# Extracellular Signal-Regulated Kinase and Growth Factor Regulation of the Fetal Ovine Cardiac Myocyte

by

Nathan Cole Sundgren

A Dissertation

Presented to the Department of Physiology & Pharmacology

and Oregon Health & Science University

School of Medicine

in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

May 2003

School of Medicine
Oregon Health & Science University

CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. thesis of

Nathan Cole Sundgren

has been approved

[redacted]
Kent L. Thornburg, Ph.D.

[redacted]
Susan P. Bagby, M.D.

[redacted]
George D. Giraud, M.D., Ph.D.

[redacted]
Peter S. Rotwein, M.D.

[redacted]
Philip J.S. Stork, M.D.

## Table of Contents

**Acknowledgements:**

I would like to acknowledge the many people who have been vital to my success and the completion of my thesis work. Working in the Thornburg lab has been a fun and challenging experience and the many people I have worked with have contributed greatly to my education. Pat Renwick and Robert Webber have been crucial members of the surgery team. Krista Wehrley taught me everything I know in molecular biology and I am especially indebted to her hard work in generating protocols for the Light Cycler. Sonnet Jonker has been a fellow student always willing to discuss experiments and think through problems. Kirsten Labudda from Dr. Phil Stork's lab taught me everything I know about western blots and I appreciate her willingness to teach me. I also thank the rest of the Stork lab for tolerating my occasional time spent in their lab running gels. There have been many people who have been a part of our surgery teams that make these experiments possible. Dr Jess Schultz and Dr. Parvez Ahmed were especially helpful to my surgeries.

I particularly thank Dr. George Olsen for his expert advice on choosing a correct dose for Long R3 IGF-1 that would be comparable to endogenous IGF-1. My thesis committee has been very helpful as both collaborators and advisors on this project. I thank them all for their guidance and ideas. Dr. George Giraud has always been helpful in analyzing my data.

Kent Thornburg has been an excellent mentor and friend during my four years of work with him. I cannot thank him enough for his leadership and example to me of the type of scientist I want to be. Thank you.

**Abstract:**

The heart matures by a regulated process of cell differentiation, cell maturation, cell division (hyperplasia), cell enlargement (hypertrophy), and apoptosis. How the heart cells are regulated to form a functioning heart with the appropriate number and size of cells is unknown. Over the last decade, it has become increasingly appreciated that adult heart disease may have its origins in fetal life. The link between fetal growth and heart disease has been called the programming hypothesis. While my thesis does not directly study the programming hypothesis, the goal of my thesis work was to study the fetal cardiac growth effects of endocrine factors that have been studied as links in the programming phenomenon. I focused on two endocrine factors, insulin-like growth factor 1 (IGF-1) and angiotensin II (Ang II), and studied the mechanisms by which they can induce fetal heart growth.

IGF-1 and Ang II are implicated in many aspects of growth and is likely to be important in developmental heart growth. IGF-1 stimulates the IGF-1 receptor (IGF1R) and downstream signaling cascades including extracellular signal-regulated kinase (ERK) and phosphoinositol 3-kinase (PI3K). Ang II is known to stimulate hypertrophy in rodent cardiomyocytes via the ERK signaling cascade. The ERK cascade regulates many fundamental cellular processes such as proliferation, differentiation, survival, hypertrophy, growth arrest, and apoptosis. The PI3K signaling cascade also regulates various cellular processes such as proliferation, hypertrophy, apoptosis, and differentiation.

*In vivo* administration of an IGF-1 analogue, Long R3 IGF-1 (LR3 IGF-1), did not stimulate cardiomyocyte hypertrophy, but led to a decreased percentage of cells that

were binucleated. In culture, LR3 IGF-1 increased myocyte BrdU uptake by 3-5 fold. Blockade of either ERK or PI3K signaling completely abolished BrdU uptake stimulated by LR3 IGF-1. Ang II increased myocyte BrdU uptake compared to serum free conditions. Blockade of ERK signaling prevented BrdU uptake in Ang II stimulated cells. Phenylephrine (PE) stimulated an increase in footprint area of binucleate cells but Ang II and LR3 IGF-1 did not. We found that no endocrine factor studied (LR3 IGF-1, Ang II, and PE) changed footprint area of mononucleated cells. Blockade of ERK signaling prevented cell hypertrophy in PE stimulated cells.

We conclude that in fetal ovine cardiomyocytes 1) IGF-1 stimulates cardiomyocyte division; 2) Ang II stimulates hyperplastic growth among mononucleate myocytes; 3) IGF-1 does not stimulate binucleation; 4) IGF-1 does not stimulate hypertrophy; 5) Ang II is not a hypertrophic agent; 6) PE is a hypertrophic agent in binucleate myocytes; 7) IGF-1 requires both ERK and PI3K signaling for proliferation of fetal sheep cardiomyocytes; 8) the ERK cascade is required for the proliferation effect of Ang II and the hypertrophic effect of PE; 9) ERK stimulation has different actions based on maturation level of the cell *within the same cell type.*

# Chapter 1:

# Introduction

**Adult Heart Disease and Fetal Growth:**

Heart disease is the number one cause of death in the United States and accounts for more deaths than the next three leading causes combined (American Heart Association: *Heart Disease and Stroke Statistics – 2003 Update*). Ischemic heart disease and cerebrovascular disease rank 1 and 2 respectively in the leading causes of mortality worldwide (World Health Organization: *World Health Report 1999*). Yet, the generally accepted risk factors for heart disease - smoking, diabetes, high cholesterol, high blood pressure, obesity, and physical inactivity - account for only about half of heart disease cases (Lynn Smaha, President American Heart Association, 1999 – personal communication to K.L. Thornburg). Over the last decade, it has become increasingly appreciated that adult heart disease may have its origins in fetal life. David Barker and his colleagues in England were the first to establish the epidemiological link between low birth weight and adult-onset heart disease (Barker *et al.*, 1989c;Barker *et al.*, 1989b;Barker *et al.*, 1989a). Subsequently, this link has been reproduced in studies of populations in Europe, China, India, and the USA. Specifically, low birth weight is associated with a cluster of metabolic disturbances including central obesity, increased blood pressure, glucose intolerance, and dyslipidemia - a constellation of symptoms that has been variously labeled metabolic syndrome, insulin-resistance syndrome, or syndrome X (Barker, 1998;Phillips, 2002). Current evidence suggests that low birth weight combined with an increased weight gain during childhood, often labeled "catch-up growth," further increases the risk of acquiring the metabolic syndrome and adult heart disease (Barker, 2002). The fetal origins of adult disease hypothesis proposes that stresses resulting from an adverse intrauterine environment "program" an individual to

have altered physiological responses, altered organ structure, and altered gene expression patterns that provide immediate adaptive benefits to the fetus while increasing the risk for chronic disease later in life. One of the tenets of the programming hypothesis is that each organ has a critical window of time during which it is especially vulnerable to an environmental insult. If an insult, like hypoxemia or malnutrition, occurs during a critical window, the organ structure and function will be permanently altered (Barker, 1998). Hence, a decrease in skeletal muscle mass may lead to insulin resistance; a decrease in nephron number may lead to hypertension; and a decrease in liver size may lead to dyslipidemia (Barker, 2002). But birth weight is a crude measure of fetal health and is not helpful in understanding the processes occurring at the organ level during fetal life that lead to heart disease. It is now clear in animal studies that organs can be programmed for adult onset disease without the insult affecting birth weight (Barker, 2002).

Fetal nutrition is a primary link between the fetal environment and birth weight. Size at birth depends more on the maternal intrauterine environment than on either maternal or parental genotype (Harding, 2001;Holt, 2002;Giussani *et al.*, 2003). Experimentally, reducing or altering maternal diet has been shown to alter fetal and adult physiology of offspring consistent with the syndrome X phenotype (Harding, 2001). In a rat model, global nutrient restriction throughout pregnancy leads to offspring with obesity, hypertension, hyperinsulinemia, and hyperleptinemia, all of which can be amplified with a hypercaloric postnatal diet (Vickers *et al.*, 2000). Maternal undernutrition in sheep also programs offspring for increased blood pressure (Edwards & McMillen, 2001).

Fetal nutrient supply is not simply a matter of maternal diet. Nutrient delivery to the fetus also depends on the maternal metabolic and endocrine status, uterine blood flow, placental transport and metabolism, umbilical blood flow, and the fetal metabolic and endocrine status (Harding, 2001). Therefore, simply assessing maternal diet may not be sufficient to determine the magnitude of insult that affects the fetus (Oliver *et al.*, 2002).

While this thesis was not designed to test the programming hypothesis, I focused on two endocrine factors, insulin-like growth factor-1 and angiotensin II, that are thought to play a prominent role in the well established fact that the prenatal hormonal environment alters organ specific growth of the fetus and affects its life long health (Harding, 2001). Thus, findings from these studies may shed light on programming mechanisms.

**IGF-1 and Fetal Growth:**

Fetal nutrition in general and the rate of glucose transport across the placenta in particular regulate insulin and insulin-like growth factors-1 (IGF-1) and -2 (IGF-2) levels in the fetal plasma (Oliver *et al.*, 1993). Insulin and IGF-1 are the most important hormonal regulators of fetal growth (Harding, 2001;Holt, 2002). IGF-1 is regulated by fetal nutrient supply (Oliver *et al.*, 1993;Oliver *et al.*, 1996) and alters protein and carbohydrate metabolism in the fetus and placenta (Harding *et al.*, 1994). Therefore, IGF-1 levels are dependent on nutrient supply, but also determine fetal demand and supply for that nutrition.

The genetic basis for controlling supply and demand for maternal nutrients is not understood, but it is known that the IGFs are expressed in fetal and placental tissues and that each growth factor type has growth regulatory functions in those tissues (Reik *et al.*, 2003). IGF-2 is a paternally imprinted gene that can regulate placental size and, therefore, nutrient transfer to the fetus (Reik *et al.*, 2003).

IGF-1, but not IGF-2, plasma levels correlate positively with birth weight in normal and growth restricted human fetuses and newborns (Lassarre *et al.*, 1991). Human children born small and thin have IGF-1 plasma levels that are higher than "normal" for their current weight and height at 4 to 9 years old (Fall *et al.*, 1995). Thus, children with the highest IGF-1 plasma levels are those born small but currently heavy for their age (Fall *et al.*, 1995). It has been concluded that those at the most risk for adult heart disease - born small with increased catch up growth - have resistance to IGF-1 analogous to insulin resistance (Fall *et al.*, 1995). But, in a 15 year prospective study, people with the highest plasma levels of free IGF-1 had the lowest risk for ischemic heart disease while those with the lowest plasma levels of free IGF-1 had a four-fold increase in risk (Juul *et al.*, 2002). The work of Fall *et al.* (1995) and Juul *et al.* (2002) appear to be quite contradictory. However, the story is not complete. While fetal growth restriction may lead to increases in plasma IGF-1 levels during childhood, it is not known whether these elevated IGF-1 levels are reduced later in adulthood or if they remain high. If it follows the pattern of insulin resistance, one may postulate that late in the resistance process IGF-1 levels fall and increase risk for ischemic heart disease.

Maternal diet can be experimentally altered in animals to induce intrauterine growth restriction with an associated decrease in fetal IGF-1 levels (Gallaher *et al.*,

1998;Jensen *et al.*, 1999;Osgerby *et al.*, 2002). Intermittent umbilical cord occlusion in the sheep model (causing intermittent hypoxia) over four days also tends to lower fetal IGF-1 levels (Green *et al.*, 2000). This suggests that chronic exposure to fetal hypoxia, known to reduce fetal size, may lower IGF-1 levels. Sheep fetuses that are growth restricted do not display the normal increase in placental lactate production in response to an IGF-1 infusion which suggests a resistance to IGF-1 (Jensen *et al.*, 1999). This model provides evidence of IGF-1 resistance in growth-restricted fetuses and mimics clinical findings in children. In a rat model of syndrome X, an IGF-1 infusion normalizes insulin and leptin plasma levels, reduces hyperphagia and obesity, and lowers blood pressure (Vickers *et al.*, 2001). This raises the possibility that altered IGF-1 physiology is not only a part of the programming problem but may become important in treatment.

IGF-1 circulates in plasma bound to a family of IGF binding proteins numbered 1-6 (IGFBP1-6) that greatly increase IGF plasma half lives by protecting IGFs from degradation. Different binding proteins can either facilitate or inhibit IGF-1 actions. In general, the smaller IGFBPs, 1 & 2, are thought to be membrane soluble to facilitate IGF action while larger IGFBPs, 3 & 5, form ternary complexes thought to sequester IGF-1 from its receptor (Delafontaine, 1995;Silha & Murphy, 2002). One mechanism that may explain lowered IGF-1 levels in plasma is an alteration in binding protein levels. Maternal diet restriction lowers fetal IGF-1 plasma levels related to a fall in fetal IGFBP3 plasma levels (Gallaher *et al.*, 1998). Intermittent cord occlusion alters IGFBP2 mRNA levels in several organs including an up-regulation of IGFBP2 levels in the heart (Green *et al.*, 2000).

Because IGF-1 regulates fetal growth, is altered by levels of nutrition, and is permanently altered by adverse changes in the uterine environment, it is an obvious endocrine factor for investigating the links between fetal growth and adult disease.

**Angiotensin II and Fetal Growth:**

While IGF-1 has experimental links in the pathogenesis of the fetal origins of adult disease, it is by no means the only hormonal system that has been studied. Angiotensin II (Ang II), best known for its endocrine role in systemic blood pressure regulation, is also believed to be important in programming adult heart disease. Since hypertension is part of the syndrome X phenotype and an independent risk factor for heart disease, Ang II has been an important focus of study for a link between birth weight and adult heart disease.

Pregnant rats fed a low protein diet have offspring that become hypertensive as adults (Langley & Jackson, 1994;Woods *et al.*, 2001a). The offspring have altered renin-angiotensin systems (RAS) with decreases in renal Ang II tissue levels as well as renin message and protein levels (Woods *et al.*, 2001a). Normal nephrogenesis requires a normal intrarenal RAS. When rats are given the angiotensin 1 receptor (AT1) blocker, losartan, during the period of nephrogenesis, nephron number is reduced and offspring develop hypertension in adulthood (Woods & Rasch, 1998). Protein restricted rat pups also develop fewer nephrons (Woods *et al.*, 2001a). However, the nephrons that do develop undergo compensatory hypertrophy, and kidney function is maintained as glomerular filtration rate (GFR), effective renal plasma flow (ERPF), and filtration fraction (FF) are normal (Woods *et al.*, 2001a). This sets up a situation of

"hyperfiltration" for each glomerulus that is associated with hypertension and renal insufficiency (Ingelfinger & Woods, 2002). This is analogous to removing a single kidney during early development which halves nephron number, causes hyperfiltration, and leads to hypertension in adult rats (Woods *et al.*, 2001b). Similar work in the sheep suggests that the final common pathway of programmed hypertension is a reduction in nephron number in the kidney. Growth restriction in fetal sheep, caused by experimental placental insufficiency, produced smaller kidneys with reduced message levels for angiotensinogen (Aogen) and renin (Zhang *et al.*, 2000). But in these animals, there was no reduction in hepatic Aogen mRNA levels, suggesting that the effect was specific to the intrarenal RAS (Zhang *et al.*, 2000).

Newborn lambs exposed to mid-gestation nutrient restriction had increases in AT1 and glucocorticoid receptor (GR) message levels in kidney, liver, lung, and adrenal gland (Whorwood *et al.*, 2001). This is opposite the effect in rats where protein restriction during fetal life decreased kidney AT1. However, a brief 48 hour exposure to dexamethasone (a cortisol analogue) during the earliest stages of sheep nephrogenesis also led to an increases in fetal kidney AT1, AT2, and Aogen message levels (Moritz *et al.*, 2002) and a reduction in nephron number postnatally (Moritz *et al.*, 2003). Fetal nutrient restriction is known to reduce the expression of 11β-hydroxysteroid dehydrogenase (11βHSD) in the placenta (Whorwood *et al.*, 2001). This enzyme inactivates maternal cortisol as it crosses the placenta preventing elevated maternal cortisol levels in the fetal compartment. Reduction of 11βHSD and resulting increases in fetal cortisol exposure is, therefore, another mechanism by which fetal undernutrition

programs for adult hypertension (Moritz *et al.*, 2003). It may be that either increases or decreases in the fetal intrarenal RAS can lead to fewer nephrons (Moritz *et al.*, 2003).

IGF-1 is also known to effect fetal kidney function and the RAS. Most of the effects of protein restriction and/or glucocorticoid exposure seem to disturb the intrarenal RAS without affecting circulating levels of RAS components. However, IGF-1 infusion ranging from 4 hours to 10 days dramatically increases plasma renin concentration and plasma renin activity (PRA) with a slight decline in plasma Aogen levels (Marsh *et al.*, 2001a;Marsh *et al.*, 2001b). While a fetal IGF-1 infusion has been shown to increase kidney size relative to body weight (Marsh *et al.*, 2001a;Lok *et al.*, 1996), plasma renin levels increase within the first 4 hours, well before chronic changes in kidney mass could be responsible for the alteration in circulating RAS components (Marsh *et al.*, 2001b).

Other RAS components, such as the AT1 and AT2 receptor, have not been studied in response to IGF-1 infusion. IGF-1 can down-regulate AT1 and Aogen levels in cardiomyocytes which reduces Ang II induced hypertrophy and apoptosis (Leri *et al.*, 1999a;Leri *et al.*, 1999b). Therefore, IGF-1 can alter the systemic and renal RAS systems and likely raise plasma levels of Ang II by increasing PRA. Alterations in the RAS, especially the intrarenal components, are implicated in fetal programming of adult disease.

**Heart Growth and Adult Disease:**

The epidemiological link between fetal development and adult heart disease is powerful. In addition, many investigators have created animal models that recapitulate the epidemiological studies with some models showing that altered physiological and

endocrine systems are a link between early development and later heart disease. What has been lacking, however, are studies that demonstrate the mechanistic roles that these endocrine systems have on heart growth and development at the cellular and molecular levels. The dogma has long persisted that cardiac myocytes are "terminally differentiated" and no longer able to divide. Recent work by Anversa and colleagues challenges that notion by showing low levels of proliferation throughout the adult myocardium (Anversa & Kajstura, 1998;Beltrami *et al.*, 2001;Kajstura *et al.*, 1998;Nadal-Ginard *et al.*, 2003;Quaini *et al.*, 2002). However, their findings do not necessarily disprove the idea that heart cell numbers at birth are important. Perhaps low levels of adult hyperplasia only match the similarly low levels of apoptosis and this is a system meant to maintain cardiomyocyte number during adult life; however, that mechanism is clearly not able to make up for a major loss of cells as occurs in a myocardial infarction. Thus, the number of cardiac myocytes one has at birth may determine the risk to later heart disease. Therefore, understanding the mechanisms by which the insulin-like growth factors and angiotensins effect fetal heart development is crucial to understanding the origins of adult heart disease. A fetal heart that is increased in size due to increased myocyte number may have a very different set of disease risks associated with it in adulthood compared to one that has increased in size due to increased myocyte size.

**Fetal Heart Growth:**

Individual organs, including the heart, mature by a regulated process of cell differentiation, cell maturation, cell division (hyperplasia), cell enlargement

(hypertrophy), and apoptosis. These processes function to form a heart that is capable of pumping blood through the body for a lifetime. How the heart cells are regulated to form a functioning heart with the appropriate number and size of cells is unknown. However, it is known that when large numbers of adult heart myocytes die, as with an infarction, they are unable to regenerate and replace themselves adequately.

