Endocrine and other physiologic modulators of perinatal cardiomyocyte endowment

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Abstract

Immature contractile cardiomyocytes proliferate to rapidly increase cell number, establishing cardiomyocyte endowment in the perinatal period. Developmental changes in cellular maturation, size and attrition further contribute to cardiac anatomy. These physiological processes occur concomitant with a changing hormonal environment as the fetus prepares itself for the transition to extrauterine life. There are complex interactions between endocrine, hemodynamic and nutritional regulators of cardiac development. Birth has been long assumed to be the trigger for major differences between the fetal and postnatal cardiomyocyte growth patterns, but investigations in normally growing sheep and rodents suggest this may not be entirely true; in sheep, these differences are initiated before birth, while in rodents they occur after birth. The aim of this review is to draw together our understanding of the temporal regulation of these signals and cardiomyocyte responses relative to birth. Further, we consider how these dynamics are altered in stressed and suboptimal intrauterine environments.

Key Words
- heart development
- cardiomyocyte growth
- terminal differentiation
- angiotensin II
- insulin-like growth factor 1
- cortisol
- thyroid hormone
- hypoxia
- hypertension
- birth

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Introduction

Since the late 20th century it has been recognized that, near the time of birth, growth of the mammalian heart transitions from proliferation of contractile cardiomyocytes to cellular enlargement (Adler 1975, Bishop & Hine 1975, Clubb & Bishop 1984, Rumyantsev 1991). The number of cardiomyocytes continuously decreases with age in adulthood and, contribution of stem cell-derived myocytes notwithstanding, progressive loss of adult myocytes is associated with heart failure (Olivetti *et al.* 1991, Pessanha & Mandarim-de-Lacerda 2000, Levkau *et al.* 2008, Laflamme & Murry 2011). Thus, perinatal cardiomyocyte endowment is consequential for life-long health. Factors in the fetal environment resulting from both normal and stressed pregnancies act on the developing heart to modulate cardiomyocyte number. Many of these regulatory factors change dramatically at or near the time of birth.

Proliferation, terminal differentiation, attrition and cellular enlargement are processes that must all be considered in the regulation of cardiomyocyte number and the size of the developing heart. Each of these cellular processes can be to some extent governed separately. Regulatory factors may effect different outcomes in a suboptimal intrauterine environment compared to normal development. Thus, it is critical to understand the dynamics of normal cardiomyocyte growth during this period before we can fully appreciate the outcomes following prenatal stress. The chronically catheterized fetal sheep model has enabled a detailed examination of how the fetal cardiac environment shapes cardiomyocyte

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growth, maturation and endowment. The purpose of this review is to summarize what is known about endocrine and other regulators of growth and maturation of cardiomyocytes in the immature ovine heart and how these factors may contribute to normal development.

Sheep as a model of cardiac development

Our knowledge about *in vivo* regulation of fetal cardiomyocytes depends heavily on animal models because cardiac biopsies are rarely available from healthy human infants. Likewise, sampling of blood and direct hemodynamic measurements of the healthy human fetus are very rarely undertaken. The fetal sheep model has filled this gap because it tolerates chronic surgical instrumentation, allowing serial blood sampling and measurement of hemodynamic factors. Additionally, altered endocrine or hemodynamic fetal environments can be experimentally produced to investigate the regulation of cardiac outcomes.

Specific interspecies differences must be borne in mind when extrapolating knowledge of sheep cardiac development to the human. Adult sheep are a good model of the adult human cardiac function (Milani-Nejad & Janssen 2014), and form the basis for much of our knowledge of cardiac function in the immature heart (Rudolph 2009). Sheep have a dissimilar placenta to humans, which may influence the materno-fetal nutritional, hemodynamic and hormonal milieu guiding cardiac development (Carter 2007, Barry & Anthony 2008). While most human pregnancies are singletons, sheep commonly bear singletons or twins; multiple pregnancy in sheep affects placental nutrient transfer, and consequently reduces fetal growth, although the magnitude of this effect is only half that as occurs in human twinning (Gardner *et al.* 2007, The *et al.* 2010, van der Linden *et al.* 2013). The same hormones are responsible for maintenance of pregnancy and initiation of parturition in humans and sheep, and some of these hormones influence heart growth (in sheep, there is late gestation systemic withdrawal of progesterone that is unmatched in humans; Challis *et al.* 2000). The gestational period of a sheep is slightly more than half that of a human, long enough for good resolution of time-dependent growth-regulating effects in experimental studies. Sheep are born somewhat more mature than humans in regards to their ability to stand and walk, but many of their physiological systems have similar relative rates of development, including kidney and brain (Hinchliffe *et al.* 1992, Gimonet *et al.* 1998, Back *et al.* 2006). Notably, in the perinatal periods of humans and sheep, cardiomyocyte numbers experience similar plateaus, cardiomyocyte cell cycle activity declines similarly, and polyploidy or binucleation increase rapidly (Adler & Costabel 1975, Adler & Costabel 1980, Kim *et al.* 1992, Huttenbach *et al.* 2001, Burrell *et al.* 2003*a*, Jonker *et al.* 2015). In this review we focus on data from humans and sheep, except where critical data is available only from other species.

Modes of growth in the fetal sheep heart

Proliferation

Proliferation of immature contractile cardiomyocytes is well established in all vertebrate orders (Rumyantsev 1991), and is responsible for increasing ovine cardiomyocyte number throughout gestation and the perinatal period (Fig. 1 and 2A and C; Burrell *et al.* 2003*b*, Jonker *et al.* 2007*a*, Jonker *et al.* 2015). It has long been thought that in mammals this process ends around the time of birth, and that cardiomyocyte endowment for life is set in the prenatal period (Thornburg *et al.* 2011). New evidence suggests that proliferation of contractile cardiomyocytes increases cell number in neonatal sheep (Jonker *et al.* 2015) and even in the juvenile human (Mollova *et al.* 2013). Although division of contractile cells in the adult heart has been highly debated, stem cell replacement of cardiomyocytes is now recognized to occur (reviewed in Laflamme & Murry 2011). Nevertheless, net cardiomyocyte number declines continuously between young adulthood and senescence in the human

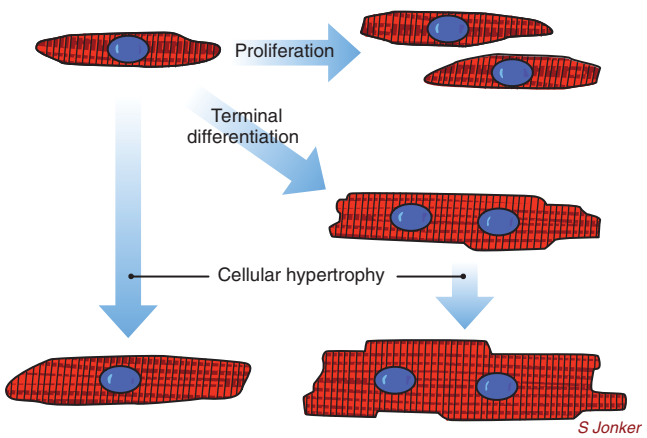

Figure 1
Modes of cardiomyocyte growth in the fetal heart. Mononucleated cardiomyocytes have the potential to proliferate. They can also become binucleated, indicative that they are unable to undergo further cytokinesis. They can, however, replicate their DNA and become polyploid (not shown). Both mononucleated and binucleated cardiomyocytes can undergo cellular enlargement, or apoptosis (not shown).

