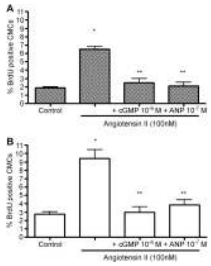

**Figure 9.**

Ang II-stimulated BrdU uptake in cardiomyocytes isolated from right (A) and left (B) fetal ventricles was inhibited by 48 hours of treatment with the cGMP analog, 8-bromo-cGMP ($10^{-6}$M) and atrial natriuretic peptide (ANP, $10^{-7}$M). Control cells were cultured in serum-free conditions. Data are means ± SEM. *n*=5 or 6 fetuses per group. *P<0.05 vs. control; **P<0.05 vs. Ang II alone. O'Tierney, P.F. *et al. J*ournal of Physiology, 2010. *588*, 2879–2889.

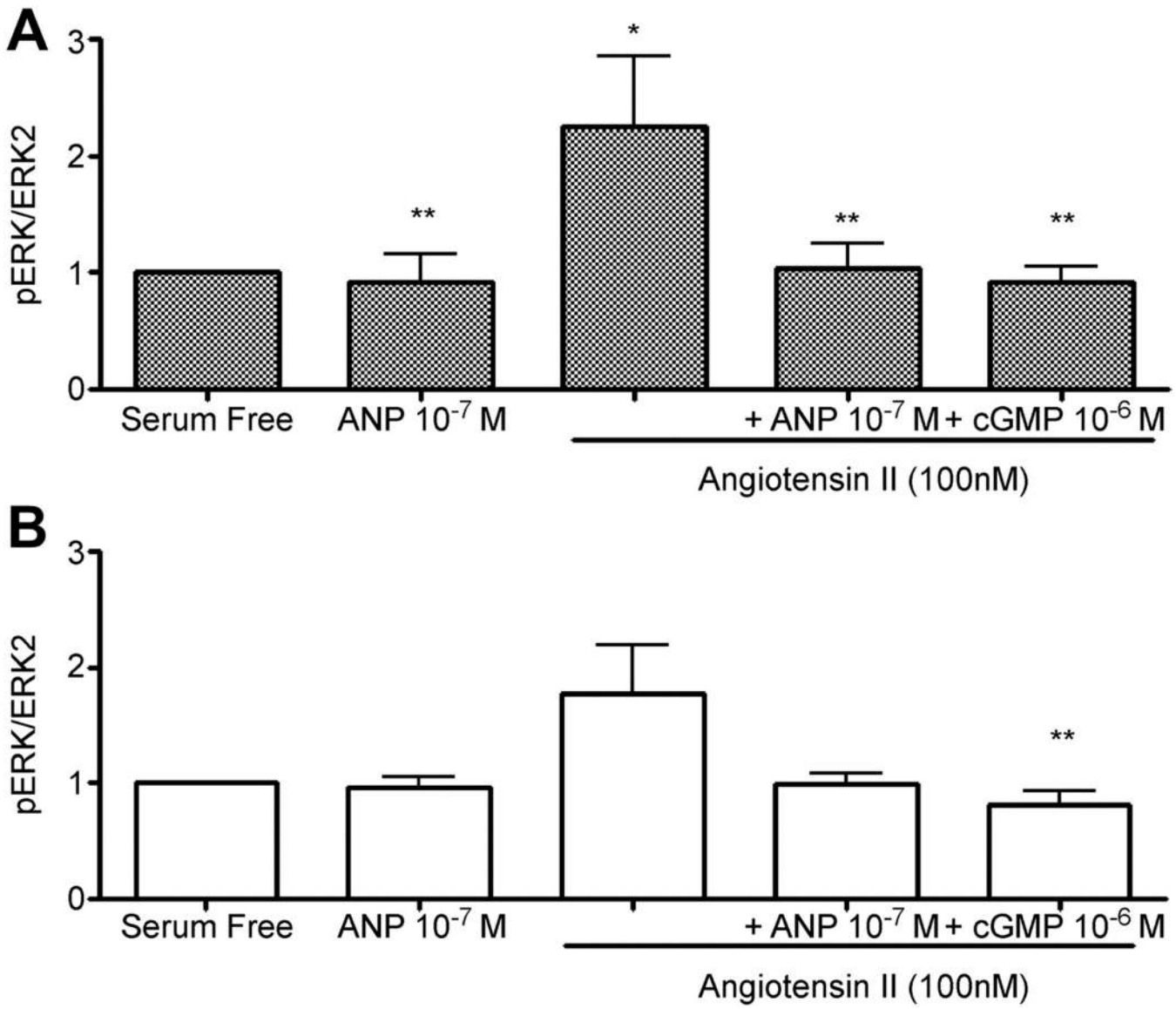


**Figure 10.**

ANP inhibits Ang II-induced ERK phosphorylation in fetal cardiomyocytes isolated from the right ventricle (A) with a similar trend for the left ventricle (B). cGMP suppresses the generation of phospho-ERK in right and left ventricular cardiomyocytes. Data are means ± SEM. *n*=5 or 6 fetuses per group. All treatments were 10 minutes. *P<0.05 vs. serum-free control; **P<0.05 vs. Ang II alone. O'Tierney, P.F. *et al. J*ournal of Physiology, 2010. *588*, 2879–2889.