Clubb and Bishop (Clubb & Bishop, 1984;Oparil *et al.*, 1984) were the first to show that one marker of terminal differentiation in the rat heart myocyte is binucleation and they concluded that binucleate cells are unable to divide and can only grow by hypertrophy. Binucleation occurs in the large mammal sheep also. From early embryonic life through mid gestation, total myocyte mass increases through cell division (myocyte hyperplasia). From mid gestation onward, there is an increasing proportion of myocytes that are differentiated (binucleate or polyploid) and total myocyte mass increases by both myocyte hyperplasia and hypertrophy. After birth and throughout adulthood, most myocytes are binucleate, terminally differentiated, and unable to divide. During adult life, total myocyte mass can only increase significantly by hypertrophy and the maximum number of myocyte cells may be set. The signals that cause the "switch" from a hyperplastic growth to a hypertrophic growth during late gestation are not known.

The ratio of binucleated to mononucleated cells can be altered by environmental factors during fetal life. An experimental systolic pressure load applied to the right ventricle in fetal sheep stimulates an increase in cardiac mass due to increased cardiomyocyte hypertrophy and hyperplasia and stimulates binucleation simultaneously, increasing the percentage of myocytes with two nuclei and the total number of myocytes

(Barbera *et al.*, 2000). However, it is unclear whether IGF-1 or Ang II are important in the regulation of myocyte maturation under these experimental conditions.

A full review of IGF-1's known and potential mechanisms for affecting heart growth will be reviewed in the introduction to chapter 3. In brief, IGF-1 has been implicated in all the modes of growth listed above. Theoretically, IGF-1 could affect heart growth through each mode of growth during late gestation, the period of time studied in this work. Ang II is also known to be a potent mediator of heart growth. The evidence and review of Ang II's known and potential roles for affecting heart growth are fully reviewed in chapter 4.

**Cell Signaling – ERK:**

The mitogen activated protein (MAP) kinase family is involved in variety of cell processes. The three primary MAP kinase cascade pathways are p38 MAPK, JNK, and p42,44 MAPK/ extracellular signal-regulated kinase (ERK). The ERK kinases ERK1 and ERK2 regulate many fundamental cellular processes such as proliferation, differentiation, migration, survival, hypertrophy, growth arrest, and apoptosis (Belcheva & Coscia, 2002). Thus, ERK signaling has many potential effects on growth of the fetal myocardium and may be critical to growth factor regulation of myocardial growth.

ERK stimulation is at the end of a cascade of protein kinases used to amplify receptor signaling from either receptor tyrosine kinases - such as IGF1R - or G-protein coupled receptors - such as AT1 (English *et al.*, 1999). Coupling to the two types of receptors is different, but they have in common most of the ERK kinase pathway (English *et al.*, 1999). The receptor is activated which creates docking sites for adapter

proteins such as Grb2 or Shc (English *et al.*, 1999;Sadoshima & Izumo, 1996). The adapter protein links the receptor to the guanine nucleotide exchange protein son of sevenless (SOS) which then catalyzes GDP release and GTP binding to p21 Ras (Egan *et al.*, 1993;Buday & Downward, 1993a;Sadoshima & Izumo, 1996;English *et al.*, 1999). GTP bound p21 Ras is activated, and GDP bound p21 Ras is inactivated (Buday & Downward, 1993b). Activated p21 Ras then binds to and activates Raf-1, a MAP kinase kinase kinase; activated Raf-1 phosphorylates and activates MEK, a MAP kinase kinase, which in turn phosphorylates and activates ERK (Stork & Schmitt, 2002). A diagram of ERK signaling is shown with some of the potential mechanisms of ERK action (Fig 1-1).

One of the key roles of ERK is regulation of the cell cycle and proliferation. Proliferation has been little studied in cardiomyocytes. This is likely due to the fact that neonatal rat cardiomyocytes, the cells most often used to study heart cell signaling *in vitro*, rapidly differentiate in culture and no longer proliferate (Sadoshima *et al.*, 1997). However, formation and maturation of skeletal myoblasts has been well documented. Skeletal myoblasts remain proliferative in culture conditions with high serum, but differentiate into myotubes with activation of the myogenin program under low serum conditions (Stewart & Rotwein, 1996). Activated ERK increases skeletal myoblast proliferation and inhibits myogenin and the differentiation into myotubes (Coolican *et al.*, 1997;Weyman & Wolfman, 1998;Adi *et al.*, 2002).

Of the three primary MAP kinase cascade pathways, the intracellular ERK cascade is thought to be a key stimulant of hypertrophy in cardiomyocytes (Aoki *et al.*, 2000;Bueno *et al.*, 2001;Bueno *et al.*, 2000;Fischer *et al.*, 1998;Sadoshima & Izumo, 1993a). Stimulation of the ERK signaling cascade may be necessary to affect cell

hypertrophy with exposure to Ang II, phenylephrine, endothelin-1, leukemia inhibitory factor, isoproterenol, sphingosylphosphorylcholine, and stretch (Clerk *et al.*, 2001;Wang & Proud, 2002;Lazou *et al.*, 1998;Sekiguchi *et al.*, 1999;Ueyama *et al.*, 2000;Yue *et al.*, 2000). Overexpression of MEK or expression of a constitutively active MEK induces hypertrophy *in vivo* or *in vitro* (Bueno *et al.*, 2000;Ueyama *et al.*, 2000). Expression of a dominant negative MEK blocks hypertrophy normally induced by phenylephrine, endothelin-1, leukemia inhibitory factor, isoproterenol, and stretch (Ueyama *et al.*, 2000).

**Cell Signaling – PI3K:**

The phosphoinositol 3-kinase (PI3K) signaling cascade also regulates various cellular processes such as proliferation, hypertrophy, apoptosis, differentiation, and cytoskeletal rearrangement (Vivanco & Sawyers, 2002).

PI3K activation is most closely associated with receptor tyrosine kinases such as IGF1R, but G-protein coupled receptors such as AT1 can also activate PI3K (Hirsch *et al.*, 2000;Li *et al.*, 2000;Sasaki *et al.*, 2000). The PI3K pathway is briefly summarized here, but has been well reviewed previously by Vivanco and Sawyers (2002). Typically, receptor activation provides docking sites for direct interaction with PI3K or for intermediate docking of phosphoproteins like insulin receptor substrate 1 (IRS1). PI3K is actually made up of two subunits, the p85 regulatory subunit which binds to IRS1 and the p110 catalytic subunit. The p110 subunit catalyzes the phosphorylation of phosphatidylinositol(4,5)$P_2$ ($PIP_2$) to phosphatidylinositol(3,4,5)$P_3$ ($PIP_3$). $PIP_3$ recruits protein kinase B (PKB or AKT) to the intracellular leaf of the cell membrane by binding to a pleckstrin homology domain on AKT. Once AKT is brought to the cell membrane, it

can be phosphorylated by 3-phosphoinositide-dependent protein kinase-1 & -2 (PDK1 & PDK2) that activate AKT. Downstream of AKT lay many targets that mediate the various cellular responses to PI3K activation. And while PI3K is closely linked with AKT phosphorylation, there are AKT independent pathways for PI3K signaling. A general diagram of PI3K signaling is given in Fig 1-2.

Much of the research into PI3K has focused on its proliferative and survival effects in cancer cells (Vivanco & Sawyers, 2002;Vemuri & Rittenhouse, 1994;Castoria *et al.*, 2001). PI3K-AKT stimulation phosphorylates and inactivates glycogen synthase kinase-3β (GSK3β) which otherwise would phosphorylate Cyclin $D_1$ and target it for degradation - the net effect being an increase in Cyclin $D_1$ (Diehl *et al.*, 1998). AKT also negatively regulates the cyclin dependent kinase inhibitors p21 and p27 (Graff *et al.*, 2000) which allows for PCNA binding and proliferation (Rossig *et al.*, 2001).

Recent work has shown that PI3K and its downstream target AKT may be critical components of the regulation of heart size (Shioi *et al.*, 2000;Shioi *et al.*, 2002;Condorelli *et al.*, 2002;Crackower *et al.*, 2002). Transgenic mice expressing a constitutively active PI3Kα construct have an increased heart size due to increased myocyte size; mice expressing a dominant negative PI3Kα construct have decreased heart size due to decreased myocyte size (Shioi *et al.*, 2000). The same results are seen when constitutively active AKT and dominant negative AKT are expressed (Shioi *et al.*, 2002;Condorelli *et al.*, 2002). Skeletal muscle hypertrophy can also be regulated by PI3K-AKT via mTOR activation (Bodine *et al.*, 2001;Rommel *et al.*, 2001). The kinase mTOR is an important regulator of protein synthesis that is sensitive to nutrient availability and ATP levels (Vivanco & Sawyers, 2002).

PI3K signaling may be important in heart differentiation and maturation because it is known to induce skeletal myoblast differentiation into skeletal myotubes (Coolican *et al.*, 1997;Kim *et al.*, 1999). Inhibitors of PI3K activity such as wortmannin or LY294002 block skeletal myoblast differentiation (Coolican *et al.*, 1997;Kim *et al.*, 1999;Kaliman *et al.*, 1996). Expression of a dominant negative p85 subunit of PI3K blocks skeletal myoblast differentiation (Kaliman *et al.*, 1998) while expression of a constitutively active p110 subunit induces myoblast differentiation (Kim *et al.*, 1999).

**Hypotheses:**

It is likely that endocrine systems perturbed in fetal undernutrition are important in fetal heart growth regulation and the onset of heart disease in the adult. Thus, it is important to understand the mechanisms by which these endocrine systems alter fetal heart development at the cellular level, because either fewer or larger cardiomyocytes could bring independent risk factors to the heart in later life. Fewer cells would mean that the work performed per cell would be increased and would represent a reduced reservoir in times of increased apoptosis - a feature of heart failure. I hypothesized that IGF-1 through IGF1R regulates heart size by increasing cellular proliferation of mitotically competent mononucleated cardiomyocytes and by promoting myocyte binucleation and hypertrophy of the more mature binucleated cells during late gestation. Based on neonatal rat cardiomyocyte culture data, I hypothesized that Ang II would induce hypertrophy of fetal sheep cardiomyocytes. I further hypothesized that binucleated myocytes would be able to enlarge in response to hypertrophic stimuli while mononucleated cells would not. Based on the balance of the evidence in skeletal

myoblasts and cardiomyocytes, I hypothesized that proliferative and hypertrophic stimuli would be dependent on ERK signaling while binucleation would be dependent on PI3K signaling.

**Specific Aims for this Project:**

1. To determine the extent to which exogenous IGF-1 stimulates heart growth through myocyte proliferation. This aim tested the hypothesis that IGF-1 causes a cardiomyocyte proliferation in immature myocardium and that this response is through IGF1R and the ERK signaling pathway.

2. To determine the extent to which exogenous IGF-1 stimulates heart maturation and myocyte binucleation. This aim tested the hypothesis that IGF-1 causes maturation and binucleation of cardiomyocytes in late gestation and that this response is through IGF1R and the PI3K pathway.

3. To determine the extent to which exogenous IGF-1 and Ang II stimulates heart growth through myocyte hypertrophy. This aim tested the hypothesis that IGF-1 and Ang II causes cardiomyocyte hypertrophy in fetal sheep myocardium and that this response is through the ERK signaling pathway.

4. To determine the myocyte maturation level required for hypertrophy. This aim tested the hypothesis that only binucleated cardiomyocytes can be stimulated to grow by cellular hypertrophy.

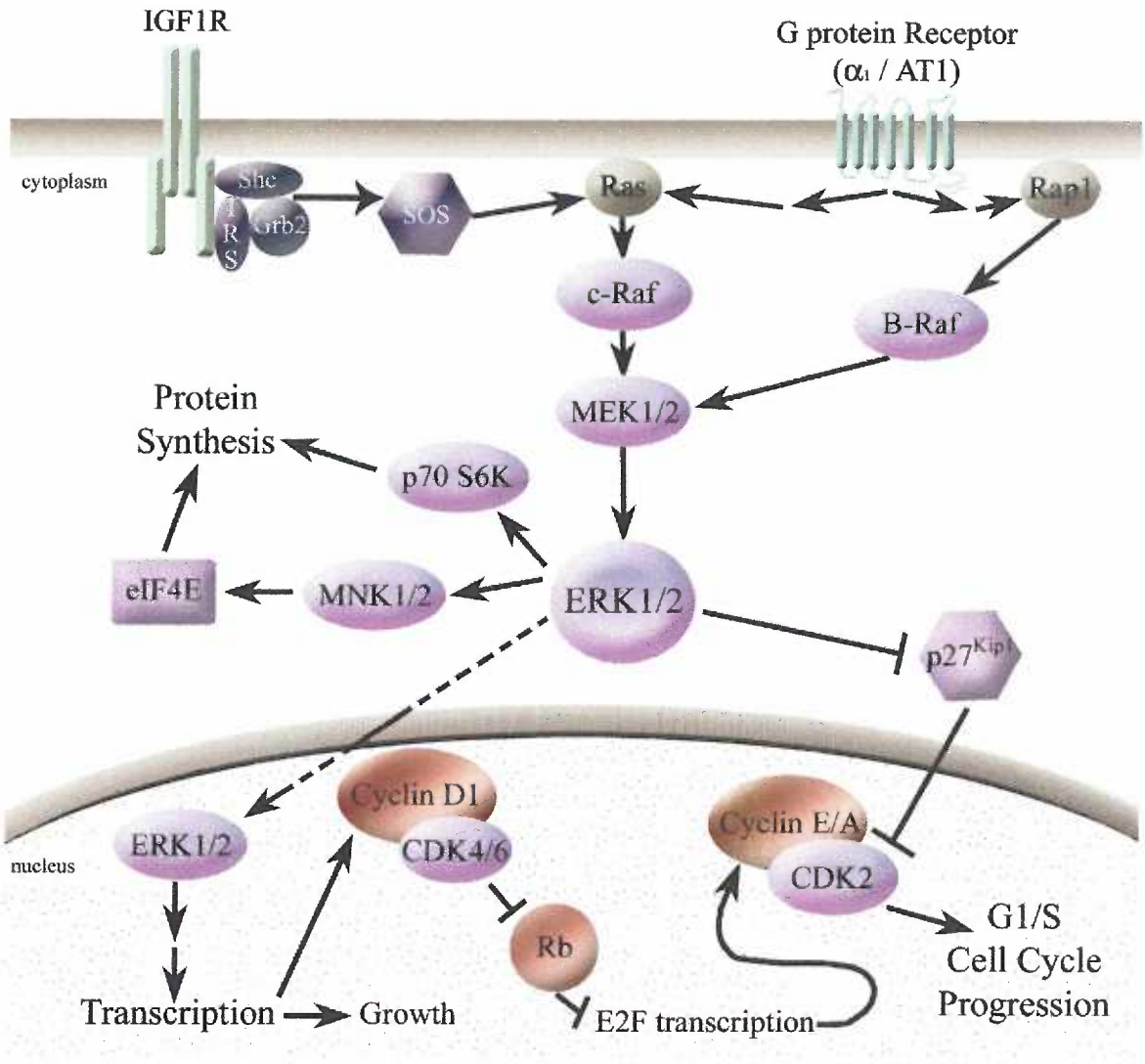


**Figure 1-1: ERK Signaling Cascade**

An abbreviated overview of ERK signaling in cardiomyocyte growth. Not defined in text: Grb2, growth factor receptor-bound protein 2; Shc, SH2-containing collagen-related proteins; Rap1, Ras related protein; p70S6K, 70kDa S6 kinase; MNK, MAP kinase interacting kinase; eIF, eukaryotic initiation factor; CDK, cyclin dependent kinase; $p27^{Kip1}$, CDK inhibitor; Rb, retinoblastoma protein; E2F, transcription factor.

# Chapter 2:

# General Methods

*Animals*: The OHSU Institutional Animal Care & Use Committee approved all procedures used in this thesis (protocol A050). On day 124.4 ± 1.4 days of gestation (mean ± s.d., term = 145d), surgery was performed on fetal sheep of mixed western breed. Our surgical protocols have been described previously.(Barbera *et al.*, 2000;Thornburg & Morton, 1983) Briefly, anesthesia was induced with diazepam (0.13 mg/kg) and ketamine (5mg/kg). The ewe was intubated and anesthesia was maintained with 1% halothane in oxygen and nitrous oxide (1:1). Maternal exhaled $CO_2$ and cutaneous $O_2$ hemoglobin saturation were monitored continuously. The neck and head of the fetus were exposed through a midline laparotomy and a uterine incision. Polyvinyl catheters of 1.3mm OD or 1.7mm OD were placed in the right atrium (via jugular vein) and the aorta (via carotid artery). In closing, catheters were brought to the exterior of the ewe and kept in a cloth pouch that was attached to the mother's back. The maternal incision was closed in anatomic layers. The fetus was given penicillin (1M units) in the amniotic fluid at the completion of surgery. Fetuses were studied, un-anaesthetized, after >5 days post surgery.

*Long R3 IGF-1 Rationale*: IGF-1 circulates largely bound to a family of at least 6 binding proteins in serum with a small fraction existing in the "free" form. The roles of the binding proteins are not entirely clear. The binding proteins may both sequester free IGF-1 and prevent binding to IGF1R or they may potentiate uptake into tissue and/or receptor binding (Delafontaine, 1995). The binding proteins themselves may also have actions independent of IGF-1 (Delafontaine, 1995). In order to isolate the actions of IGF-1 unassociated with binding protein but bound to its receptor, we infused sheep fetuses with an IGF-1 analogue, Long R3 IGF-1 (LR3 IGF-1), that binds binding protein with

much lower affinity (200-1000 fold) than endogenous IGF-1 and that circulates mostly in a free form in plasma.

LR3 IGF-1 is an IGF-1 analogue with properties that make it more potent than endogenous IGF-1 while costing less per mg. LR3 IGF-1 binds with >100 fold lower affinity for IGFBP-3, the major IGF binding protein in plasma. It binds with >250 fold lower affinity to the other minor IGFBPs (GroPep tech bulletin #2). Fetal lambs have been infused with radioactive IGF-1 and it was determined that 21% of the IGF-1 molecules circulated bound to IGFBP-3, 55% circulated bound to IGFBP-1, and 19% circulated in a free, unbound form (Bassett *et al.*, 1990). Current evidence suggests that only the free IGF-1 can bind to the IGF-1R and activate it. Interestingly, in the adult rat a dose of 278μg IGF-1/day s.c. is equivalent to 44μg LR3 IGF-1/day s.c. (16% of IGF-1 dose) measuring outcomes such as body weight, nitrogen retention, and food conversion efficiency (Tomas *et al.*, 1993). Thus, much lower doses of LR3 IGF-1 can be used as the equivalent dose of endogenous IGF-1.

The half-life and clearance has been investigated in the fetal lamb for IGF-1 but not for LR3 IGF-1. IGF-1 has the following half lives: when bound to IGFBP3, 412±104 min; when bound to IGFBP1, 202±10 min; and when unbound, 13±5 min (Bassett *et al.*, 1990). Half lives (T½) for IGF-1 and LR3 IGF-1 are roughly equal in the rat while clearance (Cl) of LR3 IGF-1 was 10 fold greater than IGF-1 (Bastian *et al.*, 1993). Using the equation T½=0.693*Vd/Cl and the assumptions that the half lives are equal and that the clearance of LR3 IGF-1 is 10 times the clearance of endogenous IGF-1, we can conclude that the volume of distribution (Vd) must also be 10 fold greater for LR3 IGF-1. The chronically infused dose is a product of Vd and plasma level (Cpss), Dose=Vd*Cpss.

Vd for LR3 IGF-1 compared to IGF-1 is 10X, but the plasma level needed is roughly 20% of that for IGF-1, which would mean that LR3 IGF-1 needs 2X the dose of IGF-1 to be equipotent, *if* they had equal potency. In fact, LR3 IGF-1 is roughly 5X more potent (GroPep Tech Bulletin #2). Thus an LR3 IGF-1 dose that is 2/5 of an IGF-1 dose should be roughly equal in effect. A previous paper has shown that a dose of 81μg/hr recombinant IGF-1 given for 10 days causes a significant increase in heart weight and heart to body weight ratio (Lok *et al.*, 1996). Therefore we used a dose of 29.8μg/hr LR3 IGF-1 ($6.6 \pm 1.2 \mu g hr^{-1} kg^{-1}$ based on weight at autopsy).