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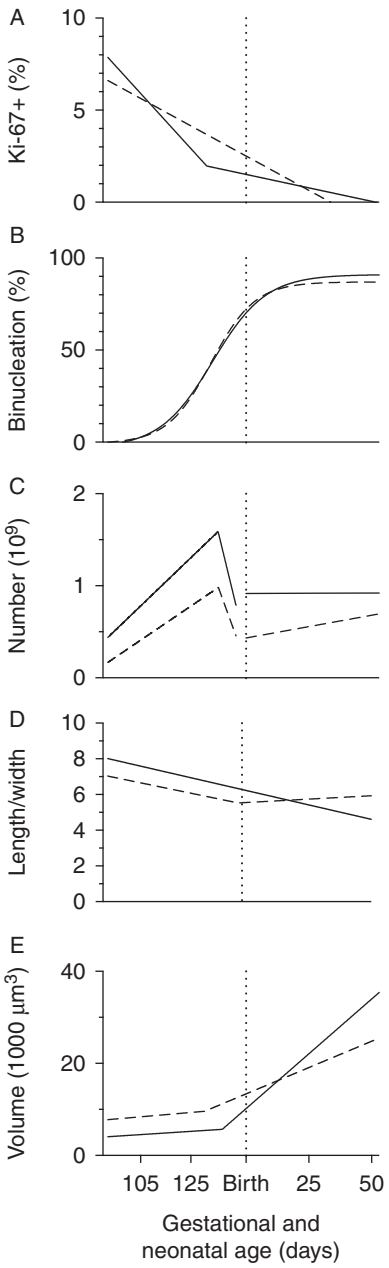

Figure 2
Regulation of cardiomyocyte growth and maturation in the normally-growing fetal and neonatal sheep left ventricle (LV; solid line) and right ventricle (RV; dashed line). Cell cycle activity as assessed by Ki-67 positivity (A) supports cardiomyocyte proliferation and terminal differentiation. Binucleation of cardiomyocytes (B) is an index of terminal differentiation, after which cells infrequently enter the cell cycle, and even less frequently undergo cytokinesis. Consequent to myocyte proliferation, cell number (C) increases rapidly in the fetus (and slowly in the neonatal RV). Cell attrition reduces myocyte number prior to birth in both ventricles. Cardiomyocytes typically grow more rapidly in width than length, decreasing their length-to-width ratio (D), except after birth in the neonatal RV. Myocyte volume (E) increases slowly in the fetus, but more rapidly after birth (much more so in the LV). Most changes in cell growth and maturation rates occur before birth. Data from Jonker *et al.* (2007*b*) and Jonker *et al.* (2015).

(Olivetti *et al.* 1991), confirming the importance of cardiomyocyte proliferation in the young heart.

Immature contractile cardiomyocytes that proliferate in perinatal mammals are typically mononucleated and diploid (2N; Clubb & Bishop 1984, Rumyantsev 1991, Jonker *et al.* 2007*a*). This cardiomyocyte phenotype predominates in the fetal human (Adler & Costabel 1980) and sheep (Burrell *et al.* 2003*a*, Jonker *et al.* 2007*a*, Bensley *et al.* 2010, Jonker *et al.* 2015). In the large mammal, it is unknown whether mononucleated, diploid cardiomyocytes at a given age are all equally proliferative. In the embryonic mouse heart, lineages of cells form related clusters layered in epicardial-to-endocardial columns (Pijnappels *et al.* 2010), implicating proliferative contribution from many or most cardiomyocytes. Cell culture experiments also suggest that most rat cardiomyocytes proliferate at mid-gestation, but fewer do so at term (Burton *et al.* 1999). This evidence suggests that many or most mononucleated cardiomyocytes increase cell number by proliferation through at least mid-gestation, after which fewer of these cells may carry out most of the proliferative activity in the heart.

Terminal differentiation

Terminal differentiation involves permanent cell cycle arrest, or senescence, after which DNA replication followed by cytokinesis no longer occurs (Figs 1 and 2B). The intracellular mechanisms regulating terminal differentiation have not been conclusively determined, but candidate genes have been described (Paradis *et al.* 2014). Terminal differentiation of cardiomyocytes appears to be tightly regulated, as evidenced by the exceeding rareness of rhabdomyoma and rhabdomyosarcoma primary cardiac tumors, which are found only in about 1 of 6000 human autopsies (Lam *et al.* 1993). Supporting this conclusion is a higher incidence of cardiac tumors found in fetuses, in which cardiomyocyte proliferation is active (Holley *et al.* 1995, Uzun *et al.* 2007).

Cell cycle activity, as detected by Ki-67, phosphohistone-3, proliferating cell nuclear antigen (PCNA) and other markers of mitosis, leads to DNA replication concluding in either cytokinesis or stable binucleation. Because of this, and the observation that binucleated cardiomyocytes are rarely noted to have reentered the cell cycle, terminal differentiation has been synonymous with binucleation in the sheep and rodent (Clubb & Bishop 1984, Soonpaa *et al.* 1996, Barbera *et al.* 2000, Burrell *et al.* 2003*a*, Jonker *et al.* 2015). In humans, failure of nuclear division following DNA replication frequently results in

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polyploid nuclei (4N, 8N or greater; Brodsky *et al*. 1994, Adler *et al*. 1996). Polyploidy of cardiomyocytes also occurs in sheep and rodents, but to a much lesser extent (Adler *et al*. 1996, Bensley *et al*. 2010). It is unknown if polyploidy also indicates terminal differentiation.

Whether binucleation (and perhaps polyploidy) are incidental or integral to terminal differentiation is also unknown. Cell culture experiments suggest that the majority of cardiomyocytes from term fetal mice have very little proliferative potential and only a subset retain the abundant potential of the mid-gestation heart (Burton *et al*. 1999). This implies that many myocytes have withdrawn from proliferative activity prior to binucleation (Burton *et al*. 1999). Further, cardiomyocyte proliferative activity in mice is temporally separated from binucleation, again suggesting that terminal differentiation may precede binucleation or polyploidy (Soonpaa *et al*. 1996).

Apoptosis

A counterpoint to cellular proliferation is the process of programmed cell death, or apoptosis. Transitory cardiomyocyte apoptosis has been described to prune the rodent heart at birth (Kajstura *et al*. 1995, Fernandez *et al*. 2001). This cell loss is described as especially heavy in the right ventricle (RV), and is thought to play a role in remodeling the heart in the context of postnatal hemodynamic patterns. Indeed, rates of myocardial apoptosis are also higher in week-old lambs than 6–8 week-old lambs (Karimi *et al*. 2004), suggestive of higher perinatal receptiveness to apoptotic signals. However, in normally-developing sheep, the perinatal bulk attrition of cardiomyocytes may occur immediately prior to birth (Fig. 2C; Jonker *et al*. 2015). Although the sheep heart is sensitive to pro-apoptotic stimuli in late gestation and in the early neonate, the ontogeny of apoptosis in the perinatal heart (especially in the large mammal) and its relative contribution to the changing cellular landscape during this transitory period remains poorly understood (Bae *et al*. 2003, Hammel *et al*. 2003, Caldarone *et al*. 2004, Karimi *et al*. 2004).