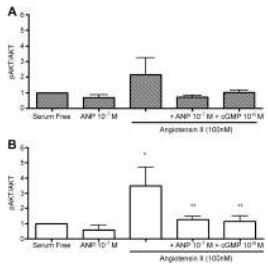


**Figure 11.**

The effects of ANP on Ang II-induced AKT phosphorylation in fetal cardiomyocytes isolated from the right (A) and left (B) ventricles. AKT phosphorylation was significantly increased in the presence of Ang II, an effect which was suppressed by ANP in the left ventricle (B) with a similar trend in the right ventricle (A). All treatments were 10 minutes. Data are means ± SEM. *n*=5 or 6 fetuses per group. *P<0.05 vs. serum-free controls; **P<0.05 vs. Ang II alone. O'Tierney, P.F. *et al. J*ournal of Physiology, 2010. *588*, 2879–2889.

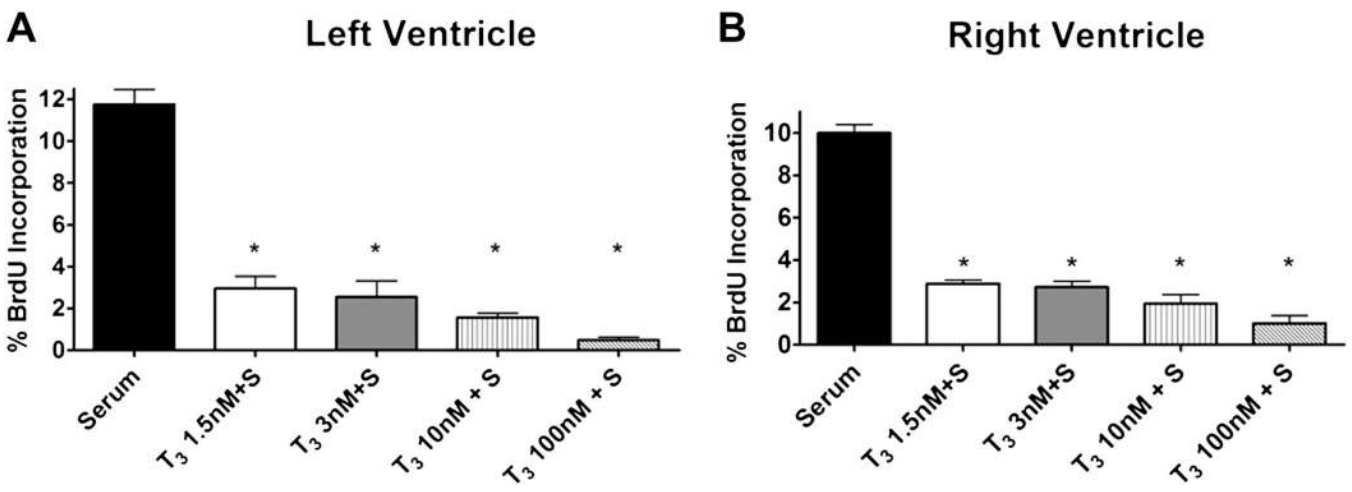


**Figure 12.**

BrdU (10uM) incorporation in isolated (A) LV and (B) RV fetal sheep cardiomyocytes with 10% FBS-serum media challenge. $T_3$ at all doses (in serum media; S) significantly inhibited BrdU uptake. Data are means ± SEM. *n*=5 fetuses per group. *P<0.001 vs serum. Chattergoon, N.N. *et al. J. Endocrinol*. 2007. 192:R1–8.