A dose of 80μg/hr IGF-1 increased plasma IGF-1 levels two to three fold at 4 hours and at 4 days (Marsh *et al.*, 2001a). We expected the estimated equivalent dose of LR3 IGF-1 to raise the plasma level even less and thus be well within the physiological range of activity.

*Experimental Plan*: Day 0 of experiments began on 130.5 ± 0.9d of gestation. Baseline hemodynamics were measured and some blood drawn from the aorta for blood gases and hematocrit. A blood sample was also obtained in EDTA on ice and the plasma was frozen for later determination of plasma IGF-1 level. An ambulatory mechanical pump was attached to a catheter placed in the right atrium via the jugular vein and secured in the cloth pouch attached to the mother. Vehicle (normal saline + 1% BSA) or a solution of LR3 IGF-1 (Gropep, Adelaide, Australia) was infused into the fetus at a rate of 10.4mL/day for a total of 7 days. Experimental fetuses were given a total of 5mg of LR3 IGF-1 over the 7 days. During infusion the mother was free to walk around in her pen. On days 4 and 7 the mother was put in a stanchion cart and the hemodynamic measurements and blood samples were taken as on day 0. After 7 days of infusion, a

lethal dose of commercial euthanasia solution containing pentobarbital sodium (Delmarva Laboratories Inc., Midiothan, VA, USA) was given to the ewe and the fetus was heparinized with 10,000 units of heparin sulfate and given 3cc of saturated KCl solution to arrest the heart in diastole. Fetal body weight, total heart weight, total liver weight (including gall bladder), and combined kidney weights were measured and the heart was taken for cell isolation.

*Plasma IGF-1 and Long R3 IGF-1*: Plasma IGF-1 levels were measured using an ELISA kit that releases the IGF-1 from its binding proteins and gives a value for the total IGF-1 (ALPCO Diagnostics, Windham, NH). Measurement of plasma LR3 IGF-1 used an ELISA kit from the makers of the LR3 IGF-1 (Gropep). Coating and capture antibodies were supplied in the kit and were used at the recommended concentrations. Anti-rabbit Ig HRP antibody was used at 1:8000 dilution (Jackson ImmunoResearch). The LR3 IGF-1 assay has no significant cross-reactivity to either IGF-1 or IGF-2 up to concentrations of 50ng/mL.

*Cell Isolation*: Our cell isolation procedure has been described previously (Barbera *et al.*, 2000). Some modifications have been made for obtaining viable live cells for culture. The hearts were hung from a perfusion apparatus by cannulating the aorta and were perfused retrograde by a series of oxygenated solutions at 39°C through the coronary arteries. The protocol was 5 - 10 min perfusion with calcium-free tyrode buffer; 10 min with Ca-free Tyrode solution containing collagenase (Worthington type II, 160 units/ml) and protease (type XIV 13 mg in 80 ml, Sigma); and 5-10 min with high potassium (KB) solution (in mM: 74 glutamic acid, 30 KCl, 30 $KH_2PO_4$, 20 taurine, 3 $MgSO_4$, 0.5 EGTA, 10 HEPES, and 10 glucose, adjusted to pH 7.37 using KOH). The

right ventricle (RV) and left ventricle (LV) free walls were separately removed with scissors, cut into chunks, and gently agitated in KB solution to release the cells. Isolated cells were poured into separate tubes removing tissue chunks, resulting in a cell slurry containing >95% isolated individual cells. Although cardiac myocytes seem to be released preferentially, endothelial cells, fibroblasts, and blood cells were also present.

*Isolated Mycoyte Size Measurements*: A portion of the isolated cell slurry was fixed in 1% paraformaldehyde in PBS, pH7.4. The suspended free-floating cells were stained with methylene blue and then wet mounted to a slide and viewed under the microscope as described previously (Barbera *et al.*, 2000). Using image analysis software (Optimas, Media Cybernetics, Seattle, Wa.) the length and the width of individual cells were measured at 400X under light microscopy (Zeiss Axiophot) and were marked as being either mononucleated or binucleated in the data output. One hundred cells were measured for size and two hundred counted for binucleation. Some cells were also stained to check cellular integrity. Dissociated cells were fixed and dried on a glass slide. A monoclonal antibody, anti-myosin (1:300, Alexis Biochemicals, CA, USA, clone A4.1025), diluted in blocking buffer (PBS plus 1% bovine serum albumin (BSA) and 0.5% Triton X-100) was added and left overnight at 4°C. Cells were washed in PBS and secondary antibody with nuclear stain added. Secondary antibody was anti-mouse rhodamine red-X-conjugate (1:200; Jackson ImmunoResearch Laboratories, Inc., PA, USA), and the nuclear stain was Hoechst 33342 (1:10,000; Molecular Probes, Eugene, OR, USA). Secondary was incubated for 1 hour at room temperature. Cells were washed with PBS and covered with a coverslip mounted with ProLong anti-fade kit (Molecular Probes).

*Cell Culture*: Cardiomyocytes used for cell culture include those from vehicle- or saline-infused instrumented fetuses, non-infused instrumented fetuses, and non-instrumented fetuses ranging from 127-139d gestation. LV and RV cells were kept separate throughout. Cells were left to rest for 30 minutes to 1 hour at room temp in KB, while the concentration of cells was measured in a counting chamber (VWR). Total number of cells was estimated from the concentration and volume (mL) of slurry. Typically, 20-50 million cardiomyocytes from LV and 10-30 million cardiomyocytes from RV were recovered. Cells were gently pelletted at 1,000 rpm (≈200g) for 3-5 minutes. KB solution was removed and cells were re-suspended to a concentration of $10^6$ cells $mL^{-1}$ in serum medium (MCDB105 medium (Sigma) supplemented with 2mM KCl, 0.2mM glycine, 5mM creatine, 5mM taurine, 2mM L-carnitine, 1% insulin-transferrin-sodium selenite medium supplement (Sigma), 0.1% antibiotic-antimycotic solution (100X, Sigma), and 10% (v/v) fetal bovine serum). The cell suspension was added to untreated tissue culture flasks for 2 hours in the incubator (39°C, 5% $CO_2$) to pre-plate fibroblasts and endothelial cells. Cells were gently poured from the first flask to a second flask and a second pre-plate interval followed for another 2 hours. After pre-plating, unattached cells were moved to a tube and spun down again at 1000 rpm. This second pelleting step helps remove any debris and possible contaminants. The medium was removed and cells were re-suspended in serum medium to the same concentration as above. Lastly, cells were plated into 35mm diameter tissue culture dishes holding sterile 22x22mm cover slips pre-treated with laminin (1-5μg/mL) or plated into 6-well plates that were laminin treated. Cardiomyocytes were plated out at 500,000 per 35mm diameter dish or 1-2 x $10^6$ per well of a 6-well plate. Additional serum medium was

added to make a final volume of 2.5mL in each well or plate. After 24 hours, the serum medium was replaced with serum free medium (as serum medium without serum). Cells were left for an additional 48 hours during which time the myocytes "plate out" on the cover slips and wells. Fresh serum free medium was added and left for 24 more hours before experiments began. Thus from the time the myocytes were first plated onto cover slips or wells, they were held in serum conditions the first 24 hours and then serum free conditions an additional 72 hours before experiments began.

*Cultured Cell Size Experiments*: Cardiomyocytes were exposed to Ang II and LR3 IGF-1 in order to determine to what degree they would cause hypertrophy. A dose response curve for Ang II was generated using concentrations ranging from 10nM-5μM. From these experiments, 100nM Ang II was chosen; this dose corresponds to the optimal dose in the rodent myocyte model (Aoki *et al.*, 2000;Sadoshima *et al.*, 1995). Because PE is believed to cause hypertrophy through α adrenergic receptor stimulation, cardiomyocytes were exposed to PE as a positive control. A dose-hypertrophic response relationship for PE was determined using concentrations of 0.2-20μg/mL PE. A dose of 10μg/mL PE was chosen to use in future experiments based upon its maximal stimulation of cell hypertrophy. Further experiments were performed in the absence of BrdU, using 1000X stocks of agonists to obtain final concentrations of 10μg/mL (49.1μM) PE, 100nM Ang II, 1μg/mL (110nM) LR3 IGF-1, 10μM UO126, and 10μM LY294002. After 48 hours, cells were fixed for staining and measuring.

*BrdU Uptake Experiments for Cultured Cells*: Media were replaced with serum free medium containing 10μM 5-bromo-2-deoxyuridine (BrdU). Experimental solutions were made by addition of 1000X stocks of agonists and inhibitors to final concentrations

of 10μg/mL (49.1μM) PE, 10nM Ang II, 100nM Ang II, 0.1μg/mL (11nM) LR3 IGF-1, 1μg/mL (110nM) LR3 IGF-1, 10μM UO126, and 10μM LY294002. Positive controls consisting of serum medium and 10μM BrdU were also used. All experiments lasted for 48 hours and cells were fixed thereafter for staining.

*Fixing Cells to Cover Slips*: The medium was removed and the cover slips were washed once with PBS in 35mm plates. Cells were fixed in 70% ethanol in 50mM glycine, pH2.0, for 30 minutes at -20°C. Cells were washed with PBS again and stored at 4°C covered with PBS and 1% sodium azide until staining.

*BrdU Immunofluorescence and Cell Counting*: Cells were stained on the cover slips. Cells were treated with 5μg/mL DNase I in a tris-buffer (66mM Tris-buffer, 0.66mM $MgCl_2$, 1mM 2-mercaptoethanol) for 30 minutes at 37°C. Three washes of PBS + 1mM EDTA were used to stop all DNase activity. Monoclonal antibodies including 1:500 anti-myosin (Alexis Biochemicals, CA, USA, clone A4.1025) and 1:500 anti-BrdU (AbCam Ltd, Cambridge, UK, clone BU1/75) were diluted in blocking buffer (PBS plus 1%BSA and 0.5% Triton X-100) and added to coverslips. Primary antibodies were left overnight at 4°C. Cells were washed in PBS and secondary antibodies added. Secondary antibodies included anti-mouse rhodamine red-X-conjugate (for detection of myosin) and anti-rat FITC-conjugate (for detection of BrdU) (1:200; Jackson ImmunoResearch Laboratories, Inc., PA, USA) and were incubated for 2 hours at room temperature. Cells were washed in PBS and the coverslips inverted and mounted to slides with ProLong anti fade kit (Molecular Probes, Eugene, Or.). Three hundred myosin positive cells were counted under a fluorescence microscope (Zeiss Axiophot) at 400x, and the number of cells positive for BrdU recorded. This ensured that only myocytes and not any

contaminating cells were counted. Data were kept as both numbers and as the percentages of the 300 cells counted.

*Cultured Cell Measurements*: The Envision + System/HRP (DakoCytomation, Carpenteria, CA, USA) was used to stain myosin with diaminobenzidine to differentiate myocytes from contaminating cells. The kit protocols were followed using the primary antibody against myosin (1:500). Cells were counterstained with hemotoxylin and dehydrated; cover slips were inverted and mounted in a xylene-based medium onto a glass slide. Optimas tissue analysis software (MediaCybernetics, Seattle, Wa, USA) was used to capture images and measure the cell sizes under light microscopy at 400X (Zeiss Axiophot). The threshold was set for each image so that the cells of interest were outlined while all other cells or non-cells were not. The software calculates area, length, width, and perimeter for each outlined cell. The areas of 50 mononucleated cells and 50 binucleated cells were saved in separate files from each experiment and the means for all values were calculated.

*Cell Stimulation for Western Blot Analysis*: After following our cell culture protocol, fresh serum free medium was added to each well and cells were left alone for 12 hours before the experiment. For RV cardiomyocytes, four wells were pre-incubated 20 minutes with 10μM UO126, four were pre-incubated 20 minutes with 10μM LY294002, and the final four were left in serum free media alone. Then both inhibited and uninhibited wells were stimulated with 1μg/mL LR3 IGF-1 for 0, 5, 10, or 20 minutes. For LV cardiomyocytes, half the wells were pre-incubated 20 minutes with 10μM UO126. Then both inhibited and non-inhibited wells were stimulated with 100nM Ang II for 0, 5, 10, or 20 minutes. In separate LV experiments, cells were stimulated

with 10μg/mL PE for 0, 5, 10, or 20 minutes along with separate wells stimulated with 100nM Ang II for the same times. The stimulation was stopped on ice and medium was removed. Cells were washed in ice cold PBS and then scraped in Bos Buffer (10% glycerol, 1% IGEPAL CA-630, 50mM Tris-HCl pH7.4, 200mM NaCl, 2mM $MgCl_2$, 0.5mM β-Glycerolphosphate) with the protease inhibitors PMSF (1mM), Aprotinin (2μg/mL), Leupeptin (1μg/mL), Trypsin inhibitor (10μg/mL), and Sodium OrthoVanadate (1mM). Cell lysate was spun down and the supernatant removed and stored at –80°C or run on a gel right away.

*Western Blot Analysis*: Chemiluminescent reactions were used to detect levels of phospho-ERK stimulation normalized to total ERK2 and phospho-AKT stimulation normalized to total AKT. Protein concentrations were calculated from a Bradford reaction read on a 96-well plate. Twenty to forty μg of total protein from lysate was run for each time point and experiment. The protein was run on a 7.5 or 12% SDS-PAGE gel and then transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 1% BSA in PBS + 0.1% Tween (PBST) for 1 hour. The primary antibody used was 1:1000 phospho-p44/42 MAP Kinase (Thr202/Tyr204) or 1:1000 phospho-(Thr308)-AKT (Cell Signaling Technology Inc., Beverly, MA, USA). Primary antibody was added to the PVDF membrane diluted in PBST + 1%BSA and left to shake overnight at 4°C. The PVDF Membrane was washed 3 times and then secondary antibody (ant-rabbit HRP 1:10,000 in PBST) was added for 1 hour at room temperature. The membrane was washed three times and then ECL western blotting detection reagent (Amersham Pharmacia Biotech, Buckinghamshire, UK) was added. A film was placed over the membrane, exposed, and then developed. The membrane was then stripped 20

minutes at 55°C in a stripping solution containing beta mercaptoethanol. Membrane was washed thoroughly. Steps were repeated with primary antibody to ERK2 (1:1000, Santa Cruz Biotechnology Inc, CA, USA, clone sc-1647) and AKT (1:1000, Cell Signaling Technology). Film images were scanned into TIFF files and analyzed on multianalyst software (Bio-Rad Laboratories, Hercules, CA, USA) and normalized to the 0 minute time point of stimulation in the absence of either inhibitor for each experiment.

*Statistics*: For western blot density of Ang II and PE stimulated ERK, non-parametric ANOVA with Dunn's post hoc test was used. For LR3 IGF-1 stimulated ERK and AKT western blot data, a constant of 0.5 was first added to all fold changes and then log-transformed to improve symmetry and enhance the normality of the data prior to analysis. A linear mixed effect model (Penheiro & Baudler, 2000) was fit to the transformed data in which the effects of time and treatment were regarded as mixed effects while the four experimental replications were regarded as a random factor performed using R version 1.6.1 (Ihaka & Gentleman, 1999). The mean on the log-transformed scale is consistent with the median on the original scale; hence, all inferences apply to the median fold change. Subsequent p-values from individual pairwise comparisons were adjusted using Holm's method (Holm, 1979) to control the error rate among all tests under consideration. All other multiple comparisons used ANOVA with Tukey's post hoc test. Student's t-test was used to compare two groups. Comparisons were considered significant at $p<0.05$.

# Chapter 3:

# Extracellular Signal-Regulated Kinase and Phosphoinositol-3 Kinase mediate IGF-1 induced proliferation of fetal sheep cardiomyocytes

**Nathan C. Sundgren, George D. Giraud, Jess M. Schultz, Michael R. Lasarev, Philip J.S. Stork, and Kent L. Thornburg**

**Submitted for Publication**

**Abstract:**

Growth of the fetal heart involves processes of cardiomyocyte enlargement, division, and maturation. Insulin like growth factor-1 (IGF-1) is implicated in many aspects of growth and is likely to be important in developmental heart growth. IGF-1 stimulates the IGF-1 receptor (IGF1R) and downstream signaling cascades including ERK and PI3K. We hypothesized that IGF-1 stimulates cardiomyocyte proliferation and enlargement through stimulation of the ERK cascade and stimulates cardiomyocyte differentiation through the PI3K cascade. *In vivo* administration of Long R3 IGF-1 (LR3 IGF-1) did not stimulate cardiomyocyte hypertrophy, but led to a decreased percentage of cells that were binucleated. In culture, LR3 IGF-1 increased myocyte BrdU uptake by 3-5 fold. Blockade of either ERK or PI3K signaling completely abolished BrdU uptake stimulated by LR3 IGF-1. LR3 IGF-1 did not increase footprint area while phenylephrine did increase binucleated cardiomyocyte size. We conclude that IGF-1 through IGF1R: 1) stimulates cardiomyocyte division *in vivo* and *in vitro*, 2) does not stimulate binucleation *in vivo*, 3) does not stimulate hypertrophy either *in vivo* or *in vitro*, 4) requires both ERK and PI3K signaling for proliferation of fetal sheep cardiomyocytes *in vitro*.

**Introduction:**

Prenatal malnutrition leads to "programming" of the fetus and increased susceptibility to adult-onset hypertension, insulin resistance syndrome, and ischemic heart disease (Harding, 2001;Barker *et al.*, 1989b). Insulin like growth factor 1 (IGF-1) is an important regulator of growth during fetal life and is regulated by fetal nutrient supply (Oliver *et al.*, 1993;Oliver *et al.*, 1996). Maternal under-nutrition lowers fetal IGF-1 levels and decreases fetal heart to body weight ratio (Osgerby *et al.*, 2002) while infusion of IGF-1 to the fetus increases heart to body weight ratio in sheep (Lok *et al.*, 1996). However, the mechanisms by which IGF-1 regulates heart size are unknown. IGF-1 is known to stimulate hypertrophy, hyperplasia, and/or cell differentiation depending upon the cell type and maturation state of the cell (Han & Hill, 1994), but its role in cardiomyocyte growth and maturation is unclear.

Transgenic and knock out mice have proven IGF-1's critical role in development and growth. Mice null for IGF-1 are born 60% of normal birth weight and mice null for the IGF-1 receptor (IGF1R) are 45% of normal birth weight; mice double null for IGF-1 and its receptor are not phenotypically different from the receptor null (Liu *et al.*, 1993). The difference between the two null models is attributed to IGF-2's ability to bind and activate IGF1R (Liu *et al.*, 1993). The IGF-1 nulls that survive birth are stunted in growth compared to littermate controls (Baker *et al.*, 1993). These data show that IGF-1 remains an important growth factor into the neonatal period in mice, a period comparable to fetal development of precocious mammals, such as sheep.

IGF-1 is an important regulator in the cell cycle progression from the $G_1$- to S-phase (Pardee, 1989;Baserga & Rubin, 1993). In primary cardiomyocyte cultures, IGF-1

causes a large increase in PCNA expression and in several cyclins involved in cell cycle progression (Liu *et al.*, 2001a). When IGF-1 is over-expressed in the heart using a transgenic approach, heart weight is increased, as is heart weight to body weight ratio, apparently due to an increase in cardiomyocyte number rather than cell size (Reiss *et al.*, 1996).

Several *in vivo* animal models have linked pressure load and hypertrophy with IGF-1 levels. In the adult pig, volume or pressure overload causes increases in IGF-1 protein and mRNA that last for the duration of loading (Modesti *et al.*, 2000). IGF-1 stimulates neonatal rat cardiomyocytes to enlarge *in vitro* and the increase in size is accompanied by increased expression of myosin light chain 2 and skeletal alpha actin (Ito *et al.*, 1993;Lavandero *et al.*, 1998). In the mouse, IGF-1 is highly expressed during adult cardiac hypertrophy caused by transverse aortic banding compared to normal controls (Schoenfeld *et al.*, 1998). However, interpreting the role of IGF-1 in promoting cardiomyocyte hypertrophy *in vivo* is difficult because studies of IGF-1 are usually performed in adult animals where most myocytes are terminally differentiated.