Cellular enlargement

Cellular enlargement, or hypertrophy, is the primary growth mode of cardiomyocytes in the adult heart, whether in response to exercise, cell loss with aging, or as part of a disease process (White *et al*. 1987, Adler *et al*. 1996, Kramer *et al*. 1998). Fetal cardiomyocytes are small compared to those of the adult and, in the normally growing fetus, change little in size until after birth (Fig. 2D and E; Smolich *et al*. 1989, Burrell *et al*. 2003*a*, Jonker *et al*. 2015). However, when stimulated, cardiomyocytes can increase both in length and width in the near-term fetus (Barbera *et al*. 2000, Jonker *et al*. 2007*b*). Increasing the width of cardiomyocytes requires development of the t-tubule system for excitation-contraction coupling (Bers 2002, Seki *et al*. 2003). The narrow diameter of immature cardiomyocytes allows them to function before development of the t-tubule network, which matures in the perinatal period (Legato 1979, Sheridan *et al*. 1979, Forsgren & Thornell 1981, Maylie 1982).

Interestingly, postnatal cardiomyocyte hypertrophy is typically associated with increased production of the contractile elements so that the enlarged cell can perform more work, but this is not necessarily the case in the fetus (Barbera *et al*. 2000). The contractile elements in immature cardiomyocytes are relatively less dense and organized near the plasma membrane (Brook *et al*. 1983, Smolich *et al*. 1989). Increasing cardiomyocyte width without proportional synthesis of contractile proteins may confer an immediate mechanical advantage by adjusting the ventricular wall thickness-to-chamber radius ratio to reduce wall stress. Most investigations that include fetal cardiomyocyte hypertrophy have focused on changes in gross cell dimensions rather than synthesis and organization of contractile machinery. Differential regulation of these processes remains to be investigated.

Non-cardiomyocyte growth

The heart is composed not only of cardiomyocytes, but fibroblasts, endothelial cells, smooth muscle, pericytes, other cell types and the matrix. Cardiomyocytes occupy an increasing percentage of the myocardium with advancing gestational age and in the neonate (Smolich *et al*. 1989). Simultaneously, the relative amount of matrix and vasculature in the myocardium declines (Wearn 1941, Smolich *et al*. 1989, Marijianowski *et al*. 1994). Factors that regulate perinatal cardiomyocyte growth may also regulate other components of the immature heart. These changes may have profound implications for life-long cardiac function, but are outside the scope of this review.

Regulatory signals associated with birth

The peripartum hormonal milieu is in a state of flux, with gestational decreases in vasoactive hormones such as angiotensin II (AII), increases in growth-promoting

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hormones such as insulin-like growth factors (IGF) and maturational hormones such as cortisol and thyroid hormones; most are magnitudes higher in the days after birth. Each of these circulating factors have individual effects on cardiomyocyte growth kinetics and, in concert with physical and local factors, balance the cellular processes that determine cardiomyocyte endowment in the perinatal period. The transition from intra- to extrauterine life is also met with the loss of the placenta and immediate increases in oxygenation, cardiac afterload and systemic arterial pressure. Changes in some factors precede birth, reflecting their critical role in preparing the fetus for the stress of parturition and extrauterine life.

Angiotensin II

The renin-angiotensin system is essential for the maintenance of normal fetal systemic arterial pressure (Scroop *et al.* 1992, Anderson *et al.* 1994, Faber *et al.* 2011). Circulating AII steadily decreases from 0.04–0.05 ng/ml in fetuses <120 days gestational age (dGA; term ~147d GA) to 0.02–0.03 ng/ml in the final week before term (Fig. 3; Rosenfeld *et al.* 1995). In the first postnatal week, these levels are more than 25 times higher than in the prepartum period but then decline soon after (Velaphi *et al.* 2007). Fetal hypoxia and cortisol can both increase circulating plasma renin activity (PRA) and AII levels (Broughton Pipkin *et al.* 1974, Forhead *et al.* 2000); thus, AII may play a role in cardiomyocyte changes associated with hypoxia and fetal stress.

AII receptor 1 (AT1), which is the most common receptor subtype in the adult heart, is very low in mid-gestation but increases abruptly around 120 days of gestation (Table 1; Burrell *et al.* 2001). In contrast, AT2 is highest in mid-gestation and declines towards term. Angiotensin converting enzyme (ACE) mRNA levels, which converts angiotensin I to AII, also increases abruptly near term and remains high in the neonate (Reini *et al.* 2009).

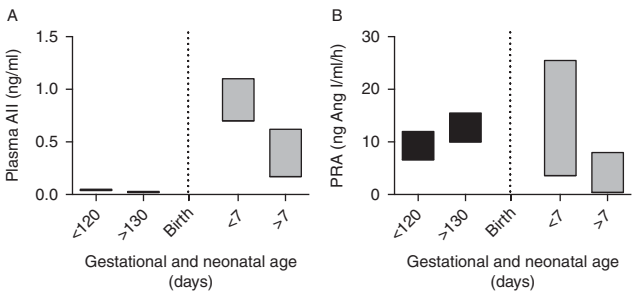

Figure 3
Published ranges for (A) angiotensin II (AII) and (B) plasma renin activity (PRA) in normal fetal (black) and newborn (grey) sheep. Owing to the short half-life of AII, PRA is often measured instead of AII (renin converts angiotensinogen to angiotensin I). Data from Broughton Pipkin *et al.* (1974), Fleischman *et al.* (1975), Louey *et al.* (2000), Louey *et al.* (2007), Rosenfeld *et al.* (1995), Siegel and Fisher (1980), Velaphi *et al.* (2007).

The outcome of AII signaling in fetal sheep cardiomyocytes is complex, in part depending on whether the experiment is conducted in isolated cells or *in utero*. AII causes near-term fetal sheep cardiomyocytes to proliferate in culture, contrary to its hypertrophic effect in cultured neonatal rat cardiomyocytes (O'Tierney *et al.* 2010*a*, Sundgren *et al.* 2003*a*). In contrast, AII infusion *in utero* increases ovine heart growth via myocyte hypertrophy and maturation (Segar *et al.* 2001, Norris *et al.* 2014, Sandgren *et al.* 2015). Atrial natriuretic peptide (ANP) inhibits AII-stimulated fetal cardiomyocyte proliferation in culture (O'Tierney *et al.* 2010*a*); increased arterial pressure resulting from *in utero* AII infusion may simultaneously inhibit proliferation by stimulating ANP release while stimulating cellular enlargement and terminal differentiation via increased wall stress. When the increase in systemic pressure induced by exogenous AII is mitigated by co-infusion with a nitric oxide donor, cardiac hypertrophy, cellular hypertrophy and accelerated terminal differentiation are all eliminated (Sandgren *et al.* 2015). Interestingly, cardiomyocyte PCNA staining remains elevated, suggesting cellular proliferation despite lack of gross cardiac hypertrophy (Sandgren *et al.* 2015). Chronic blockade of ACE inhibits fetal cardiac proliferative growth, although interpretation of this effect is complicated by dramatically lowered arterial pressure (O'Tierney *et al.* 2010*b*). The findings of these studies taken together suggest that the primary effect of AII on cardiomyocyte growth *in utero* is a result of altered systemic arterial pressure load.

Insulin-like growth factors

IGF1 and IGF2 are required for normal fetal growth (Fowden 2003, Brown 2014). Circulating IGF1 steadily increases with gestation, while IGF2 levels remain relatively high throughout gestation (Fig. 4; Carr *et al.* 1995). After birth, high IGF1 levels are maintained (Crespi *et al.* 2006, Long *et al.* 2011). Interestingly, ontogenic trends in the cardiac mRNA levels of *IGF1* and *IGF2* do not mirror circulating levels, with expression decreasing towards term for both genes (Table 1; Cheung *et al.* 1996, Reini *et al.* 2009). IGF1 and IGF2 appear to be regulated by oxygen and nutrition levels; thus, prenatal stresses such as

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Table 1 Cardiac receptors and factors and circulating binding proteins responsible for signal transduction and modulation in the fetal sheep heart.