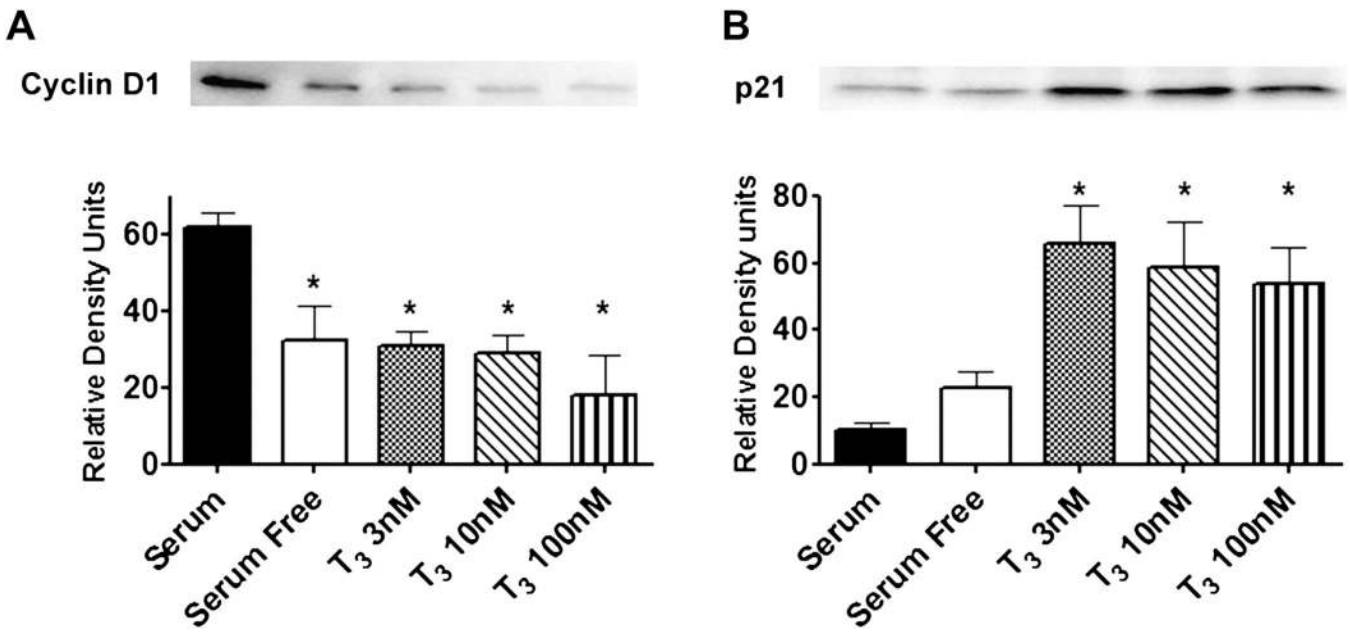


**Figure 13.**

Cyclin D1 and p21 expression (A, B respectively) in LV cardiomyocytes following $T_3$ treatment. The same trend is seen in RV cardiomyocytes. A: Cyclin D1 expression was significantly decreased in all $T_3$ treated cardiomyocytes vs serum controls. B: p21 expression significantly increases in $T_3$ treated cells compared to Serum. Data are means ± SEM. *n*=4 fetuses per group.*P<0.05 vs. Serum. Chattergoon, N.N. *et al. J. Endocrinol.* 2007; 192:R1–8.

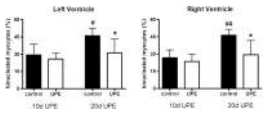

**Figure 14.**

Proportion of binucleated cardiomyocytes in the left and right ventricles of control fetuses (n=6 in both age groups) and those subjected to umbilicoplacental embolization (UPE, n=5 in both age groups) at 125d GA (after 10d UPE) and 136d GA (after 20d UPE). Control fetuses demonstrate the normal increase in the proportion of cardiomyocytes that are binucleated; UPE fetuses do not show this normal gestational increase and thus after 20d of UPE fetuses have a relatively immature myocardium. Data are means ± S.D., *P<0.05 *versus* age-matched control, #P<0.05, ##P<0.01 *versus* 125d GA control. Louey, S. et al. *J Physiol*. 2007; 580: 639–648.