IGF-1 and 2, acting through the IGF1R, can lead to either the proliferation or differentiation of skeletal muscle myoblasts (Stewart & Rotwein, 1996;Stewart *et al.*, 1996;Quinn *et al.*, 1994) through different signaling pathways (Coolican *et al.*, 1997). However, the binucleation of cardiac myocytes during fetal or early postnatal growth, while called "terminal differentiation," is quite different from developmental tissue differentiation. Cardiomyocyte binucleation occurs by DNA replication that is followed by karyokenesis without the cytokenesis that would normally follow in the mitotic cycle (Li *et al.*, 1997a;Li *et al.*, 1997b). Reiss, et al (1996) found in transgenic mice over-

expressing IGF-1 that the proportions of mononucleated and binucleated cells in the heart were unchanged. However, the cardiac myocytes appear to have been beyond the age that binucleation normally occurs. The role of IGF-1 in heart maturation in the large mammal has not been examined.

IGF-1 through IGF1R stimulates a multitude of signaling pathways; the two best studied are the Extracellular Signal-Regulated Kinase (ERK) and Phosphoinositol-3 Kinase (PI3K) cascades. Distinctive and complementary roles for these IGF-1 stimulated signaling cascades have been found. In skeletal myoblasts, IGF-1 induced proliferation through ERK signaling and differentiation through PI3K signaling (Coolican *et al.*, 1997). However, in a neuroblastoma cell line (SH-SY5Y cells), IGF-1 induced growth and differentiation required both ERK and PI3K (Kurihara *et al.*, 2000). PI3K but not ERK signaling mediates IGF-1 stimulated endothelial cell migration (Liu *et al.*, 2001b). In these cells, however, both ERK and PI3K signaling induced NF-kappa-B dependent transcription (Liu *et al.*, 2001b). Both signaling cascades are required to protect cardiomyocytes from apoptosis during exposure to hypoxia (Mehrhof *et al.*, 2001) or oxidative stress (Hong *et al.*, 2001). ERK signaling is necessary in IGF-1 induced protein synthesis and hypertrophy in neonatal rat cardiomyocytes (Lavandero *et al.*, 1998) consistent with the ERK cascade's obligatory role in many forms of agonist induced hypertrophy (Ueyama *et al.*, 2000;Yue *et al.*, 2000). Given the importance of these cascades in various forms of growth and differentiation, it is important to dissect their roles in IGF-1 induced growth in the fetal myocardium of the large mammal.

Thus, there are many forms of evidence that IGF-1 plays a major role in heart development. We are interested in the intermediate phase of late fetal growth when

working cardiac myocytes transition from being entirely mononucleate to 100% binucleate. This phase of heart growth includes the transition from the early fetal hyperplastic to adult-type hypertrophic mode of growth. We set out to determine the role of IGF-1 and its signaling cascades in heart growth and maturation of fetal cardiomyocytes of sheep. Because we discovered recently that binucleate but not mononucleate cardiomyocytes are capable of enlargement under the stimulus of phenylephrine (Sundgren *et al.*, 2003), we tested the hypothesis that IGF-1 would lead to increased binucleation followed by hypertrophy simultaneously with proliferation in the mononucleate population of fetal sheep cardiomyocytes. We hypothesized that proliferation and hypertrophy would be controlled by ERK stimulation while binucleation would require PI3K stimulation.

**Specific Methods:**

*Animals*: Day 0 of experiments began on 130.5 ± 0.9d of gestation. Vehicle (normal saline + 1% BSA) or a solution of Long R3 IGF-1 (Gropep Ltd, Adelaide, Australia) was infused into the fetus at a rate of 10.4mL/day for 7 days. Experimental fetuses were given 5mg of LR3 IGF-1 over 7 days (29.8μg/hr, 6.6 ± 1.2$\mu g hr^{-1} kg^{-1}$ based on weight at autopsy). This dose was calculated based on published pharmacological data to be equivalent to 81μg/hr recombinant IGF-1 that has been used previously to increase fetal sheep heart weight.(Lok *et al.*, 1996) After 7 days of infusion, fetal body weight, total heart weight, total liver weight (including gall bladder), and combined kidney weights were measured and the heart was taken for cell isolation.

*BrdU uptake and Cell Size experiments*: Media was replaced with serum free media containing 10μM BrdU. Experimental solutions were made from addition of 1000X stocks of agonists and inhibitors to final concentrations of 0.1μg/mL (11nM) LR3 IGF-1, 1μg/mL (110nM) LR3 IGF-1, 10M UO126 and 10μM LY294002. Positive controls consisted of serum media and 10μM BrdU. Cells were fixed for staining 48 hours after stimulation began. Doses for experiments for cell size were the same as for BrdU uptake experiments but in the absence of BrdU. 10μg/mL phenylephrine (PE) was used as a positive control. After 48 hours, cells were fixed for staining and measuring.

*Cell Stimulation and Western Blot Analysis*: At experiment, four wells were pre-incubated 20 minutes with 10μM UO126, four were pre-incubated 20 minutes with 10μM LY294002, and four were left in serum free media alone. All wells were stimulated with 1μg/mL LR3 IGF-1 for 0, 5, 10, or 20 minutes. Data is normalized to the 0 minute time point of stimulation in the absence of either inhibitor for each experiment.

*Statistics*: For western blot density a constant of 0.5 was first added to all fold changes and then log-transformed to improve symmetry and enhance the normality of the data prior to analysis. A linear mixed effect model (Penheiro & Baudler, 2000) was fit to the transformed data in which the effects of time and treatment were regarded as mixed effects while the four experimental replications were regarded as a random factor performed using R version 1.6.1 (Ihaka & Gentleman, 1999). The mean on the log-transformed scale is consistent with the median on the original scale; hence, all inferences apply to the median fold change. Subsequent p-values from individual pairwise comparisons were adjusted using Holm's method (Holm, 1979) to control the error rate among all tests under consideration. All other multiple comparisons used ANOVA with Tukey's posttest. Student's t-test was used to compare two groups. Comparisons were considered significant at $p<0.05$.

**Results:**

Table 3-1 shows the arterial blood gas, pH, hematocrit, and hemodynamic data of experimental and vehicle control animals before infusion (Day 0), after 4 days (Day 4), and on the final day of infusion (Day 7). Of note is the gradual decline in $Pa_{O2}$ and $O_2$ content over the 7 days of infusion with LR3 IGF-1. Other groups have reported this $Pa_{O2}$ decline with infusion of recombinant IGF-1 over several days, but the cause for this has not been found (Lok *et al.*, 1996;Marsh *et al.*, 2001a). Experimental animals also showed an increase in heart rate on Day 7 compared to vehicle controls (Table 3-1). Otherwise the animals were healthy and in the expected ranges for all parameters.

Plasma levels of endogenous IGF-1 are shown in Table 3-1. At day 0, experimental plasma IGF-1 levels were not different from vehicle control plasma IGF-1 levels. Animals infused with vehicle showed no change in plasma IGF-1 levels over 7 days. Experimental animals showed a dramatic decrease in endogenous IGF-1 levels in response to LR3 IGF-1 compared to vehicle at both days 4 and 7 (Table 3-1). LR3 IGF-1 plasma levels were increased on Day 7 in experimental animals over vehicle infused controls (Table 3-1).

Whole animal experiments were performed on 5 animals infused with LR3 IGF-1 (experimental group), 5 un-instrumented twins of the experimental group (twin control group), and 5 vehicle infused animals (vehicle control group) of which 3 were twins and 2 were singletons. A seven-day infusion of LR3 IGF-1 did not alter body weight of experimental animals (4.69 ± 0.44 kg, mean ± s.e.) compared to vehicle infused controls (4.61 ± 0.24 kg) or twin controls (4.28 ± 0.28 kg, Fig 3-1A). Heart weight increased by 35% in experimental animals (41.4 ± 2.6g) compared to vehicle control animals (30.6 ±

2.3g). Previous work showed that IGF-1 infusion increased kidney and liver weight as well as heart weight (Lok *et al.*, 1996). We measured these organ weights to assess the effectiveness of our dosing protocol. Combined kidney weight appeared to increase in experimental animals (31.0 ± 1.4g) compared to vehicle control animals (25.1 ± 2.3g), but was not quite significant until normalized to body weight (Fig 3-1B). Liver weight was not different in experimental animals (83.7 ± 11.3g) compared to vehicle control animals (88.4 ± 7.6g). Vehicle control animals were not statistically different from twin control animals in any measurement (heart weight = 26.6 ± 2.3g, kidney weight = 22.2 ± 2.1g, liver weight = 78.9 ± 13.3g)

Morphometric measurement of dissociated cells is summarized in Table 3-2. LR3 IGF-1 infusion did not change cell sizes. Experimental animals did, however, show a significant decrease in percent of cells that were binucleated, or alternately stated, an increase in percent of cells that were mononucleated.

We surmised that the increase in percent of mononucleated cells resulting from LR3 IGF-1 infusion *in vivo* indicated a proliferation of mitotically competent mononucleated cells. Therefore, we used an *in vitro* cell culture system of ovine cardiomyocytes to measure BrdU uptake as an assay of proliferation. In culture, cardiomyocytes in serum free conditions have a low rate of BrdU uptake over 48 hours (1.11 ± 0.23%, n=9) that is increased in serum enriched media conditions (8.26 ± 2.32%, n=9, $p<0.05$). Figure 3-2 is an image of BrdU labeling (green) in a cardiomyocyte labeled for myosin (red). Two doses of LR3 IGF-1 were used in serum free conditions and BrdU uptake measured. 0.1μg/mL LR3 IGF-1 increased BrdU uptake (3.59 ± 0.50%, n=9, $p<0.05$) that was further increased by 1μg/mL LR3 IGF-1 (5.54 ± 0.77%,

n=8, $p<0.05$, Fig 3-3). The BrdU uptake, stimulated by LR3 IGF-1, was dependent on both ERK and PI3K signaling cascades as demonstrated by the inhibition of BrdU uptake in the presence of either UO126 or LY294002 (Fig 3-4). Neither of the inhibitors alone nor in combination with high dose LR3 IGF-1 stimulated BrdU uptake above the serum free levels ($p>0.05$, ANOVA).

Though LR3 IGF-1 had not increased ovine cardiac mycoyte cell size *in vivo*, IGF-1 has been shown to increase rat cardiomyocyte size in culture (Ito *et al.*, 1993). We have previously shown that PE causes an increase in ventricular binucleated cell size while not changing cell size in mononucleated cells (Chapter 4, (Sundgren *et al.*, 2003). In cardiomyocytes from either ventricle, there was no change in mononucleated cardiomyocyte cell sizes with any treatment (Serum free LV = 908.51 ± 64.02$\mu m^2$, RV = 1056.90 ± 76.06$\mu m^2$, n=7, Fig 3-5). In left ventricle cardiomyocytes, LR3 IGF-1 did not stimulate an increase in cell size (1612.04 ± 125.21$\mu m^2$) compared to cells grown in serum free conditions (1599.10 ± 96.48$\mu m^2$) while PE did increase cell size (2125.03 ± 124.36$\mu m^2$, $p<0.05$, Fig 3-5A). In right ventricle cardiomyocytes, known to be larger than LV cardiomyocytes (Smolich *et al.*, 1989), the same pattern was true; LR3 IGF-1 did not cause hypertrophy (2162.05 ± 138.08$\mu m^2$) compared to serum free conditions (2001.86 ± 186.10$\mu m^2$), but PE did increase cell size (2618.73 ± 124.90$\mu m^2$, $p<0.05$, Fig 3-5B).

We have previously shown that PE induces cell enlargement in an ERK dependent manner (Chapter 4;Sundgren *et al.*, 2003). We tested the hypothesis that ERK signaling in the absence of PI3K signaling from IGF1R activation could induce hypertrophy. However, LR3 IGF-1 given in combination with LY294002 did not

increase binucleated cell size over baseline serum free conditions in LV or RV cells (LV = 1562.32 ± 368.12μm$^2$, n=3, RV = 2120.06 ± 288.95μm$^2$, n=4, Fig 3-6).

BrdU uptake assays in culture show that both ERK and PI3K signaling are required for LR3 IGF-1 to increase proliferation. To rule out a non-specific effect of an inhibitor or cross talk between cascades as the reason for the dual signaling necessity, we measured phospho-ERK and phospho-AKT levels in response to LR3 IGF-1 stimulation. A representative blot of LR3 IGF-1 stimulation of phospho ERK is shown in Fig 3-7A. Differences across treatment were highly significant ($F_{2,33}$ = 164.13; $p$<0.0001). Addition of UO126 produced uniform decreases in median fold changes compared to LR3 IGF-1 stimulation at all time points (p<0.005) and addition of LY294002 produced a significant increase in baseline p-ERK (p<0.05, Fig 3-7B). Changes were also observed across the four time points ($F_{3,33}$ = 5.21; p=0.005). LR3 IGF-1 alone significantly increased stimulation over time relative to baseline (Fig 3-7B). A representative blot of LR3 IGF-1 stimulation of phospho-AKT is shown in Fig 3-8A. Median fold changes are significantly affected by treatment ($F_{2,33}$ = 30.86; p<0.0001) but not by time ($F_{3,33}$ = 1.33; $p$ = 0.2802, Fig 3-8B). Addition of UO126 resulted in median fold changes that were not significantly different from LR3 IGF-1 alone (p≥0.174 for all time points).

**Discussion:**

We have described experiments designed to elucidate the effects of IGF1R on fetal sheep heart growth and to determine the importance of ERK and PI3K signaling cascades as mediators of those effects. We hypothesized that IGF1R stimulation would simulate the changes seen in a systolic pressure load by increasing proliferation, differentiation, and cell size (Barbera *et al.*, 2000).

Previous work established a dose of IGF-1 that increased fetal sheep heart weight by 26% and heart to body weight by approximately 15% (Lok *et al.*, 1996). This dose was sufficient to increase kidney and liver weight while not increasing overall body weight (Lok *et al.*, 1996). We here report a dose of LR3 IGF-1 that similarly affected heart weight, increasing heart weight by 35% and heart weight to body weight by 37% (Fig 3-1). We also saw an increase in combined kidney weight, but did not see any effect on liver weight (Fig 3-1). Whereas infusion of IGF-1 increases plasma levels of IGF-1 (Marsh *et al.*, 2001a;Lok *et al.*, 1996) our infusion of LR3 IGF-1 decreased endogenous levels of IGF-1 while increasing levels of LR3 IGF-1 (Table 3-1) suggesting that the administration of LR3 IGF-1 stimulated a negative feedback to reduce endogenous IGF-1 levels. The proportion of the total IGF-1 in the unbound form was not determined. However, we measured LR3 IGF-1 plasma levels at the conclusion of the infusion period to show that they were within the expected physiological range.

While we hypothesized that IGF1R stimulation would increase the percentage of myocytes that were binucleated, we found the opposite - a decrease in the percentage of cardiomyocytes that were binucleated (Table 3-2). The increase in heart weight, but not cell size, suggests an increase in the population of myocytes. Consistent with that

interpretation, LR3 IGF-1 induced a 5-fold increase in BrdU uptake in normal fetal cardiomyocytes *in vitro* (Fig 3-3). This increase in proliferation was dependent on both ERK and PI3K signaling (Fig 3-4). While several IGF-1 effects require both signaling cascades (Mehrhof *et al.*, 2001;Hong *et al.*, 2001;Kurihara *et al.*, 2000), others do not (Coolican *et al.*, 1997;Liu *et al.*, 2001b;Lavandero *et al.*, 1998;Pene *et al.*, 2002). In our studies, the cardiomyocyte proliferation response was dependent on both ERK and PI3K.

Cross talk between these two cascades has been described previously at the level of AKT (a downstream target of PI3K) and Raf-1 (downstream target of Ras and upstream of ERK) (Moelling *et al.*, 2002;Zimmermann & Moelling, 1999). AKT can directly phosphorylate Raf-1 and decrease its activity in skeletal myotubes, but not skeletal myoblasts (Zimmermann & Moelling, 1999;Rommel *et al.*, 1999). In our studies, LR3 IGF-1 increased ERK phoshorylation over 20 minutes of stimulation (Fig 3-7B). In the presence of PI3K inhibition by LY294002, baseline ERK phosphorylation is increased (0 minutes, Fig 3-7B) suggesting that in fetal sheep cardiomyocytes *in vitro* PI3K has a tonic inhibition of ERK activity perhaps by AKT phosphorylation of Raf-1. However, if proliferation were solely dependent on ERK, then we would predict that PI3K inhibition would increase ERK activity and therefore proliferation. Instead, PI3K inhibition abolished the increase in BrdU uptake stimulated by LR3 IGF-1 (Fig 3-4). It is also reported that ERK activation can inhibit PI3K activation (Yu *et al.*, 2002). Blockade of ERK signaling by UO126 in fetal sheep cardiomyocytes may change LR3 IGF-1 induced AKT phosphorylation causing an increase in AKT stimulation at 5 minutes though this was not quite significant ($p=0.067$, Fig 3-8B). Here again, if proliferation depended only on PI3K, then ERK inhibition would be expected to increase BrdU uptake

by increasing PI3K activity. However, as with PI3K inhibition, ERK inhibition abolished an increase in BrdU uptake (Fig 3-4). Therefore, it appears that the increased BrdU uptake from LR3 IGF-1 stimulation represented the synergistic activity of ERK and PI3K signaling.

Dissociated mononucleate and binucleate cells from LR3 IGF-1 treated fetal sheep were no larger than control cells (Table 3-2). This is consistent with the absence of cardiomyocyte enlargement in transgenic mice over-expressing IGF-1 (Reiss *et al.*, 1996). In contrast, others have seen hypertrophy of rat cardiomyocytes in culture in response to IGF-1 (Ito *et al.*, 1993). LR3 IGF-1 stimulation did not increase the size of fetal sheep cardiomyocytes in culture, while PE stimulation did cause hypertrophy (Fig 3-5). PE induced hypertrophy is dependent on ERK signaling (Sundgren *et al.*, 2003;Ueyama *et al.*, 2000;Yue *et al.*, 2000). Other work has proposed that IGF-1 induced hypertrophy was dependent on ERK but not on PI3K signaling (Lavandero *et al.*, 1998). We hypothesized that PI3K signaling in combination with ERK signaling would lead to hyperplasia and blocked hypertrophy, while ERK signaling alone would cause hypertrophy. We found, however, that IGF1R stimulation in the presence of PI3K blockade did not increase cell size either (Fig 3-6).

It is often assumed that neonatal rat cardiomyocytes are similar to late fetal cardiomyocytes of more mature species such as the sheep. However, neonatal rat cardiomyocytes lose there ability to increase BrdU uptake after just 48 hours in culture (Sadoshima *et al.*, 1997) while we have shown that fetal sheep cardiomyocytes in culture retain their ability to increase BrdU uptake after a total of 96 hours in culture (Fig 3-3).

Therefore IGF-1 may only be able to induce hypertrophy of cardiomyocytes after some further maturation event that occurs later in gestation or after birth in the sheep.