Growth factor	Pathway component	Change from mid to late gestation	Source
AII	AT1	Very low, rapid increase from about 120 days of gestation	Burrell *et al.* (2001)
	AT2	High and decreasing	Burrell *et al.* (2001)
	ACE (cardiac mRNA)	Low, increasing abruptly just before term	Reini *et al.* (2009)
IGF	*IGF1* (cardiac mRNA)	Increases slightly from second trimester, decreasing through second half of gestation	Cheung *et al.* (1996)
	IGF2 (cardiac mRNA)	Decreasing continuously from second trimester	Cheung *et al.* (1996)
	IGF1R (cardiac mRNA)	Slight decline with advancing gestational age	Cheng *et al.* (1995), Reini *et al.* (2009)
	IGF2R (cardiac mRNA)	Decline with advancing gestational age	Reini *et al.* (2009)
	IGFBP-2 (circulating)	Increasing throughout, peaking before birth	Carr *et al.* (1995), Crespi *et al.* (2006)
	IGFBP-2 (cardiac mRNA)	Dramatic decline with advancing gestational age	Reini *et al.* (2009)
	IGFBP-3 (circulating)	Unchanged throughout	Carr *et al.* (1995), Crespi *et al.* (2006)
Cortisol	*GR* (cardiac mRNA)	Declining in the LV; unchanged in the RV	Reini *et al.* (2009)
	MR (cardiac mRNA)	Declining in the LV; unchanged in the RV	Reini *et al.* (2009)

placental insufficiency and intrauterine growth restriction are usually associated with decreased circulating IGFs (Owens *et al.* 1994).

Developmental regulation of IGF receptors (IGFR) probably modifies cardiomyocyte responses to circulating IGF levels. *IGFR1* mRNA in the left ventricle (LV) declines with advancing gestational age (Table 1; similar to maturational changes found in the rat heart), but changes little in the RV (Cheng *et al.* 1995, Reini *et al.* 2009). The mRNA for *IGFR2*, the clearance receptor, is highest in mid-gestation and declines towards term and in the neonate (Reini *et al.* 2009). Nutrient restriction increases fetal cardiac IGF2R levels at a post-transcriptional level (Dong *et al.* 2005), suggesting that our understanding of normal cardiac *IGFR* levels in development may be incomplete without knowing the protein levels. Further, insulin receptor (IR) may share the pro-survival signaling of IGFR1, and new data in the rodent heart suggests an important role for insulin receptor substrates 1 and 2 downstream of both IR and IGFR1 for the regulation of cardiomyocyte metabolism and survival (Qi *et al.* 2013). IGF binding proteins (IGFBP) also substantially modify IGF system signaling by sequestering IGF, modifying the interaction of IGF with the cell, and through IGF-independent cell signaling (Baxter 2014). IGFBP-2 can potentiate IGF signaling and is the primary binding protein in the fetus; serum levels progressively increase throughout gestation and peak before birth (Carr *et al.* 1995, Crespi *et al.* 2006). In contrast, cardiac *IGFBP-2* mRNA levels decline dramatically with advancing age (Reini *et al.* 2009). IGFBP-3 is the primary form in the adult, acting to stabilize IGF and prolong its circulating half-life. Serum levels are largely stable *in utero* (Carr *et al.* 1995, Crespi *et al.* 2006). Together, these data suggest a complex *in utero* IGF regulatory environment.

Overall, studies support the pro-mitogenic role of IGF1 in the fetal sheep heart. IGF1 stimulates proliferation in cardiomyocytes cultured from both mid-gestation and near-term fetal sheep (Sundgren *et al.* 2003*b*, Chattergoon *et al.* 2014), although whether it also causes cellular enlargement at term is debated (Sundgren *et al.* 2003*b*, Wang *et al.* 2012). When chronically infused into the fetal circulation, IGF1 causes massive cardiac enlargement by the stimulation of cellular proliferation without increased cellular enlargement or terminal differentiation (Sundgren *et al.* 2003*b*). After a short, transient exposure, gross cardiac hypertrophy was not evident, although there was

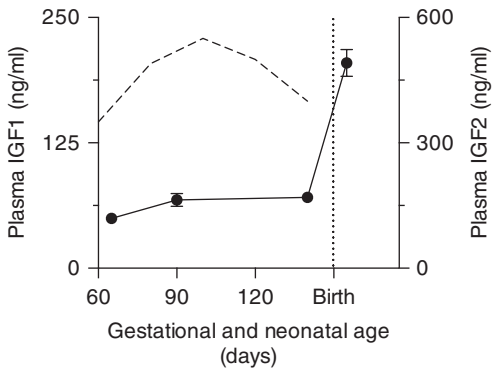

Figure 4
Circulating insulin-like growth factor 1 (IGF1; solid line, mean ± S.E.M.) and insulin-like growth factor 2 (IGF2; dashed line, mean) in normal fetal and newborn sheep. Data from Carr *et al.* (1995) and Crespi *et al.* (2006).

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a suggestion of sex-specific regulation of cellular enlargement (Lumbers *et al*. 2009). Transient exposures complicate interpretation because the fetal heart will subsequently adjust growth to normalize heart weight, probably to match heart size to somatic circulatory demand (Jonker *et al*. 2011*a*).

Cortisol

Circulating levels of cortisol are negligible in the sheep (<10 ng/ml) until the last 10 days before term, when levels rapidly increase (Fig. 5). The surge in the final days of gestation is believed to be a push to mature key organ systems in preparation for the transition to extrauterine life. Increased fetal cortisol is essential to initiating parturition at least in the sheep (Liggins 1968), it can be seen that the prepartum surge consistently commences 7–10 days before birth (Magyar *et al*. 1980). Prenatal stresses such as placental insufficiency are known to increase fetal cortisol levels (Joyce *et al*. 2001, Morrison *et al*. 2007), and may be sufficient to induce preterm birth in subsets of growth-restricted fetuses (Cock *et al*. 2001*a*), whereas those with normal cortisol levels will be born at term (Louey *et al*. 2000). Exogenous cortisol elevates arterial pressure (Wood *et al*. 1987, Tangalakis *et al*. 1992), renin and AII (Forhead *et al*. 2000), and triiodothyronine (T_3; see next section), thus these other factors must be taken into consideration when designing and interpreting experiments.

Glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) mRNA levels (encoded by *NR3C1* and *NR3C2* respectively) in the LV decline from mid- to late gestation, but change relatively little thereafter (Table 1; Reini *et al*. 2009). In contrast, neither mRNA changes in the RV, save for a transient depression of *MR* at 100 days of gestation. There can be post-transcriptional regulation

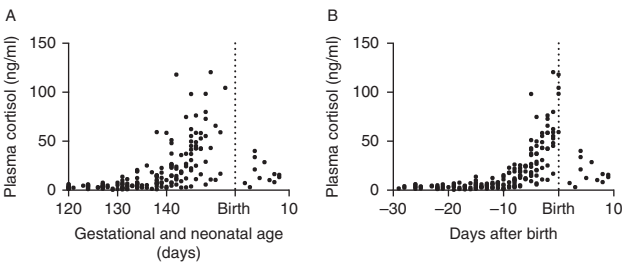

Figure 5
Circulating cortisol levels in normal fetal and newborn sheep expressed relative to (A) gestational age, or (B) the time of birth. Data from Louey *et al*. (2000), Magyar *et al*. (1980).

levels for both receptors, as for example in the developing sheep kidney (Hantzis *et al*. 2002); therefore, caution is urged in interpreting this data in the absence of receptor binding or protein levels.

Although clearly a critical hormone for the initiation of parturition and maturation of organs such as the lungs, the direct role of cortisol in regulating perinatal cardiomyocyte growth and maturation remains unclear. Infusion of sub-pressor levels of cortisol into the near-term fetal sheep coronary artery leads to cardiomyocyte proliferation, but not maturation or hypertrophy (Giraud *et al*. 2006). In contrast, intact adrenal hormone signaling inhibits anemia-induced cardiomyocyte proliferation (Jonker *et al*. 2011*b*). The key to whether cortisol has a maturational effect on the heart may depend on the dose and resulting circulating levels. At higher levels that increase arterial pressure, and mimicking levels that would induce labor, exogenous cortisol induces cardiomyocyte enlargement (Lumbers *et al*. 2005). Maternal cortisol administration also causes cardiac enlargement in fetal sheep with minimal changes in fetal cortisol levels (Reini *et al*. 2008). This growth occurs via cellular proliferation, and can be blocked by intrapericardial administration of an MR blocker (Feng *et al*. 2013). Cortisol also induced apoptosis of cells of the cardiac conduction system through the GR. This picture of glucocorticoids inducing fetal cardiomyocyte proliferation through MR, and hypertrophy via increased arterial pressure, contrasts with findings in other mammals. In fetal mice, lack of cardiac glucocorticoid receptor leads to small hearts with small myocytes that are structurally immature (Rog-Zielinska *et al*. 2013), and in the pig at 80% of gestation, maternal betamethasone administration reduces fetal cardiomyocyte proliferation while increasing terminal differentiation and apoptosis (Kim *et al*. 2014).

Thyroid hormones

Thyroid hormones are essential for normal fetal growth and maturation (Forhead & Fowden 2014). Circulating thyroxine (T_4) continuously increases over the latter two-thirds of gestation, reaching a peak and maintaining high levels (10–12 μg/dl) after birth (Polk 1995). The prepartum cortisol surge stimulates a similar surge in T_3 by stimulating deiodination of T_4 while inhibiting placental clearance of T_3 (Liggins 1994, Forhead *et al*. 2006); T_3 levels remain high (>300 ng/dl) after birth (Fig. 6; Polk 1995).

Ontogenetic changes in cardiac thyroid receptor (THR) concentrations have not been studied, to our knowledge, in the developing sheep heart. In rats, fetal

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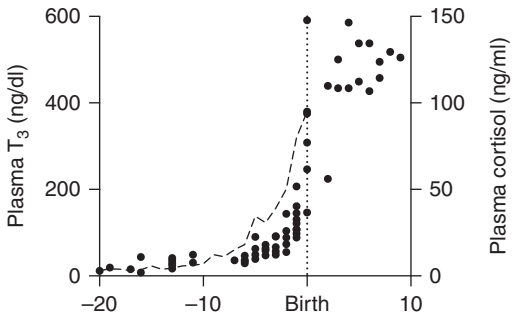

Figure 6
Circulating triiodothyronine (T_3; symbols) levels in normal fetal and newborn sheep expressed relative to the timing of birth; the prepartum T_3 surge is driven by the prepartum cortisol surge (dashed line, mean data from Fig. 5). Data from Klein *et al.* (1978) and Mathur *et al.* (1980).

T_3 binding is similar in the nuclear extract of heart and liver (Perez-Castillo *et al.* 1985), and developmental liver and brain T_3 binding profiles are similar between the rat and sheep (Polk *et al.* 1989). We hypothesize that, in the fetal sheep, cardiac THR follows the same developmental profile as liver THR: increasing slowly from mid-gestation and much more rapidly near term to levels maintained in the neonate and adult (Polk *et al.* 1989). Differential regulation of the isoforms is unclear; in rats, *THRA* mRNA expression is less than *THRB* (Strait *et al.* 1990), but THRA actively represses transcription of THRB via aporeceptor action in the fetal mouse heart (Mai *et al.* 2004).

Exogenous T_3 reduces proliferation and increases terminal differentiation of near-term sheep cardiomyocytes *in utero* (Chattergoon *et al.* 2012*a*) and in culture (Chattergoon *et al.* 2007). Interestingly, thyroidectomy also reduces cell cycle activity (Chattergoon *et al.* 2012*a*, Segar *et al.* 2013); however, this reduction in cell cycle activity may not be solely due to reduced proliferation, because binucleation, which requires at least one last round of cell cycle activity for DNA replication, was also reduced. The proliferation-suppressing actions of T_3 can be observed in cardiomyocytes cultured from mid-gestation sheep, long before endogenous thyroid hormones increase and proliferation slows, but this effect in younger cardiomyocytes is not present in the rodent (Burton *et al.* 1999, Chattergoon *et al.* 2012*b*). Both T_3 supplementation and thyroidectomy cause a small increase in cardiomyocyte size (Chattergoon *et al.* 2012*a*). Interestingly, T_3 is required for the fetal sheep cardiac growth response to increased systolic stress imposed by pulmonary artery banding (Segar *et al.* 2013). This may be mediated by a lower heart rate reducing cardiac workload in the context of thyroidectomy, or T_3 may be required in a permissive capacity. Adrenergic signaling may play a role, as T_3 regulates β-adrenergic receptors in fetal sheep (Padbury *et al.* 1986, Birk *et al.* 1992).

T_3 clearly has the capacity to be a major regulator of terminal differentiation, but the timing of its rise (5 days prepartum; Fig. 6) in relation to terminal differentiation (initiated as early as 40 days prepartum, most rapid at 20 days prepartum; Fig. 2B; Jonker *et al.* 2015) calls into question its role in the normally-developing sheep heart. Its effect may be enhanced by a simultaneous rise in receptor levels, and the relief from aporeceptor actions provided with ligand binding. The *in vivo* regulation of terminal differentiation may be more nuanced. An intrinsic cell cycle timer ends proliferation of fetal cardiomyocytes in culture (Burton *et al.* 1999, Ball & Levine 2005), but this arrest is reversible without addition of T_3 (Burton *et al.* 1999). If also true in the sheep heart, this may imply that binucleation first appears during the reversible cell cycle arrest preceding true terminal differentiation. Further, one might wonder how exogenous T_3 can bring proliferation to an early conclusion if it depends on the cessation of proliferation via an intrinsic timer. The answer may be found in differences in cell cycle behavior *in vitro* versus *in utero*, or it may be the result of interspecies differences, with sheep cardiomyocytes more frequently withdrawing into G_0 between proliferative cycles. Further experiments are required to determine the endogenous biological mechanisms, including T_3, regulating terminal differentiation in the heart of the large mammal.

Oxygen and metabolic substrates

All metabolic substrates required for fetal growth and development must be obtained from the mother via the placenta. The placenta itself is a metabolic organ and thus disruptions in maternal supply or placental function impact the supply of oxygen, glucose and amino acids to the fetus. After birth, the supply of these substrates is no longer dependent on the placenta or (to an extent) the mother. Birth is associated with an immediate increase in partial pressure of oxygen in arterial blood from ~25 mm Hg to more than 60 mm Hg (Comline & Silver 1972). Plasma glucose increases from ~20 mg/dl (1.1 mmol/l) to 40–60 mg/dl within minutes of birth; plasma free fatty acids increase fivefold in this same time (<0.2–1.0 mEq/l; Comline & Silver 1972). These substrates are made available through the actions of catecholamines, cortisol, and thyroid hormone (Jones & Ritchie 1978, Polk *et al.* 1987, Carstens *et al.* 1997, Fowden *et al.* 1998, Fowden

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et al. 2001). Circulating levels of epinephrine and norepinephrine (Jones *et al*. 1983, Fowden *et al*. 1998), cortisol (Fig. 5), and thyroid hormone (Fig. 6) increase rapidly in the last 5–10 days before birth and remain high in the newborn period.