Recent work has shown that PI3K and its downstream target AKT may be critical components of the regulation of heart size (Shioi *et al.*, 2000;Shioi *et al.*, 2002;Condorelli *et al.*, 2002;Crackower *et al.*, 2002). Transgenic mice expressing a constitutively active PI3Kα construct have an increased heart size due to an increase in myocyte size and mice expressing a dominant negative PI3Kα construct have decreased heart size due to decreases in myocyte size (Shioi *et al.*, 2000). The same results are seen when constitutively active AKT and dominant negative AKT are expressed (Shioi *et al.*, 2002;Condorelli *et al.*, 2002). It is important to note that in these transgenic mice the transgene is on an α myosin heavy chain promoter that induces expression in mice 7 days or more after birth which is past the time of mice cardiomyocyte multinucleation (Reiss *et al.*, 1996;Shioi *et al.*, 2000). What role PI3K may have on regulation of fetal heart size when the cardiomyocytes are still able to divide at a high rate cannot be deduced from these experiments. Curiously, transgenic mice expressing IGF-1 with the same α myosin heavy chain promoter showed no increase in cell size while total heart size was increased (Reiss *et al.*, 1996). These transgenic mice showed very low, but increased levels of BrdU uptake in culture and it was concluded that the increase in heart weight was due to an increase in the total number of myocytes (Reiss *et al.*, 1996). While LR3 IGF-1 does stimulate PI3K in fetal sheep cardiomyocytes (Fig 3-8), the stimulation is not sufficient to cause hypertrophy (Fig 3-5). Our *in vitro* experiments suggest that IGF1R stimulation during fetal life leads to an increase in heart weight due to hyperplasia, not hypertrophy. Contrary to our hypothesis, IGF1R stimulation does not lead to an increase in maturation

as measured by the percentage of binucleate cardiomyocytes. It follows that those born with higher IGF-1 levels may have more heart myocytes than those born with lower IGF-1 levels. It is not known when or how the optimal number of cardiomyocytes is set. However, if that number is set during the antenatal period, regardless of the fact that new cardiomyocytes can be generated during adult life (Nadal-Ginard *et al.*, 2003), the number of cells at birth may be important for life. One consequence of growth restriction during fetal life may, therefore, be a reduction in the total number of cardiomyocytes at birth with associated risks for disease in adult life.

**Table 3-1: Hemodynamic measures, arterial blood gases, hematocrit, pH, and IGF-1 plasma levels.**

| | Vehicle control | | | Experimental | | |
|---|---|---|---|---|---|---|
| | Day 0 | Day 4 | Day 7 | Day 0 | Day 4 | Day 7 |
| MAP, mmHg | 41.4±2.2 | 45.6±7.0 | 45.6±5.9 | 46.6±1.7 | 49.0±2.6‡ | 40.4±4.8 |
| Systolic BP mmHg | 53.4±5.6 | 56.0±6.3 | 56.2±7.7 | 57.4±4.7 | 61.7±7.4 | 51.0±6.4 |
| Diastolic BP mmHg | 32.0±3.5 | 36.0±4.4 | 35.2±8.8 | 39.0±2.5 | 40.0±1.6‡ | 33.4±5.9 |
| HR, beats/min | 154±6 | 156±9 | 147±10 | 166±15 | 161±14 | 176±16† |
| $Pa_{O2}$, mmHg | 20.2±1.1 | 19.8±1.1 | 21.4±0.9 | 20.0±1.9 | 17.4±1.8 | 15.6±2.7*,† |
| Hematocrit, % | 32.4±2.7 | 34.0±1.0 | 33.2±5.9 | 31.2±5.6 | 29.2±7.2 | 28.8±7.6 |
| $O_2$ Content, mL/dL | 7.2±0.6 | 7.2±0.9 | 8.4±1.0 | 7.0±1.3 | 5.5±1.8 | 4.0±1.5*,† |
| $Pa_{CO2}$, mmHg | 47.1±3.6 | 46.9±3.6 | 47.0±2.3 | 48.2±4.1 | 50.2±4.4 | 51.3±3.8 |
| pH | 7.35±0.04 | 7.34±0.02 | 7.34±0.03 | 7.35±0.02 | 7.34±0.02 | 7.33±0.01 |
| IGF-1, ng/mL | 58.1±22.0 | 51.7±19.9 | 58.4±22.3‡ | 41.6±11.2 | 11.9±5.6† | 12.3±4.8† |
| LR3 IGF-1, ng/mL | | | 0.4±0.1‡ | | | 2.3±1.6† |

Values are means ± SD. MAP, mean arterial pressure; BP, blood pressure; HR, heart rate. *$p<0.05$ compared to Day 0; † $p<0.05$ compared to same day control value, n=5 for all values except ‡ n=4.

**Table 3-2: *In vivo* Myocyte Size Data**

| | Vehicle Control (n=5) | Experimental (n=5) | *p* Value |
|---|---|---|---|
| Binucleation, % | | | |
| Left Ventricle | 61.3 ± 8.1 | 45.8 ± 5.3 | <0.05 |
| Right Ventricle | 61.9 ± 8.7 | 50.5 ± 4.9 | <0.05 |
| Binucleated Length, μm | | | |
| Left Ventricle | 87.3 ± 4.3 | 90.5 ± 5.0 | NS |
| Right Ventricle | 95.0 ± 7.4 | 98.9 ± 7.9 | NS |
| Binucleated Width, μm | | | |
| Left Ventricle | 13.7 ± 1.1 | 15.1 ± 0.6 | NS |
| Right Ventricle | 16.5 ± 1.7 | 17.3 ± 1.9 | NS |
| Mononucleated Length, μm | | | |
| Left Ventricle | 66.8 ± 3.2 | 70.4 ± 3.4 | NS |
| Right Ventricle | 73.3 ± 4.7 | 78.0 ± 5.5 | NS |
| Mononucleated Width, μm | | | |
| Left Ventricle | 11.7 ± 1.0 | 12.6 ± 0.5 | NS |
| Right Ventricle | 13.3 ± 1.3 | 14.0 ± 1.6 | NS |

Values are means ± SD. NS, not significant

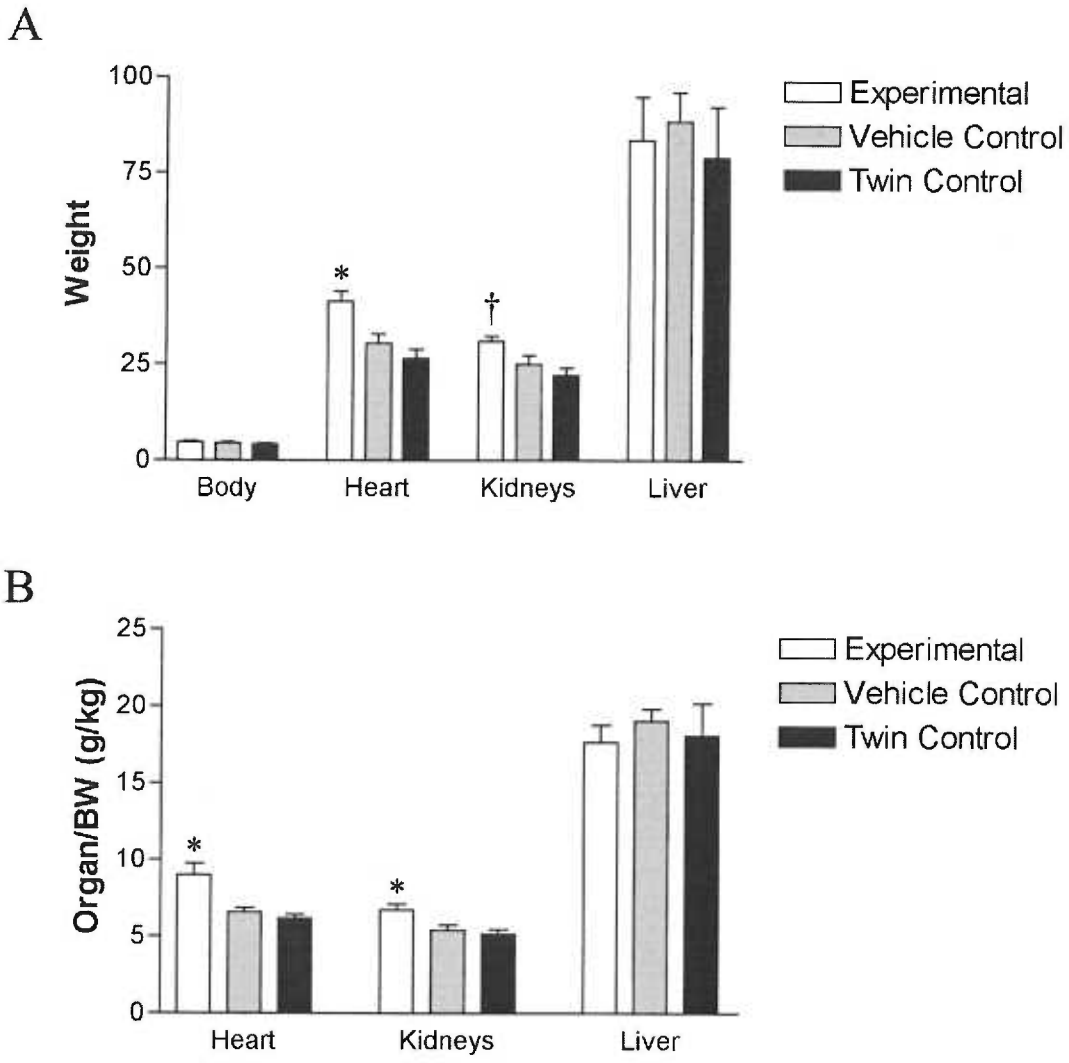


**Figure 3-1: Organ weights in response to LR3 IGF-1**

A) Graph of body weights (kg) and organ weights (g) for experimental animals compared to two sets of control animals and B) organ weights normalized to body weight (mean±s.e.). *$p<0.05$ compared to vehicle and twin controls, † $p<0.05$ compared to twin control, n=5, ANOVA.

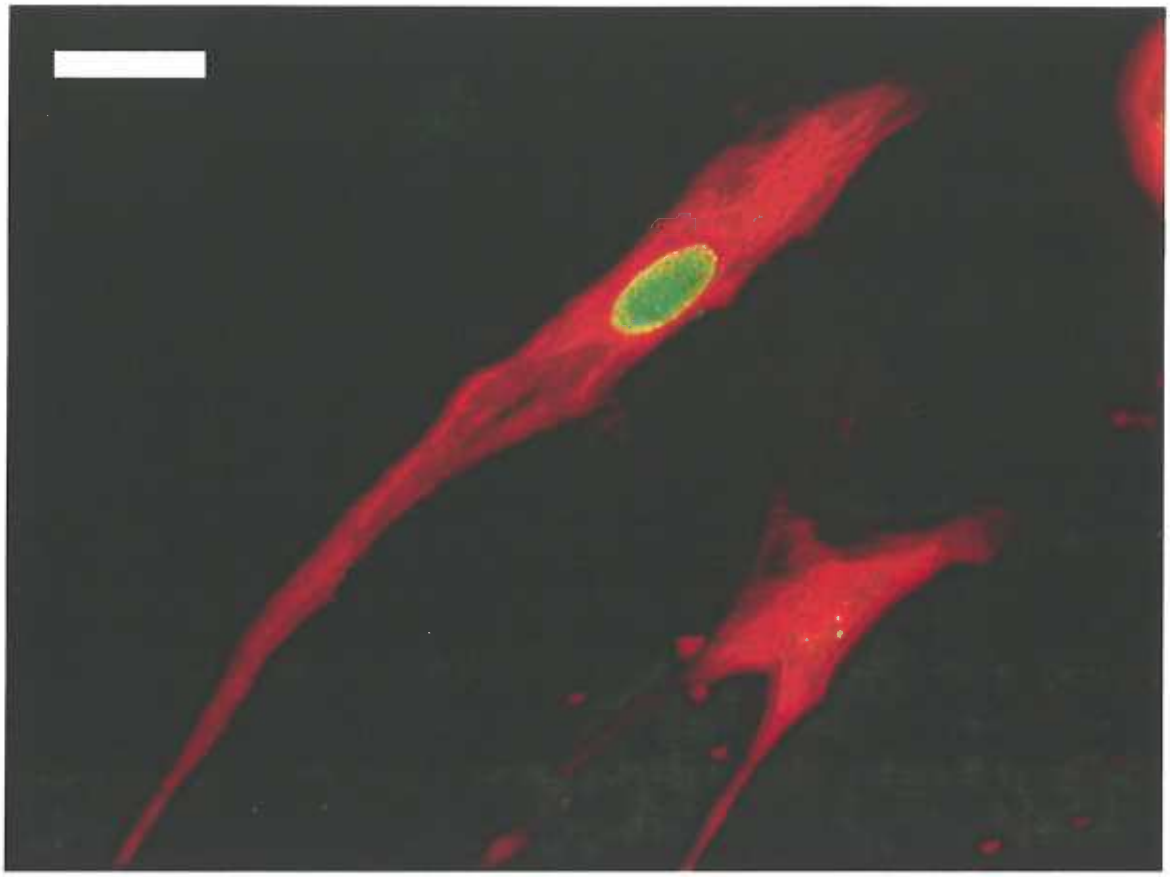

**Figure 3-2: Cardiomyocyte BrdU uptake**

An image of cardiomyocytes after stimulation with 0.1μg/mL LR3 IGF-1 shows the presence of BrdU uptake. One myocyte (myosin = red) has incorporated BrdU (green) into the nucleus. Bar = 20μm.

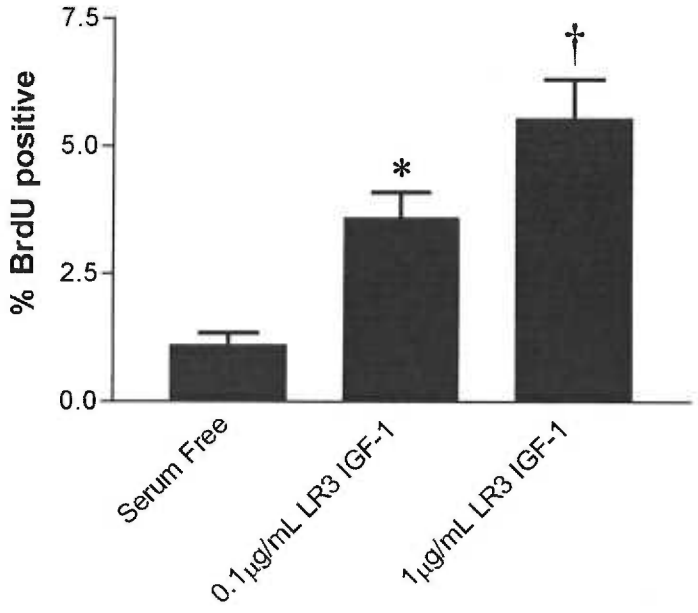


**Figure 3-3: BrdU uptake *in vitro***

BrdU uptake is increased after 48 hours in response to two different doses of LR3 IGF-1 (mean±s.e.). *$p<0.05$ compared to serum free, † $p<0.05$ compared to serum free and 0.1µg/mL LR3 IGF-1, ANOVA

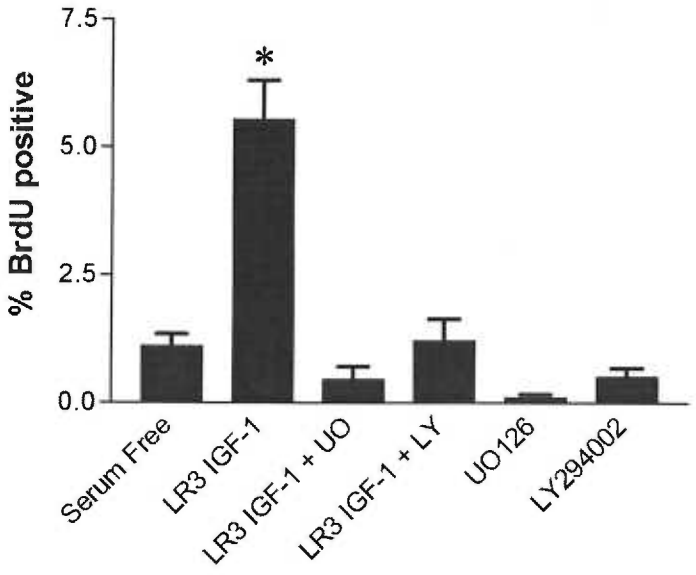


**Figure 3-4: LR3 IGF-1 induced BrdU uptake is ERK and PI3K dependent**

10μM UO126 and 10μM LY294002 abolish the increase in BrdU uptake induced by 1μg/mL LR3 IGF-1 (mean±s.e.). *$p<0.05$ compared to serum free, ANOVA

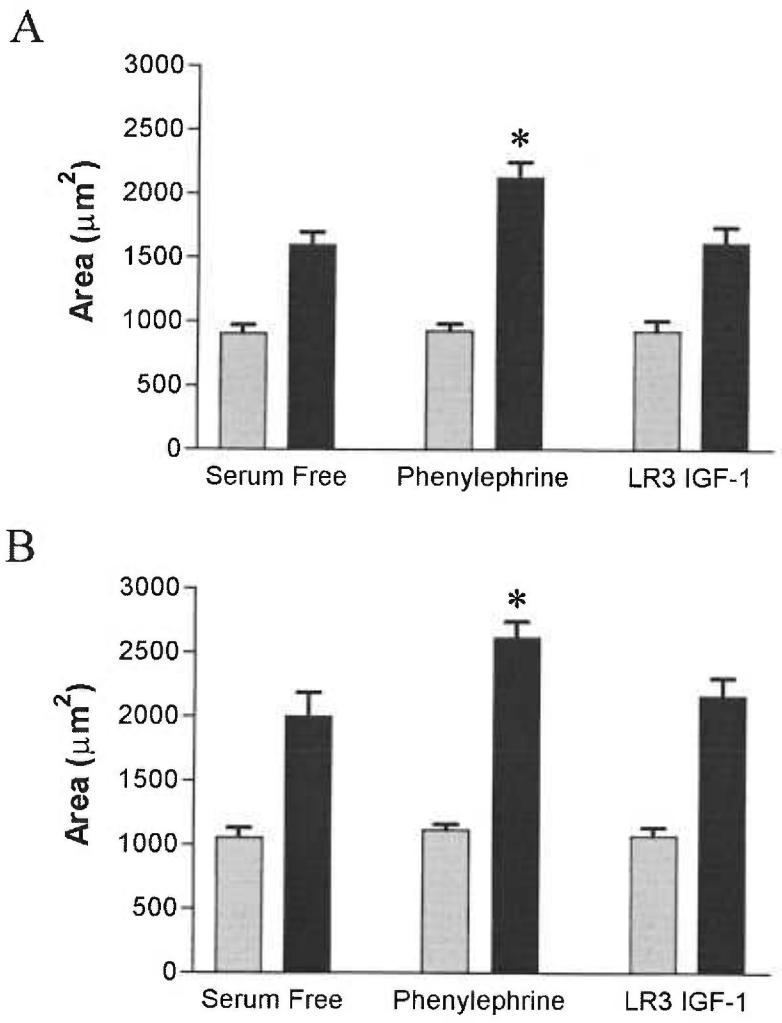


**Figure 3-5: Hypertrophy of cardiomyocytes in culture**

Area of LV (A) and RV (B) mononucleated (gray bars) and binucleated (black bars) cardiomyocytes is plotted (mean±s.e.). Doses were 10μg/mL PE and 1μg/mL LR3 IGF-1. *$p<0.05$ compared to serum free binucleated, n=7, paired ANOVA