Most *in utero* studies on the regulatory role of oxygen on cardiomyocyte growth are achieved by experimental placental insufficiency and are complicated (or complemented) by simultaneous reductions in other metabolic substrates. Placental insufficiency induced by placental embolization is typically associated with only a transient increase in fetal arterial pressure, and leads to asymmetrical intrauterine growth restriction, with cardiac mass growth restriction that is nonetheless appropriate for body size. Proliferation and terminal differentiation are retarded in these hearts (Bubb *et al*. 2007, Louey *et al*. 2007). In some cases, intrauterine growth restriction may be associated with sustained fetal hypertension and cardiac hypertrophy (Murotsuki *et al*. 1997), although the mode of heart growth has not been studied. Hypoxia stimulates ANP synthesis and release (Chen 2005), which can inhibit fetal cardiomyocyte proliferation (O'Tierney *et al*. 2010*a*). This is potentially one mechanism of growth inhibition in the hypoxic fetal heart.

Fetal anemia is another method that has been used to study the role of oxygen on fetal cardiomyocyte growth. Although anemia reduces the oxygen carrying capacity but not oxygen partial pressure of the blood, it does stabilize HIF-1-α in the myocardium (Martin *et al*. 1998). The effect of fetal anemia is unlike that of intrauterine growth restriction associated with placental insufficiency. Chronically anemic fetuses have grossly enlarged hearts as a result of cardiomyocyte proliferation, maturation and cellular enlargement (Jonker *et al*. 2010).

Maternal nutrition has also been manipulated in order to determine what nutrient deprivation or excess does to fetal growth, although cardiomyocyte-specific measures have not been reported. In fetuses of nutrient-restricted ewes, somatic and heart growth are proportionally slowed (Dong *et al*. 2005, Dong *et al*. 2008). In fetuses of over-fed ewes, heart growth is proportional or accelerated relative to somatic growth (Dong *et al*. 2008, George *et al*. 2010, Fan *et al*. 2011). Although the cellular basis for these changes have not been reported, we speculate that the primary mechanism is through proliferation, because circulating fetal IGF1 levels are regulated by maternal feeding (Dong *et al*. 2005, Dong *et al*. 2008). It is noteworthy that over-nutrition is also associated with disturbed placental hemodynamics (Frias *et al*. 2011). Thus, the picture that emerges with regards to regulation of cardiac growth by oxygen and metabolic substrates in the fetus is complex.

Arterial and venous pressure

In the fetus, the *ductus arteriosus* permits the RV to eject blood into the aorta while the *foramen ovale* enables blood entering the right atrium to bypass the pulmonary circulation and directly fill the left atrium (Anderson *et al*. 1985). Because the placenta is responsible for fetal nutrient transfer and the lungs are unventilated, fetal pulmonary resistance is high and receives little of the RV output. After birth, the fetal vascular shunts close, leaving the RV to eject into the pulmonary circulation and the LV to eject into the systemic circulation. Fetal systemic arterial pressure increases slowly and heart rate decreases slowly with advancing gestation. The greatest change to perinatal hemodynamics occurs at birth, when systemic arterial pressure and heart rate suddenly rise and pulmonary artery pressure plummets (Fig. 7). These changes are driven by the closure of fetal vascular shunts, loss of the placenta (a large, low-resistance vascular bed), decreased pulmonary vascular resistance secondary to pulmonary ventilation, loss of lung water and oxygenation (Iwamoto *et al*. 1987), and resetting of the baroreflex.

Proliferation increases the cardiomyocyte number to an estimated 1.5–2.5 billion in the sheep LV just prior to birth (Fig. 2C; Burrell *et al*. 2003*b*, Jonker *et al*. 2015). Despite the higher wall stress of the RV in the fetus (due to a larger radius of curvature and thinner wall; Pinson *et al*. 1987), myocyte number in the RV free wall at the same age is only 1–1.5 billion (Fig. 2C; Jonker *et al*. 2015). The fibrous skeleton of the myocardium modifies its experience of wall stress, but although the collagen content of the immature heart is high relative to that of the adult (Marijianowski *et al*. 1994), there is no notable difference between the fetal ventricles (Jackson *et al*. 1993). It is interesting that the RV does not grow or remodel to normalize this relatively higher wall stress, although it does respond to experimentally elevated loads. Despite this apparent tolerance to higher wall stress, fetal RV myocytes are larger (Burrell *et al*. 2003*b*, Jonker *et al*. 2015), likely as a result of relatively increased load in either systole or diastole. No differences are noted in cell cycle activity or timing of terminal differentiation between the two ventricles.

Experimentally increased cardiac load stimulates growth of both the fetal LV and RV (Fishman *et al*. 1978, Segar *et al*. 1997, Barbera *et al*. 2000, Samson *et al*. 2000, Olson *et al*. 2006, Eghtesady *et al*. 2007, Jonker *et al*.

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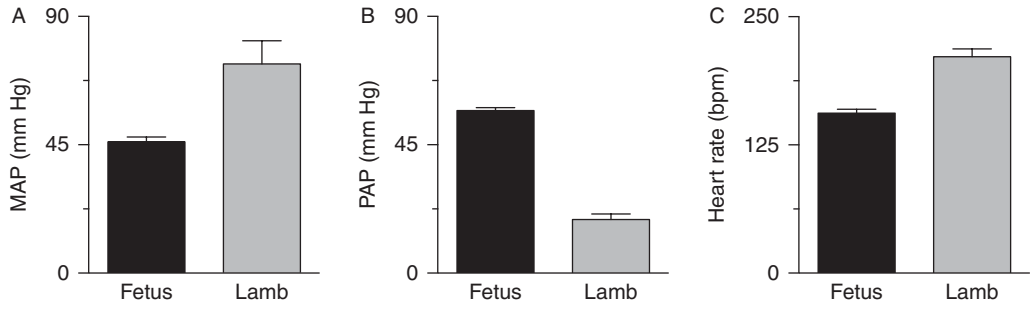

Figure 7
(A) Resting mean arterial pressure (MAP), (B) pulmonary arterial pressure (PAP), and (C) heart rate in normal fetal (118-142d GA, black) and newborn (2–28 days old, grey) sheep. Data from Black *et al.* (2002), Dawes *et al.* (1980), Fineman *et al.* (1994), Iwamoto *et al.* (1987), Jaillard *et al.* (2001), Louey *et al.* (2000), Morin and Egan (1992) and Stahlman *et al.* (1967), data are mean ± S.E.M.

2007*b*). Excessive LV systolic load from banding of the ascending aorta (fetal ventricular outflow banding restricts renal perfusion and is a high-AII model), initiated early in the second trimester, leads to either dilated ventricles with poor cardiac function and fetal hydrops or to very high LV systolic pressure generated by a grossly hypertrophic LV (Eghtesady *et al.* 2007). Third trimester aortic banding leads to compensated LV hypertrophy (Fishman *et al.* 1978, Samson *et al.* 2000). The immediate cardiomyocyte growth response to increased cardiac load (in a low-AII model) in the near-term fetus is increased cell cycle activity to increase the cell number (Jonker *et al.* 2007*b*). After about a week of aortic occlusion or plasma protein infusion (leading to fetal hypertension), however, robust cellular hypertrophy and terminal differentiation have become the predominant modes of growth (Barbera *et al.* 2000, Jonker *et al.* 2007*b*, Norris *et al.* 2014).