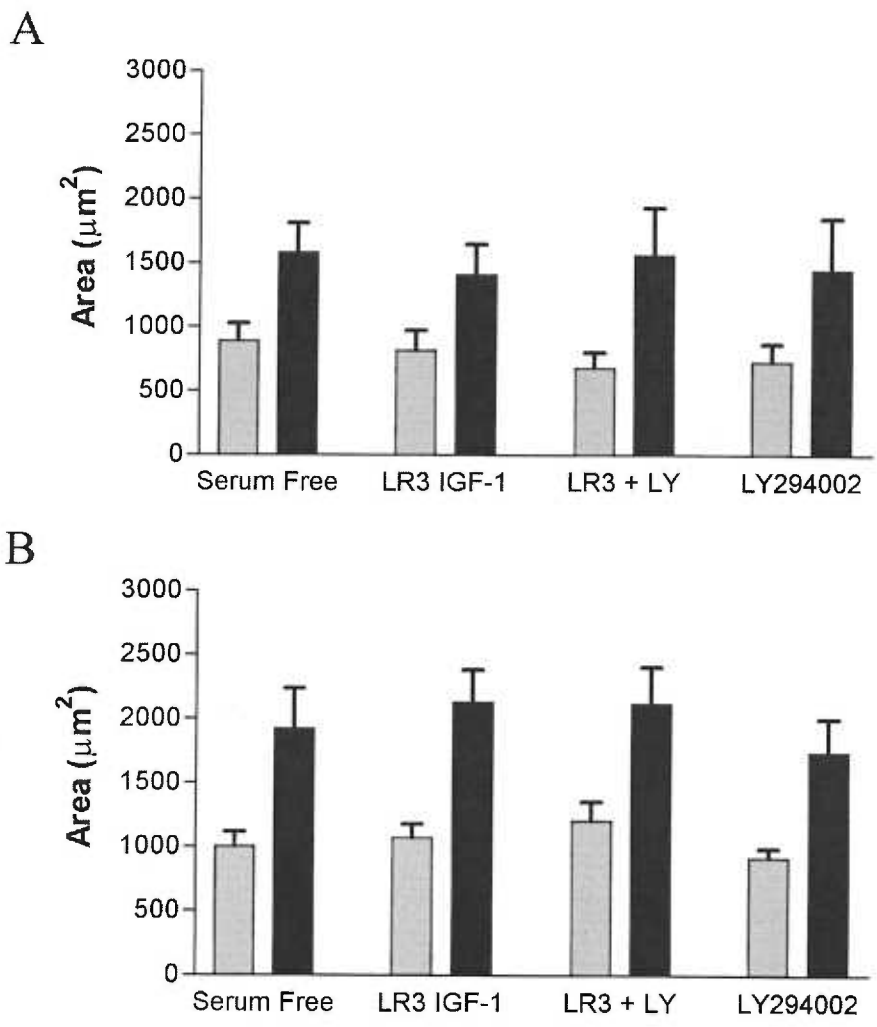


**Figure 3-6: ERK-forced stimulation by LR3 IGF-1 does not cause hypertrophy**

Area of LV (A) and RV (B) mononucleated (gray bars) and binucleated (black bars) cardiomyocytes is plotted (mean±s.e.). Doses were 1μg/mL LR3 IGF-1 and 10μM LY294002. LV n=3, RV n=4, $p>0.05$, paired ANOVA

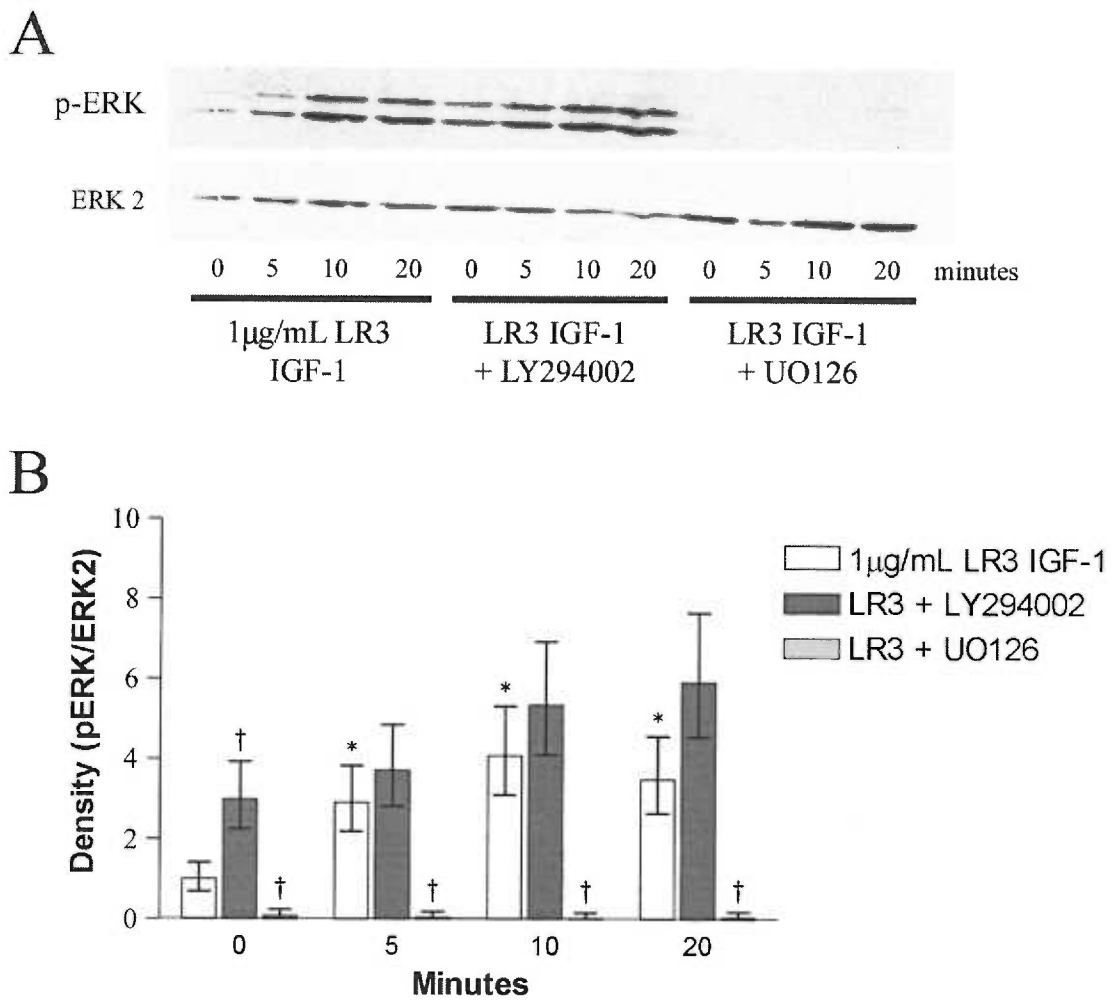


**Figure 3-7: IGF1R ERK signaling in cardiomyocytes**

A) A typical western blot of ERK stimulation is shown. B) Values are plotted as median fold change ± s.e. (n=4) of blot density normalized to baseline LR3 IGF-1 stimulation (0 min). Density is plotted in normalized units of p-ERK/ERK2. *$p<0.05$ compared to within treatment baseline, †$p<0.05$ compared to LR3 IGF-1 treatment at same timepoint.

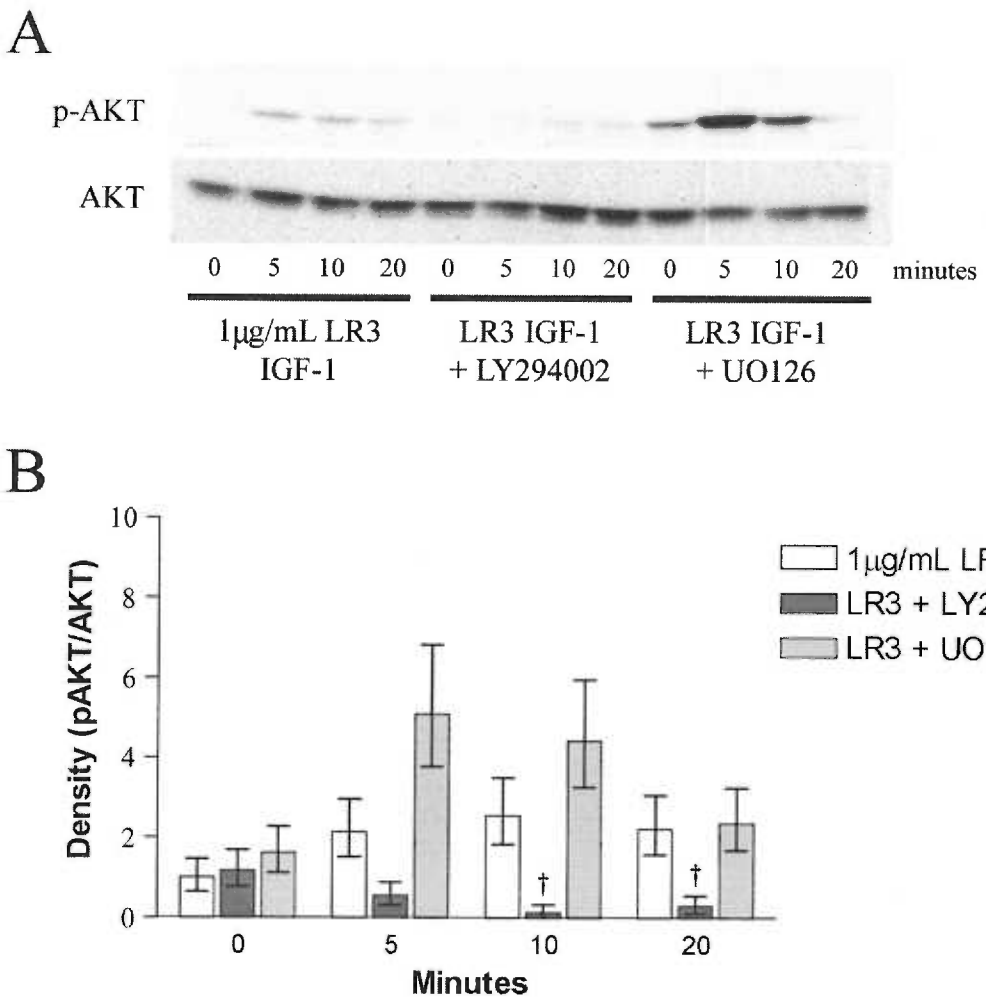


**Figure 3-8: IGF1R PI3K & AKT signaling in cardiomyocytes**

A) A typical western blot of AKT stimulation is shown. B) Values are plotted as median fold change ± s.e. (n=4) of blot density normalized to baseline LR3 IGF-1 stimulation (0 min). Density is plotted in normalized units of p-AKT/AKT. †$p<0.05$ compared to LR3 IGF-1 treatment at same timepoint.

# Chapter 4:

# Angiotensin II stimulates hyperplasia but not hypertrophy in immature ovine cardiomyocytes

**Nathan C. Sundgren, George D. Giraud, Philip J.S. Stork, James G. Maylie, Kent L. Thornburg**

**Journal of Physiology (548) p881-891, 2003**

**Abstract:**

Rat and sheep cardiac myocytes become binucleate as they complete the "terminal differentiation" process soon after birth and are not able to divide thereafter. Angiotensin II (Ang II) is known to stimulate hypertrophic changes in rodent cardiomyocytes under both *in vivo* and *in vitro* conditions via the $AT_1$ receptor and extracellular signal-regulated kinase (ERK) signaling cascade. We sought to develop culture methods for immature sheep cardiomyocytes in order to test the hypothesis that Ang II is a hypertrophic agent in the immature myocardium of the sheep. We isolated fetal sheep cardiomyocytes and cultured them for 96 hrs, added Ang II and phenylephrine (PE) for 48 hrs, and measured footprint area and proliferation (5-bromo-2-deoxyuridine (BrdU) uptake) separately in mono- vs. bi- nucleate myocytes. We found that neither Ang II nor PE changed footprint area of mononucleated cells. PE stimulated an increase in footprint area of binucleate cells but Ang II did not. Ang II increased myocyte BrdU uptake compared to serum free conditions, but PE did not affect BrdU uptake. The MEK inhibitor UO126 prevented BrdU uptake in Ang II stimulated cells and prevented cell hypertrophy in PE stimulated cells. This paper establishes culture methods for immature sheep cardiomyocytes and reports that: 1) Ang II is not a hypertrophic agent; 2) Ang II stimulates hyperplastic growth among mononucleate myocytes; 3) PE is a hypertrophic agent in binucleate myocytes; 4) the ERK cascade is required for the proliferation effect of Ang II and the hypertrophic effect of PE.

**Introduction:**

Fetal cardiomyocytes are able to use both hyperplastic (cell division) and hypertrophic (cell enlargement) mechanisms to affect growth and maturation of the myocardium (Clubb & Bishop, 1984;Oparil *et al.*, 1984). During early fetal life, growth is primarily hyperplastic but as myocytes mature during late prenatal or early postnatal life, they switch from a proliferative mode to the hypertrophic mode of growth. As working myocytes become mature and "terminally differentiated" they appear to be unable to divide (Clubb & Bishop, 1984). Under these conditions, the working myocardium could increase its mass only by increasing myocyte size for the remaining life of the individual. However, the terminal differentiation concept may be oversimplified. Recent evidence from Anversa and co-workers challenges the assumption that cardiomyocytes escape the cell cycle irreversibly for life (Anversa & Kajstura, 1998;Kajstura *et al.*, 1998;Beltrami *et al.*, 2001;Quaini *et al.*, 2002).

In rodents, the maturation process is marked by binucleation of cardiomyocytes over postnatal days 4 to 12 (Li *et al.*, 1996;Kellerman *et al.*, 1992;Soonpaa *et al.*, 1996). Both fetal and early neonatal rats exposed to hypertension through carbon monoxide exposure clearly demonstrate cardiomyocyte hyperplasia before terminal differentiation (Clubb *et al.*, 1986). Sheep undergo a similar process whereby the cardiac myocyte population goes from being 100% mononucleated at around day 75 of gestation (term=145d gestation) to 100% binucleated soon after birth (Thornburg KL, Barbera A, Giraud GD, Maylie JG, unpublished). The ratio of binucleated to mononucleated cells can be altered by environmental factors during fetal life. For example, an experimental systolic pressure load applied to the right ventricle in fetal animals stimulates

cardiomyocyte hypertrophy, hyperplasia and binucleation simultaneously, increasing the percentage of myocytes with two nuclei and the total number of myocytes (Barbera *et al.*, 2000). However, it is unclear what mechanical and chemical mechanisms regulate the maturation of myocytes *in vivo*.

Angiotensin II (Ang II) has been established by myriad investigators to stimulate hypertrophic changes in rodent cardiac myocytes both *in vivo* and *in vitro*. When exogenous Ang II is administered to rats *in vivo*, heart weight increases independently of increased afterload (Dostal & Baker, 1992;Geenen *et al.*, 1993;Kim *et al.*, 1995;Susic *et al.*, 1996). The isolated whole heart also shows an increase in protein synthesis in response to Ang II (Schunkert *et al.*, 1995). Ang II is well known as a stimulant of protein synthesis and expression of the early and late genes in association with a hypertrophic response in cardiomyocytes *in vitro* (Booz & Baker, 1996;Gray *et al.*, 1998;Miyata & Haneda, 1994;Sadoshima & Izumo, 1993b;Takahashi *et al.*, 1997;Liu *et al.*, 1998). Studies have established that the AT1 receptor and not the AT2 receptor are responsible for these actions (Booz & Baker, 1996;Dostal & Baker, 1992;Gray *et al.*, 1998;Miyata & Haneda, 1994;Sadoshima & Izumo, 1993b;Liu *et al.*, 1998). By contrast, most investigators have shown little increase in proliferation of cardiomyocytes in response to Ang II (Sadoshima & Izumo, 1993b;Miyata & Haneda, 1994). However, Ang II has been shown to increase proliferation of mitotically capable cardiomyocytes overexpressing the AT1 receptor (Fukuda & Izumo, 1998), and Ang II does have mitotic actions on non-myocytes (Sadoshima & Izumo, 1993b;Schorb *et al.*, 1993). It, therefore, remains unclear to what degree Ang II stimulates mitotically capable cardiomyocytes to proliferate in species other than rodents.

There is an extensive body of data showing that the local renin angiotensin system (RAS) is important in regulating hypertrophic growth in the mammalian myocardium (Barlucchi *et al.*, 2001;Wollert & Drexler, 1999). Periodic stretch up-regulates the RAS in cardiac cells (Malhotra *et al.*, 1999) and induces Ang II production (Sadoshima *et al.*, 1992;Sadoshima & Izumo, 1993b). Blockade of Angiotensin Converting Enzyme (ACE) and/or AT1 receptor block can attenuate pressure load hypertrophy (Baker *et al.*, 1990;Dussaillant *et al.*, 1996;Grimm *et al.*, 1998;Jalil *et al.*, 1991;Linz *et al.*, 1989;Ogawa *et al.*, 1996;Scholkens *et al.*, 1991). Volume overload induced hypertrophy can also be attenuated by Ang II blockade (Ruzicka *et al.*, 1994;Brodsky *et al.*, 1998;Everett *et al.*, 1994). Thus, inhibition of the RAS has become an important clinical tool in amelioration of hypertension and the concomitant hypertrophic pathological alterations in the myocardium (Sleight, 2000;Thurmann *et al.*, 1998).

While the case for Ang II induced hypertrophy is well established in the rodent, it has not been shown conclusively to mediate hypertrophy in large adult mammals. In adult pigs, the angiotensinogen gene is up-regulated in the setting of pressure or volume overload hypertrophy, but the up-regulation is early and goes away after 24 hours even while the pressure or volume overload stimulus remains (Modesti *et al.*, 2000). In the adult dog, mitral regurgitation (volume overload) induces ACE and chymase-like activity (Dell'Italia *et al.*, 1995), but ACE inhibitor therapy and AT1 blockade are unable to attenuate ventricular remodeling and hypertrophy (Dell'Italia *et al.*, 1997;Perry *et al.*, 2002). In the sheep fetus, Segar et al. has shown that development of right hypertrophy by pressure overload was not attenuated by blockade of the AT1 or AT2 receptor (Segar *et al.*, 1997;Segar *et al.*, 2001). This is consistent with the report showing that pressure

load induced hypertrophy in an isolated adult rat heart was not attenuated by AT1 blockade (Thienelt *et al.*, 1997). When pressure overload was produced by Ang II infusion into fetal sheep, the left but not right ventricular mass increased (Segar *et al.*, 2001). However, it is not known whether Ang II directly stimulates hypertrophy at the cellular level in the large mammal.

Ang II stimulates the three primary mitogen activated protein (MAP) kinase cascade pathways: p38 MAPK, JNK, and p42/44 MAPK/ ERKs (Izumi *et al.*, 2000;Wei *et al.*, 2000). Of these, the intracellular ERK cascade is thought to be a key stimulant of hypertrophy in cardiomyocytes (Aoki *et al.*, 2000;Bueno *et al.*, 2001;Bueno *et al.*, 2000;Fischer *et al.*, 1998;Sadoshima & Izumo, 1993a). Indeed stimulation of the ERK signaling cascade may be necessary to affect cell hypertrophy with exposure to phenylephrine, endothelin-1, leukemia inhibitory factor, isoproterenol, sphingosylphosphorylcholine, and stretch (Clerk *et al.*, 2001;Wang & Proud, 2002;Lazou *et al.*, 1998;Sekiguchi *et al.*, 1999;Ueyama *et al.*, 2000;Yue *et al.*, 2000). Stretch up-regulates the renin-angiotensin system in cardiac cells (Malhotra *et al.*, 1999) and also activates p21 Ras, which is upstream of ERK (Sadoshima & Izumo, 1993a). Ras activates Raf, which in turn activates MEK. Overexpression of MEK or expression of a constitutively active MEK induces hypertrophy *in vivo* or *in vitro* (Bueno *et al.*, 2000;Ueyama *et al.*, 2000). Expression of a dominant negative MEK blocks hypertrophy normally induced by phenylephrine, endothelin-1, leukemia inhibitory factor, isoproterenol, and stretch (Ueyama *et al.*, 2000). MEK phosphorylates the ERKs that affect the signaling program.

The goals of this present study were: 1) develop a primary cell culture method using cardiomyocytes from fetal sheep; 2) determine the response of these cultured cells to the AT1 agonist Ang II; 3) elucidate the role of ERK signaling of Ang II actions in cultured sheep myocytes. We tested the hypothesis that Ang II would behave as a hypertrophic agent and not a hyperplastic agonist as predicted from immature rat myocyte findings. We reasoned that Ang II would induce hypertrophy of the more mature binucleated myocytes and not the immature mononucleated myocytes based on our previous work with load-induced hypertrophy (Barbera *et al.*, 2000). We further hypothesized that the p42/44 MAP kinase cascade would be essential to mediate the actions of Ang II.