In contrast, reduced ventricular filling inhibits free wall growth, leading to dramatically smaller chamber volumes (Fishman *et al.* 1978). Reduced near-term fetal systemic arterial pressure similarly inhibits cardiomyocyte proliferation and cardiac growth (O'Tierney *et al.* 2010*b*, Norris *et al.* 2014). Increased preload in chronic fetal anemia may contribute to the increased cardiomyocyte proliferation, terminal differentiation and enlargement that lead to ventricular dilation and compensatory wall thickening (Jonker *et al.* 2010).

Many hormones can alter fetal hemodynamics, which must be considered when studying the influence of the hormone on fetal cardiac growth. For instance, AII-induced fetal cardiac growth is dependent on its hypertensive effect (Sandgren *et al.* 2015), despite causing fetal sheep cardiomyocyte proliferation in culture (Sundgren *et al.* 2003*c*). Similarly, the effects of cortisol depend on the site of administration and fetal hemodynamic effects (Lumbers *et al.* 2005, Giraud *et al.* 2006, Reini *et al.* 2008). Oddly, in one experiment phenylephrine increased blood pressure but did not cause heart growth (Segar *et al.* 2001). It is clear that fetal and postnatal cardiomyocytes are very sensitive to changes in ventricular wall stress and many endocrine perturbations alter cardiac load.

Integration of experimental evidence

Fetal cardiac growth patterns can be altered by *in utero* manipulation of endocrine, hemodynamic or nutritional factors (including oxygen), providing clues to underlying regulatory processes. These experiments have brought us to understand how *in utero* signals interact and establish precedence to regulate cardiomyocyte growth and maturation.

In experimental models, sustained increased systemic or pulmonary arterial pressure initially increases cellular proliferation, but leads ultimately to cardiomyocyte maturation and cellular hypertrophy (Barbera *et al.* 2000, Jonker *et al.* 2007*b*). This growth response, including gross cardiac hypertrophy and the maturational effect, is dependent on intact thyroid hormone signaling, despite low circulating levels (Sandgren *et al.* 2015). The curtailment of the proliferative response may be effected by the hypertension-induced rise in circulating ANP (Rosenfeld *et al.* 1992). In models in which increased cardiac load is imposed by placement of a constricting band on the main pulmonary artery or aorta, the renin-angiotensin system is activated. However, AII does not mediate the growth-stimulating effects of fetal cardiac load (Segar *et al.* 1997, Segar *et al.* 2001, Jonker *et al.* 2007*b*, Sandgren *et al.* 2015). In fact, the growth-stimulating effects of AII *in utero* are mediated by AII-induced cardiac load (Sandgren *et al.*

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2015). Indeed, the cardiomyocyte enlargement and maturational response to cardiac load predominates in fetal sheep models in which arterial pressure is increased (Murotsuki *et al.* 1997, Lumbers *et al.* 2005).

The nutritional environment may trump endocrine regulators of cardiomyocyte growth. Increased circulating cortisol is a stereotypic response in oxygen- and nutrient-restricted fetuses. However, this is driven by a premature activation of the hypothalamic-pituitary-adrenal axis (Braems *et al.* 1996, Murotsuki *et al.* 1996), often resulting in preterm birth (Cock *et al.* 2001*a*), rather than elevation of baseline levels of cortisol. Regardless of when the placental insufficiency was initiated, elevated levels of cortisol are not routinely observed prior to 128dGA in placentally-restricted fetuses (Gagnon *et al.* 1994, Murotsuki *et al.* 1996, Cock *et al.* 2001*b*, Louey *et al.* 2007). Cortisol is associated with the opposite effect in a low-nutrient versus normal intrauterine environment: proliferative growth, hypertrophic growth and maturation are all delayed or inhibited in placental insufficiency (although growth effects are manifest prior to differences in cortisol; Bubb *et al.* 2007, Louey *et al.* 2007, Morrison *et al.* 2007). Low IGF levels may mediate placental restriction's effects, but nutrients as substrate for growth may also be limiting. Alternatively, ANP is increased by acute and chronic anemic hypoxia (Silberbach *et al.* 1995, Chen 2005) and may mediate reduced proliferation, but it is unknown if ANP levels are sustained in chronic hypoxia stemming from placental insufficiency. Further, the inhibitory effect of ANP has only been shown to occur in the presence of exogenous stimulation of proliferation (O'Tierney *et al.* 2010*a*).

Cortisol is at the center of a web of interconnecting physiologic signals. At sub-pressor elevations of cortisol that resemble the levels from the early phase of the prepartum cortisol surge, cortisol stimulates cardiomyocyte proliferation (Giraud *et al.* 2006); these levels may not be sufficient to significantly increase T_3, but proliferation may be mediated via another effector such as AII (Forhead *et al.* 2000). Cortisol can increase fetal systemic arterial pressure via AII (Forhead *et al.* 2000), in which case the typical cardiomyocyte enlargement and maturation growth response to elevated cardiac load ensues (Lumbers *et al.* 2005). This higher level of cortisol also increases fetal T_3 levels (Sensky *et al.* 1994), which may suppress cardiomyocyte proliferation in the context of elevated pressure.

Integration of normal development

While experimental studies have helped us understand cardiomyocytes in stressed and suboptimal pregnancies, this knowledge has not translated easily into an understanding of how normal perinatal heart growth and maturation is regulated. Ontogenetically normal changes in endocrine, hemodynamic and nutritional factors are not necessarily correlated with cardiomyocyte growth and maturation outcomes that would be predicted by experimental studies (Fig. 8).

Altered fetal cortisol, T_3, oxygen and nutrients all modulate cardiac growth and maturation *in utero*. However, their roles (beyond permissive at normal levels) in normal perinatal cardiomyocyte growth and maturation remain unclear. The prepartum timing of the cortisol surge coincides with mass cardiomyocyte attrition (Fig. 8; Jonker *et al.* 2015). Cortisol administration to pregnant ewes leads to apoptosis of cardiomyocytes of the conduction system (Feng *et al.* 2013). Glucocorticoids have been shown to augment apoptosis in myocardial infarction (Mihailidou *et al.* 2009), and to cause calcium-mediated apoptosis in other cell types (Harr & Distelhorst 2010), but little is known about this potential effect in fetal or neonatal cardiomyocytes. Cardiomyocyte enlargement accelerates 10 days before birth (Fig. 2E), also coinciding with the cortisol surge (Fig. 5). However, there is no experimental evidence that cortisol drives cardiomyocyte hypertrophy in the absence of simultaneous hypertension. T_3 clearly has a regulatory role in the immature cardiomyocyte. Both too little and excess hormone decrease cardiomyocyte cell cycle activity, but have differential effects on terminal differentiation depending on gestational age (Chattergoon *et al.* 2012*a*). The role in normal maturation may be more subtle; the steepest increase in binucleation rate is largely completed prior to the prepartum T_3 surge. On the other hand, although the number of mononucleated myocytes in the neonatal heart is very low (in both absolute number and relative proportion), this population generates relatively more binucleated cells than in the fetus, consistent with increased neonatal T_3 levels (Fig. 8). In the fetus, oxygen and nutrient restriction limits cardiac growth and maturation. There is no evidence, however, to suggest that increases in oxygen with ventilation and changes in circulating nutrients following birth and with oral feeding drive increased cardiac growth in the neonate.