**Specific Methods:**

*Cell Size experiments*: A dose response curve for Ang II was generated using concentrations ranging from 10nM-5μM. From these experiments, 100nM Ang II was chosen; this dose corresponds to the optimal dose in the rodent myocyte model (Aoki *et al.*, 2000;Sadoshima *et al.*, 1995). Because PE is believed to cause hypertrophy through the α adrenergic receptor mechanism, cardiomyocytes were exposed to PE as a positive control. A dose response for PE was determined using concentrations of 0.2-20μg/mL PE. A dose of 10μg/mL PE was chosen for its maximal stimulation of cell hypertrophy. Further experiments were performed in the absence of BrdU, using 1000X stocks of agonists to obtain final concentrations of 10μg/mL (49.1μM) PE and 100nM Ang II. Cells were also stimulated with 10μg/mL PE in the presence of 10μM UO126. After 48 hours, cells were fixed for staining and measuring.

*BrdU uptake experiments*: Media were replaced with serum free media containing 10μM BrdU. Experimental solutions were made by addition of 1000X stocks of agonists and inhibitors to final concentrations of 10μg/mL (49.1μM) PE, 10nM and 100nM Ang II, and 10μM UO126. Positive controls consisting of serum medium and 10μM BrdU were also used. All experiments lasted for 48 hours and then cells were fixed for staining.

*ERK Stimulation*: After following our cell culture protocol, fresh serum free medium was added to each well and cells were left alone for 12 hours before the experiment. Half the wells were pre-incubated 20 minutes with 10μM UO126. Then both inhibited and non-inhibited wells were stimulated with 100nM Ang II for 0, 5, 10, or 20 minutes. In separate experiments, cells were stimulated with 10μg/mL PE for 0, 5, 10,

or 20 minutes along with separate wells stimulated with 100nM Ang II for the same times.

*Western Blot Analysis*: 20-40μg of total protein from lysate was run for each time point and experiment. The protein was run on a 12% SDS PAGE gel and then transferred to a PVDF membrane. Film images were scanned into TIF files and analyzed on multianalyst software (BioRad) and normalized to the 0 minute timepoint of Ang II stimulation for each experiment.

*Statistics*: Multiple comparisons were tested using ANOVA with Tukeys post hoc test except for western blot density where non-parametric ANOVA with Dunn's post hoc test was used. Student's t-test was used to compare two groups. Comparisons were considered significant at $p<0.05$.

**Results:**

One of the goals in this study was to develop a culture method for immature cardiomyocytes from sheep. Figure 4-1 shows an example of freshly isolated myocytes stained with anti-myosin antibody conjugated to rhodamine red-X and with Hoechst stain to visualize the nuclei. The cells were predominately cardiomyocytes in good health and able to contract if placed in the appropriate solution. Freshly isolated myocytes were normally ~60% binucleated and ~40% mononucleated cells at age 135d of gestation. These cells were maintained as a primary culture for up to eight weeks.

The effect of PE and Ang II on cardiac size was studied *in vitro* on cardiomyocytes from the LV and RV separately. Not surprisingly binucleated cardiac cells were found to be larger than mononucleated cardiac cells. In cardiomyocytes from either ventricle, there was no change in mononucleated cardiomyocyte cell areas with any treatment (Serum free LV = 919.07 ± 72.28 $\mu m^2$, mean ± s.e., RV = 1069.58 ± 89.59 $\mu m^2$, Fig 4-2). In left ventricle cardiomyocytes, Ang II did not stimulate an increase in cell area (1461.30 ± 147.09 $\mu m^2$) compared to cells grown in serum free conditions (1613.28 ± 69.83 $\mu m^2$, Fig 4-2A). In right ventricle cardiomyocytes, the same pattern was true, Ang II did not cause hypertrophy (2342.06 ± 286.60 $\mu m^2$) compared to serum free conditions (2006.38 ± 172.19 $\mu m^2$, Fig 4-2B). As previously reported, binucleated cardiomyocytes from the RV tend to be larger than binucleated cardiomyocytes from the LV (Barbera *et al.*, 2000;Smolich *et al.*, 1989). In order to determine whether these immature cardiomyocytes were capable of hypertrophy under culture conditions, the cells were exposed to PE as a positive control. PE increased binucleated cardiomyocyte cell

area for both left (2116.46 ± 118.83 $\mu m^2$) and right (2456.99 ± 121.80 $\mu m^2$) ventricles (Fig 4-2).

It has been reported that PE requires ERK stimulation to increase cardiomyocyte size (Ueyama *et al.*, 2000;Wang & Proud, 2002). To determine whether ERK stimulation is required for PE induced hypertrophy in immature sheep cardiomyocytes, we inhibited the ERK pathway using the MEK inhibitor UO126 in the presence of PE stimulation. In left ventricle cardiomyocytes, PE stimulation increased average cell size (1977.89 ± 255.13 $\mu m^2$) over serum free conditions (1457.84 ± 206.20 $\mu m^2$), but PE stimulation in the presence of UO126 had no effect on cell size (1426.22 ± 178.73 $\mu m^2$) and UO126 alone did not affect cell area (1203.59 ± 137.71 $\mu m^2$) compared to serum free conditions (Fig 4-3A). The same was true in right ventricle cardiomyocytes; PE stimulation increased cell size (2666.25 ± 218.38 $\mu m^2$) over serum free conditions (1926.34 ± 310.46 $\mu m^2$), but PE stimulation in the presence of UO126 had no effect on cell size (2051.89 ± 226.88 $\mu m^2$) and UO126 alone did not affect cell area (2001.63 ± 318.47 $\mu m^2$) compared to serum free conditions (Fig 4-3B).

These experiments did not support the hypothesis that Ang II was an important stimulant of hypertrophy in these fetal cardiomyocytes, so we explored the ability of Ang II to stimulate hyperplasia by measuring BrdU incorporation. We first tested the ability of cardiomyocytes to incorporate BrdU in response to serum conditions versus in serum free conditions (Fig 4-4). With regard to BrdU incorporation we saw no differences between LV and RV cells and the data were combined. Forty-eight hours of exposure to serum medium increased BrdU uptake (8.26% ± 2.32, mean ± se, n=9) compared to that in serum free medium (1.11% ± 0.23, n=9, $p<0.05$). These experiments showed that

cardiomyocytes were readily able to proliferate *in vitro*, so we tested the ability of Ang II and PE to increase BrdU uptake. Figure 4-5 is a double immunofluorescence image of cardiomyocytes showing myosin staining red and incorporated BrdU staining green. Only myosin rich cardiomyocytes were counted; contaminating cells such as fibroblasts did not stain for myosin and were thus not included in analyses. Figure 4-6 shows the incorporation of BrdU in response to PE and to two doses of Ang II. Phenylephrine did not stimulate BrdU incorporation (1.75% ± 0.43, n=8) while both doses of Ang II significantly increased BrdU incorporation (10nM = 3.38% ± 0.41, n=8; 100nM = 3.95% ± 0.71, n=7). The BrdU uptake stimulated by Ang II was dependent on the ERK signaling cascade (Fig 4-7) as demonstrated by the inhibition of BrdU uptake in the presence of the MEK inhibitor, UO126. Neither 100nM Ang II plus UO126 nor UO126 alone stimulated BrdU uptake above the serum free levels ($p>0.05$, ANOVA).

To further explore Ang II stimulation of the ERK cascade, we ran immunoblots of stimulated cell lysates with labels for anti-phospho-ERKs and anti-total ERK 2 (Fig 4-8). Phospho-ERK (p-ERK) stimulation with Ang II was significantly increased over baseline levels at 5 and 10 minutes, while PE induced stimulation was significant at 5 minutes (Fig 4-8C). The addition of UO126 abolished the Ang II induced p-ERK stimulation (Fig 4-8A). There was no significant difference between Ang II and PE induced stimulation of ERK ($p>0.05$). It is noteworthy that the basal level of p-ERK was further decreased by the presence of UO126, which is consistent with the non-significant decrease in BrdU incorporation in the presence of UO126 compared to serum free conditions (Figs 4-7 & 4-8). This suggests that basal serum free BrdU incorporation may also be dependent on ERK stimulation.

**Discussion:**

This study yielded four primary findings: First and contrary to our hypothesis, angiotensin II did not behave as a hypertrophic agent for fetal sheep cardiomyocytes in culture. Second, the pro-hypertrophic action of phenylephrine was dependent on the state of cardiomyocyte maturity as indicated by the number of nuclei. Third, Ang II stimulated hyperplastic growth in cardiomyocytes under the similar culture conditions. Fourth, the ERK signaling cascade was necessary for Ang II induced hyperplasia and PE induced hypertrophy, but ERK stimulation does not appear to be sufficient for either hypertrophy or hyperplasia.

A dose of 100nM Ang II has been routinely used by other investigators to induce hypertrophy in neonatal rat cardiomyocytes *in vitro* (Sadoshima & Izumo, 1993b), but the same dose did not stimulate proliferation in those cells (Sadoshima *et al.*, 1997). In fetal sheep cardiomyocytes, Ang II had no effect on the size of cells from either the LV or RV at the stage of maturity that we studied (Fig 4-2). It is possible that with further maturation, Ang II would stimulate binucleate cardiomyocytes to undergo hypertrophy in neonatal or adult sheep. However, our results are consistent with previous findings that Ang II is not important for fetal sheep in generation of cardiomyocyte hypertrophy during fetal life (Segar *et al.*, 1997).

During ovine fetal development, mitotically competent mononucleated cells co-exist with terminally differentiated binucleated cells (Barbera *et al.*, 2000). Our work establishes for the first time a difference in the response of sheep cardiomyocytes to a hypertrophic stimulus that is dependent on the state of myocyte maturation. We report here that binucleated cells increased in size in the presence of PE while mononucleated

cells were unresponsive (Figs 4-2 & 4-3). Clubb *et al.* (1986) were the first to suggest that binucleation was a marker for the transformation from hyperplastic growth to hypertrophic growth. In future studies we plan to determine whether the actions of other hypertrophic agents are limited to increasing the size of binucleated cardiomyocytes.

The fact that Ang II can stimulate hypertrophy is well established in neonatal rat cardiomyocytes and we were surprised to discover in our system that sheep cardiomyocytes are not enlarged under the influence of Ang II. However, the finding that Ang II can induce proliferation is not novel. Angiotensin II has been shown to cause proliferation in cardiac fibroblasts (Sadoshima & Izumo, 1993b;Schorb *et al.*, 1993), vascular smooth muscle cells (Mueller *et al.*, 2002;Watanabe *et al.*, 2001), and other cell types as well (Muscella *et al.*, 2002;Rossi *et al.*, 2002). However, cardiomyocytes have not been shown to proliferate in culture in response to Ang II (Miyata & Haneda, 1994;Sadoshima & Izumo, 1993b) unless transformed (Fukuda & Izumo, 1998). We first tested the ability of fetal sheep cardiomyocytes to proliferate in culture. We stimulated cardiomyocytes with either a serum free or serum-enriched medium for 48 hours in the presence of BrdU (Fig 4-4). We obtained the same positive response for BrdU uptake in sheep cardiomyocytes to that of neonatal rat cardiomyocytes exposed to fetal calf serum for 48 hours in Izumo's laboratory (Sadoshima *et al.*, 1997). However, in their experiments the neonatal rat cells failed to increase BrdU uptake in response to Ang II over serum free levels during the window of time before terminal differentiation in culture (Sadoshima *et al.*, 1997). In our system, a 100nM dose of Ang II increased BrdU uptake by nearly 4 fold above serum free levels and about half as much as with serum-enriched media (Fig 4-6). In one study Ang II was shown to increase LV mass and not

RV mass, while PE increased mass in neither ventricle (Segar *et al.*, 2001). Our data are consistent with the explanation that, in their experiments, the LV increased mass by increasing cell numbers in their experiments. However, it is not clear why RV mass did not increase. We found no difference in LV and RV response to Ang II *in vitro* and we found profound RV hypertrophy with increased mechanical systolic load (Barbera *et al.*, 2000). Nor is it clear why PE was unable to induce an increase in ventricular mass in the experiments of Segar, *et al.* (2001).

BrdU uptake was counted in all cardiomyocytes as we were not able to distinguish mononucleated from binucleated cells that were BrdU negative. It could be argued then that the increase in BrdU uptake was leading only to binucleation and not proliferation. BrdU positive binucleated cells were seen, but the vast majority of BrdU positive myocytes were mononucleated (≈90% of all BrdU positive myocytes). We expect that only mononucleated cells are capable of proliferation and that the more mature binucleated cells are not. If true, we expect that all of the BrdU positive binucleated cells that were detected were the result of mononucleated cells undergoing binucleation. If binucleated cells are able to divide in the fetal myocardium they do not fit the definition of being "terminally" differentiated. However, division in binuclear cells is highly unlikely because Ang II treatment did not affect the proportion of binucleated BrdU positive cells (data not shown) and thus, evidently did not lead to increased DNA synthesis in these cells. While the constant proportion of binucleation is not proof that binucleate cells cannot divide, it seems more likely and consistent with our data, that the mononucleated cells in the fetal myocardium are capable of proliferation in response to Ang II, while binucleated cells are unable to proliferate but are able to

increase cell size in response to PE. The fact that Ang II is not capable of inducing enlargement of even a binucleated cell at this stage of gestation suggests a species difference between sheep and rats. It may indeed indicate that there is a general difference in myocardial growth regulation between larger mammals and rodents (Dell'Italia *et al.*, 1997;Modesti *et al.*, 2000;Perry *et al.*, 2002;Segar *et al.*, 1997).

ERKs are involved in diverse cellular responses including proliferation and hypertrophy. We set out to determine the roles that ERKs were playing in Ang II signaling in immature ovine cardiomyocyte. Thus, we studied the effect of MEK inhibition on BrdU uptake. Inhibition with 10μM UO126 completely inhibited all phospho-ERK stimulation even reducing basal p-ERK stimulation from the serum free medium conditions (Fig 4-8). BrdU uptake and ERK stimulation by Ang II were completely inhibited in the presence of the MEK inhibitor (Figs 4-7 & 4-8). We conclude that ERK stimulation is necessary for Ang II action in sheep cardiomyocytes.

Phenylephrine induced hypertrophy is also reported to require ERK stimulation in rodents (Ueyama *et al.*, 2000;Yue *et al.*, 2000). We have shown that ERK stimulation is required for the induction of hypertrophy by PE in binucleate cells (Fig 4-3). It is curious that ERK stimulation is required for both PE-induced hypertrophy and Ang II-induced proliferation, two mutually exclusive processes. However, since both agonists stimulate ERK and are only capable of hypertrophy or hyperplasia, but not both, the outcome of ERK stimulation must depend upon the maturation state of the myocyte. If this is true, it is likely that the cascades themselves are significantly "remodeled" with maturation. It is also possible that PE, together with ERK, stimulates a separate signaling cascade that leads to hypertrophy, while Ang II does not, and that Ang II stimulates a separate cascade

together with ERK that is required for hyperplastic growth that is not stimulated by PE. The focus of this study was limited to ERK signaling. Further investigation of PE and Ang II signaling in the maturing cardiomyocyte will be required to distinguish between these possibilities.

In summary, this paper reports on the establishment of culture methods for immature sheep cardiomyocytes. It also reports on several novel findings regarding Ang II and its effects on cardiomyocytes. It is shown here that Ang II stimulates the ERK cascade in fetal sheep cardiomyocytes without stimulating hypertrophy. Angiotensin II is able to stimulate hyperplasia in fetal sheep cardiomyocytes and the stimulation of the ERK cascade is required for the increase in the proliferative index of BrdU uptake. It is also shown that the ability of the cardiomyocyte to enlarge in response to PE is dependent on its state of maturation. Binucleation is considered to be a marker for the transformation from hyperplastic growth to hypertrophic growth. Our studies support this hypothesis by showing that, in the immature myocardium containing a mix of immature mononucleated cells and differentiated binucleated cells, it is possible for both hyperplasia and hypertrophy to take place but in a maturation state-dependent manner. This may reflect the situation *in vivo*, as augmented afterload conditions will induce both hyperplasia and hypertrophy in the fetal right ventricle (Barbera *et al.*, 2000).

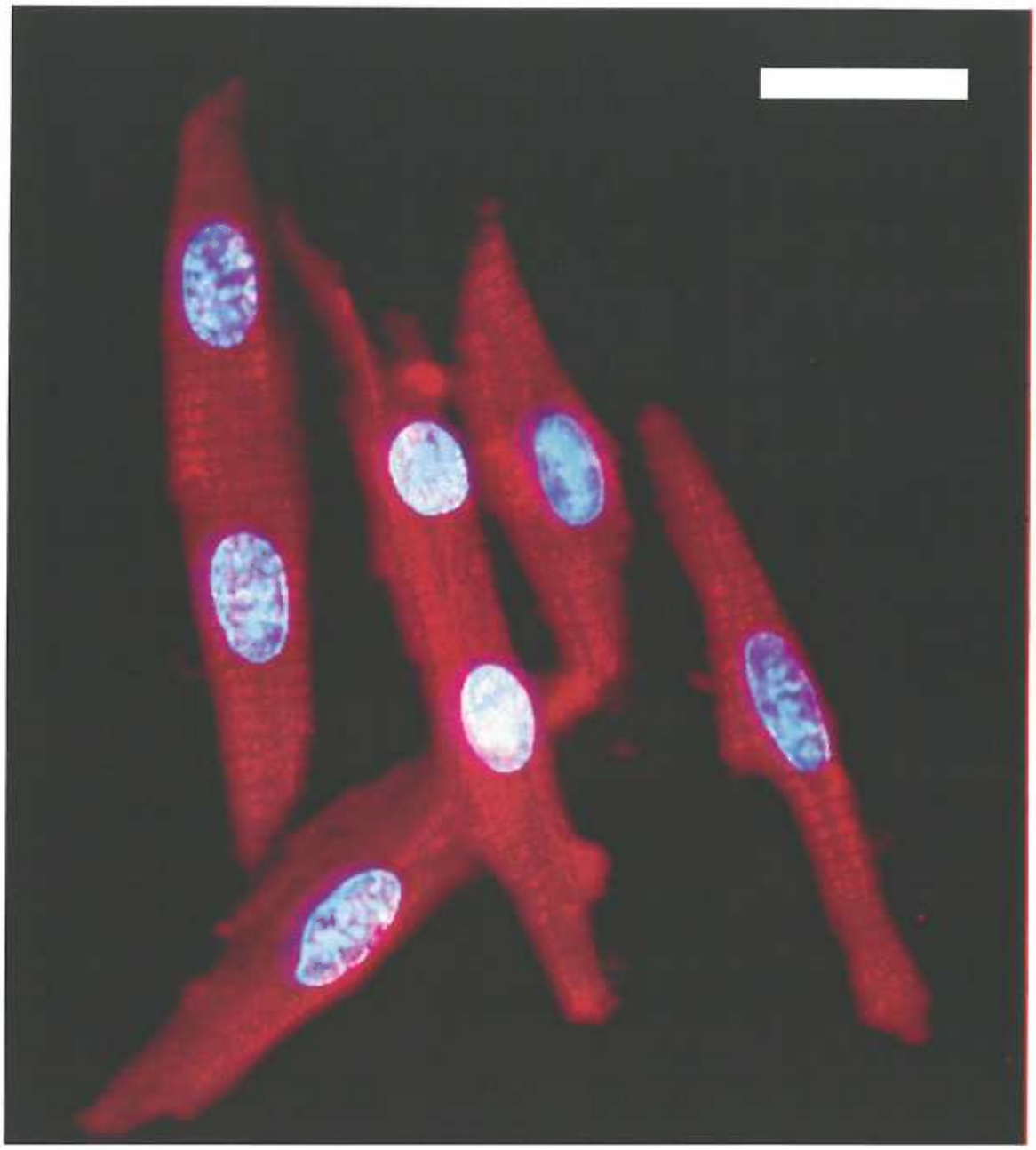

**Figure 4-1: Dissociated cardiomyocytes from fetal sheep.**

Cells were fixed after dissociation and viewed under 400X power (Zeiss Axiophot). Myosin was stained with a secondary antibody conjugated to rhodamine red-X and the nuclei were stained with Hoechst 33342 (Molecular Probes). Mononucleated and binucleated cells appear healthy and intact after dissociation. Bar = 20μm.