Better evidence, perhaps, supports the roles of IGF1, AII and arterial pressure in normal cardiac growth and maturation *in utero*. Changed cardiac load drives the type of cardiomyocyte growth in both fetuses (summarized above) and adults (Carabello 2002). This is so much so, that endocrine influences on prenatal cardiac growth can be predicted by their effect on arterial pressure. In the

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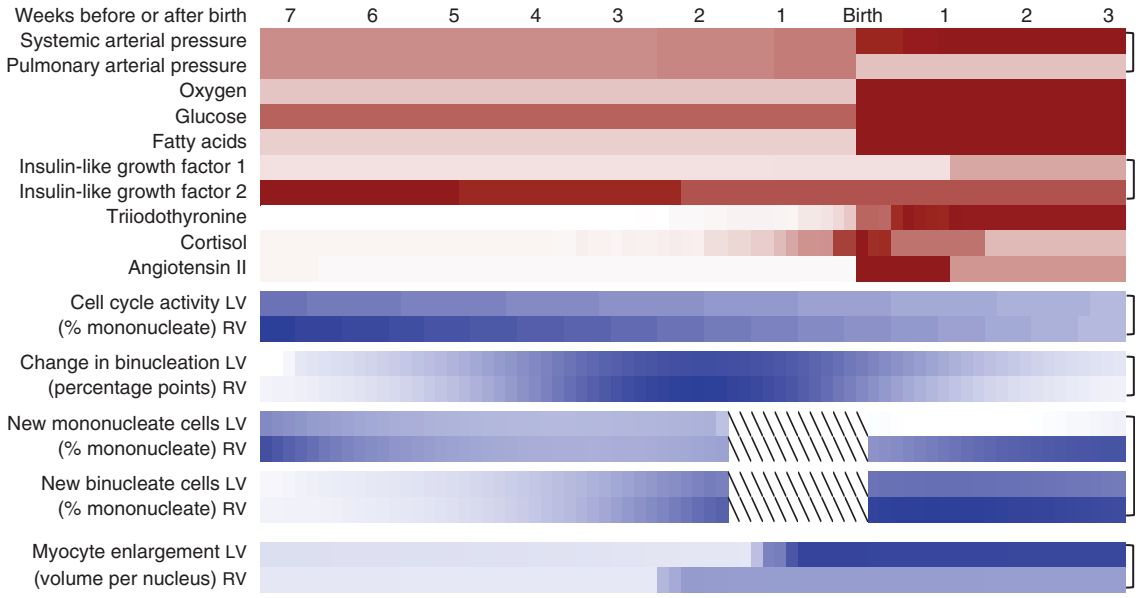

Figure 8
Overview of growth-regulating factors (red) and cardiomyocyte growth kinetics (blue) in the perinatal sheep heart. Proliferation and maturation are expressed to emphasize changing rates of growth processes in the LV and RV, rather than the accumulated outcome of these processes, and how these kinetics might relate to changing endocrine and other physiological modulators of growth. Daily number of new mononucleated and binucleated myocytes are shown normalized to mononucleated cell number (because these are the cells that enter the cell cycle to proliferate or terminally differentiate). Myocyte volume enlargement is expressed relative to nuclear number because DNA content appears to critically mediate magnitude of hypertrophic response in cardiomyocytes. Each parameter is expressed as a monochrome heat map (light=low, dark=high) and is derived from data described in this manuscript. Related data sets (bracketed) share a saturation scale and are comparable across rows. The values within the hatched area cannot be estimated because, while proliferation and terminal differentiation almost certainly continue, cell number declines in this period.

fetus, systemic and pulmonary arterial are similar. It is notable that, after birth, there are dramatic but opposite changes in arterial pressures of the systemic circulation (rises) and pulmonary circulation (decreases), but the rate of cardiomyocyte enlargement is accelerated in both ventricles compared to before birth (Fig. 8). Continued proliferation in the RV may be explained if low pulmonary arterial pressure is permissive, while sustained elevation in systemic arterial pressure inhibits LV cardiomyocyte proliferation. Increasing levels of IGF1 may support cardiomyocyte proliferation through birth. Increased systemic arterial pressure may inhibit IGF1-stimulated proliferation in the neonatal LV, while lower pulmonary arterial pressure permits proliferation in the RV (Fig. 8). Alternatively, it is unknown in sheep whether IGF1 is a mitogen or stimulates cardiomyocyte enlargement after birth, as occurs for IGF2 in the developing rat (Liu *et al.* 1996). The prepartum decline in AII correlates with the increase in cardiomyocyte terminal differentiation (Fig. 8), although the maturational effect of AII appears experimentally to be mediated through increased arterial pressure. Similar to IGF1, the tremendous increase in AII at birth is concomitant with a doubling of systemic arterial pressure and dramatic decline in pulmonary arterial pressure, which may explain dimorphic ventricular growth patterns in this same period (Fig. 8). Thus, AII may (alternatively to IGF1) drive proliferation in the low-load RV, but be inhibited from similar action in the high-load LV.

Intracellular integration of regulatory signals

The intracellular signaling pathways activated by endocrine and physiologic signals in the fetal ovine heart are beyond the scope of this review. Many intracellular proteins have been identified as contributing to regulation of cardiomyocyte growth and maturation (some topics reviewed in Heineke & Molkentin (2006) and Oyama *et al.* (2014)); much of this work has been accomplished in the rodent. It is clear that stable withdrawal from the cell cycle can be overcome by over-expression or knockout of many genes (Poolman *et al.* 1999, Chaudhry *et al.* 2004, Heber-Katz *et al.* 2004, Li *et al.* 2008). These regulatory elements, and those contributing to other aspects of

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cardiomyocyte growth, are legitimately identified as regulating cardiomyocyte endowment and morphology, and thus lifelong health. However, current approaches have failed to yield an understanding of how these elements are integrated and prioritized to generate specific cellular outcomes. Perhaps new approaches, such as interactome network analysis, will lead to a more comprehensive understanding (Menche *et al*. 2015). Indeed, transcriptomic modeling has recently identified discrete regulatory patterns in the ovine heart between 130 days of gestation, term and 14 days after birth, which may shed light on how intracellular processes interact to determine cardiomyocyte growth and maturation (Richards *et al*. 2015).

Conclusion

There are substantial disparities between perinatal changes in endocrine factors and the timing of cardiomyocyte growth and maturation. A number of gaps remain in our knowledge about the regulation of growth and maturation in perinatal ovine cardiomyocytes. First, what is the stimulus for and consequence of mass cardiomyocyte attrition in the perinatal heart? It could be deliberate remodeling, as previously proposed, or perhaps its role is to eliminate most proliferative cardiomyocytes as part of the tight cell cycle control evidenced in the postnatal heart. Secondly, can neonatal cardiomyocytes be stimulated to proliferate? Myocyte number continues to increase slowly in the neonatal RV, suggesting that if ventricular load is minimized, proliferation may be permitted. On the other hand, new evidence suggests a burst of cardiomyocyte generation in adolescence: which cells participate and what regulates their proliferation? Thirdly, what drives accelerated RV cardiomyocyte enlargement in the neonate, given low pulmonary arterial pressure? All experimental evidence suggests that cardiac load is the strongest growth modulator in the heart. Fourthly, whether terminal differentiation occurs and can be detected prior to binucleation is a problem limiting current research. Finally, our understanding of the intrinsic differences between fetal LV and RV cardiomyocytes is lacking. There are clear divergences in growth trajectories that begin *in utero* and set the stage for cardiac function after birth.

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

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