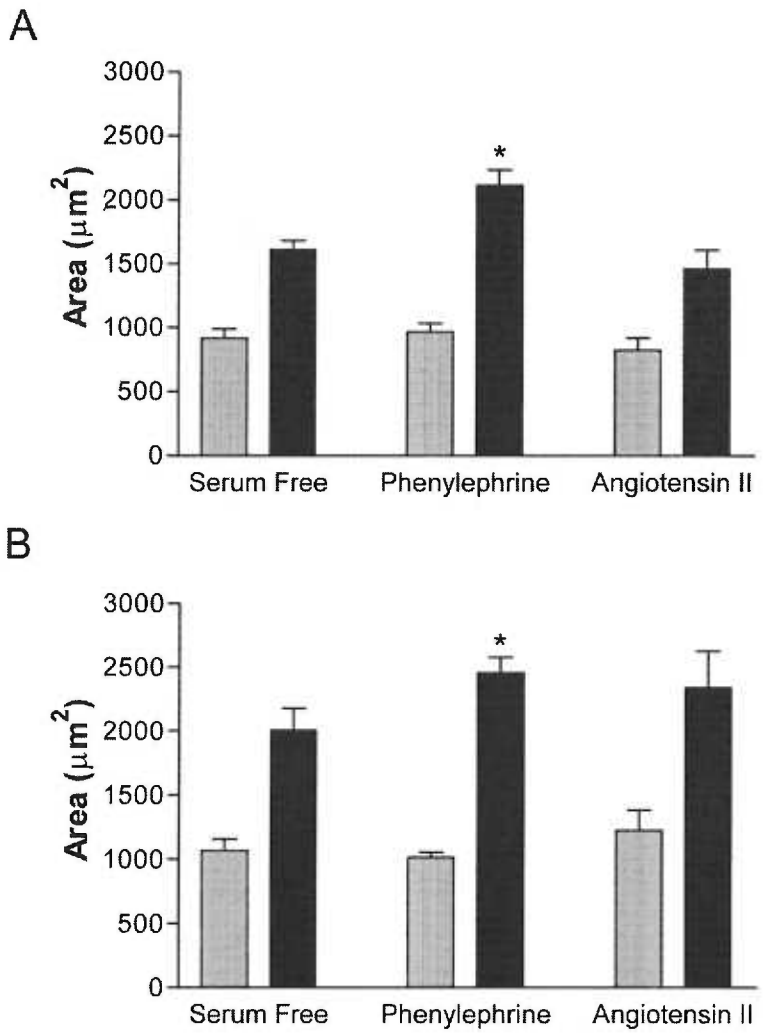


**Figure 4-2: Hypertrophy of Cardiomyocytes in Culture**

Cardiomyocyte area of LV (A) and RV (B) mononucleated (gray bars) and binucleated (black bars) cardiomyocytes from 4 separate fetuses. Cells were stimulated with 10μg/mL PE or 100nM Ang II for 48 hours. *$p<0.05$ compared to serum free binucleated, n=4, paired ANOVA.

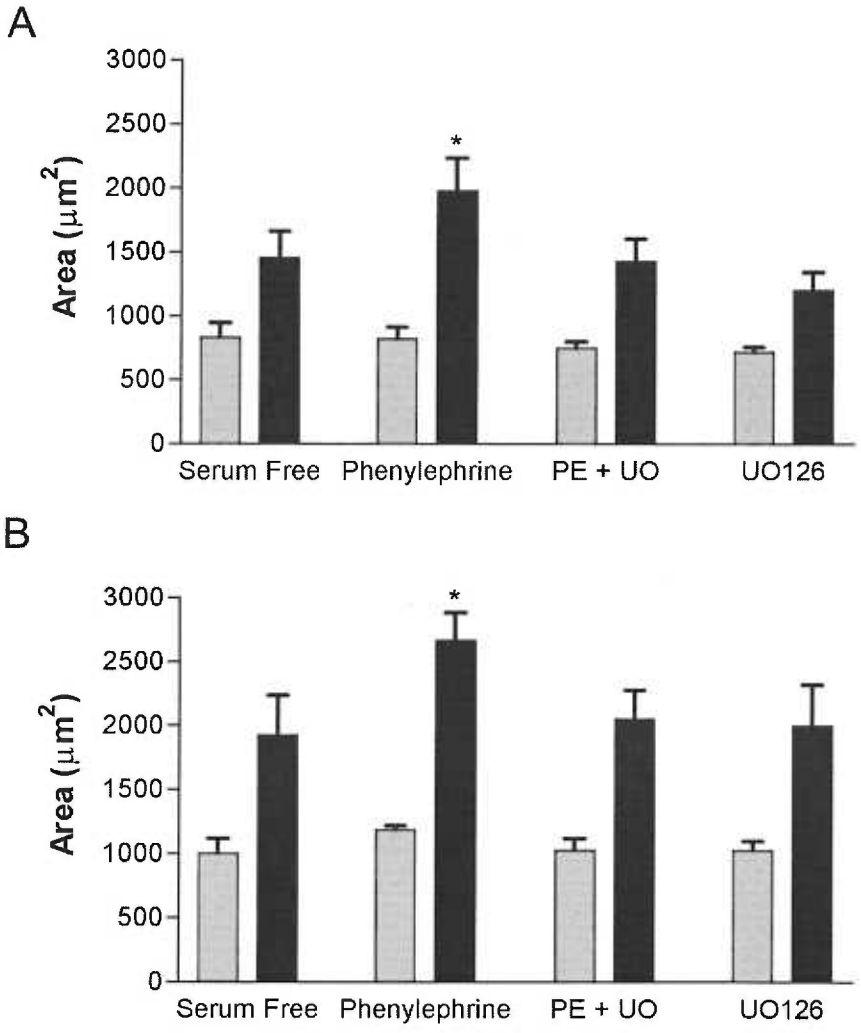


**Figure 4-3: PE induced Hypertrophy is ERK Dependent**

Cardiomyocyte area of LV (A) and RV (B) mononucleated (gray bars) and binucleated (black bars) cardiomyocytes from 4 separate fetuses. MEK inhibition with UO126 abolishes PE induced hypertrophy and is not different from serum free conditions. *$p<0.05$ compared to serum free binucleated, n=4, paired ANOVA.

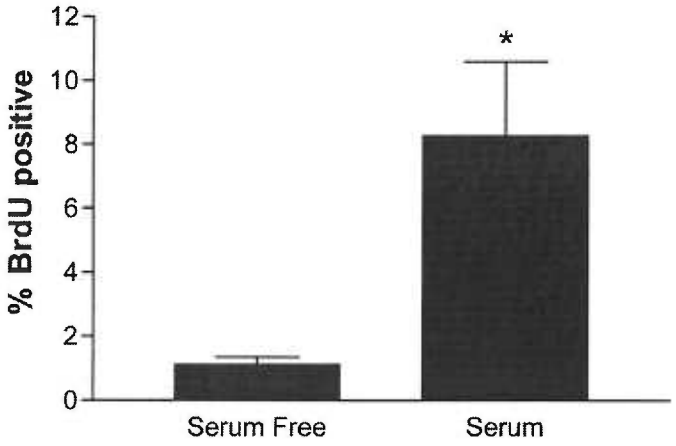


**Figure 4-4: BrdU uptake after 48 hours in Serum Free or Serum media conditions.**

*$p<0.05$, student's t-test, n=9.

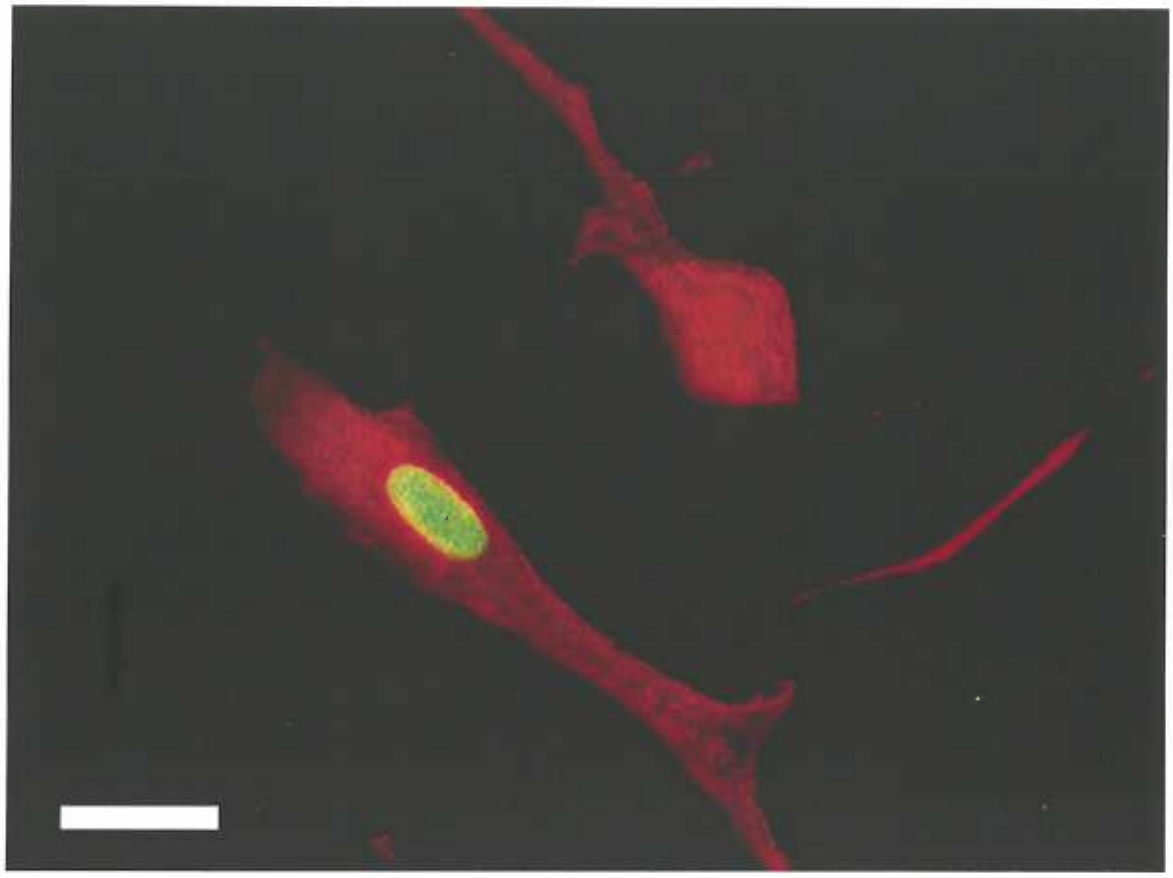

**Figure 4-5: Plated cardiomyocytes after stimulation with 100nM Ang II in the presence of BrdU for 48 hours**

Note that one cell has incorporated BrdU into the nucleus (green). Myosin was stained with a secondary antibody conjugated to rhodamine red and nuclear BrdU was stained with a secondary antibody conjugated to FITC. Negative controls ( without primary antibodies) did not stain for either myosin or BrdU (not shown). Bar = 20μm.

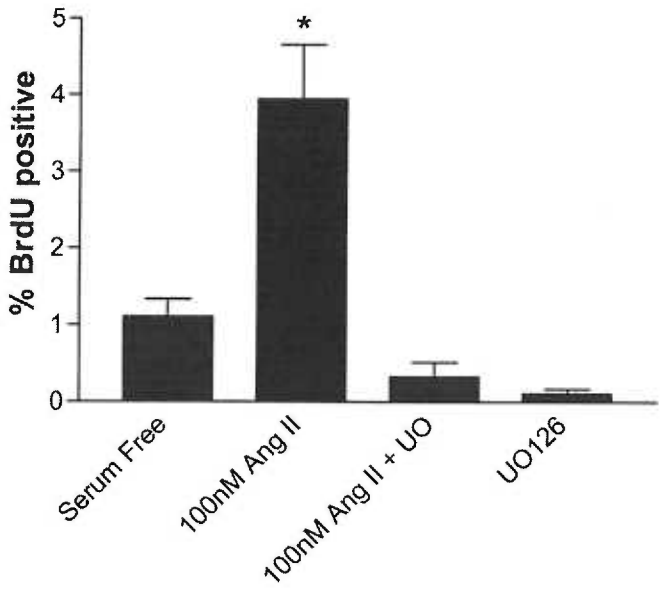


**Figure 4-7: BrdU uptake after 48 hours with Ang II in the presence of the MEK inhibitor UO126**

UO126 abolishes the increase in BrdU uptake that 100nM Ang II induces in the absence of the MEK inhibitor. *$p<0.05$ compared to serum free, ANOVA.

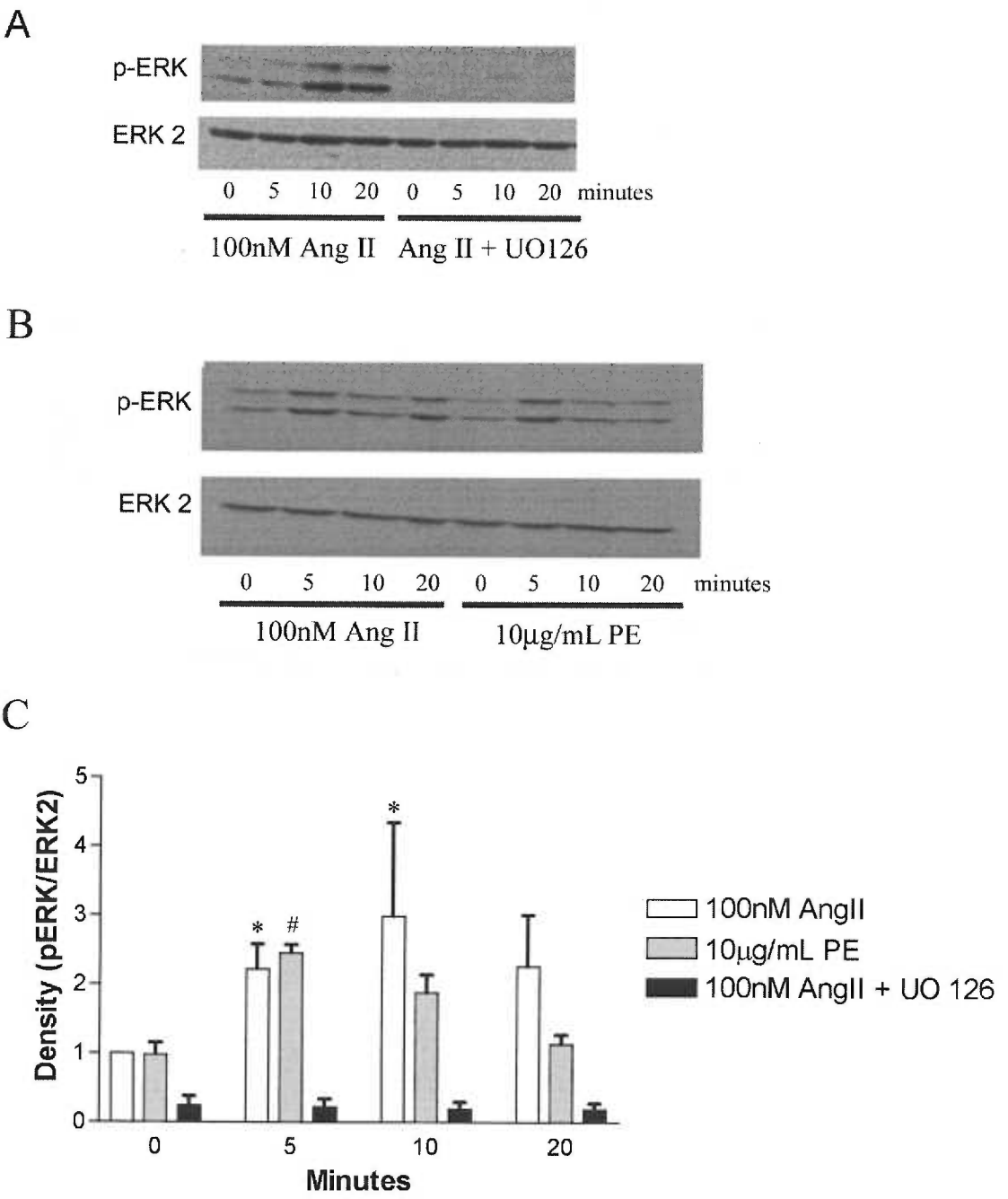


**Figure 4-8: ERK Stimulation**

A, shows a typical western blot (n = 4). Results shown in the left 4 columns were from cells stimulated with 100nM Ang II in the absence of UO126 for the indicated times.

The right 4 columns are from cells pre-treated 20 minutes with UO126 before stimulation with Ang II. B, a typical western blot of Ang II and PE stimulation (n = 4). The left 4 columns are from cells that were stimulated with 100nM Ang II and the right 4 columns from cells treated with 10μg/mL PE. All membranes were probed for phospho-ERKs, then stripped and probed for ERK2. C, average band density of western blots normalized to baseline Ang II stimulation (0 min). Density is plotted in normalized units of p-ERK/ERK2 levels. At 5 and 10 minutes, phospho-ERK levels were increased over basal unstimulated levels with Ang II (*$p<0.05$, non-parametric ANOVA). At 5 min PE significantly increases phospho-ERK levels over 0 minutes of PE stimulation (#$p<0.05$, non-parametric ANOVA).

# Chapter 5:

# Kidney RAS

**Abstract:**

IGF-1 has been shown to increase kidney weight relative to body weight in fetal sheep by others and us. The renin-angiotensin system (RAS) plays a critical role in regulating normal kidney development and function. IGF-1 has also been shown to increase plasma renin activity, but components of the intrarenal RAS have not been studied. We infused fetal sheep with LR3 IGF-1 for seven days and extracted RNA from their kidneys. Messenger RNA levels were determined for angiotensin II-type 1 receptor (AT1), angiotensin II-type 2 receptor (AT2), angiotensinogen (Aogen), and renin using real time quantitative RT-PCR. We discovered that LR3 IGF-1 apparently down-regulates the intrarenal RAS. LR3 IGF-1 significantly reduced AT1, AT2, and Aogen RNA levels, but renin levels were unchanged. Further work is needed to establish the functional and structural impact LR3 IGF-1 and the down-regulation of the RAS has in fetal sheep.

**Introduction:**

IGF-1 is known to affect fetal kidney function and to alter the function of the renin-angiotensin system (RAS). After 4 hours of IGF-1 infusion, fetal sheep have a small decrease in renal blood flow (RBF), but no changes in glomerular filtration rate (GFR) and filtration fraction (FF) (Marsh *et al.*, 2001b;Marsh *et al.*, 2001a). After 4 days of IGF-1, fetal sheep have no change in RBF, but increased GFR and FF (Marsh *et al.*, 2001a). These functional changes are simultaneous with dramatic increases in plasma renin concentration and plasma renin activity at both 4 hours and 4 days (Marsh *et al.*, 2001b;Marsh *et al.*, 2001a). Furthermore, IGF-1 has been shown to directly stimulate renin gene expression in fetal sheep renal cortical cells in culture (Liu & Rose, 2003). IGF-1 increases kidney size relative to body weight when infused intravenously into the fetal sheep (Marsh *et al.*, 2001a;Lok *et al.*, 1996) causing plasma renin levels to increase by 4 hours, well before changes in size could be responsible for the alteration in circulating RAS components. Similarly, a seven-day infusion of Long R3 IGF-1 (LR3 IGF-1) increases kidney weight relative to body weight (Fig 3-1B). Other RAS components, such as the AT1 and AT2 receptor, have not been studied in response to fetal IGF-1 infusion. In contrast to the up-regulation of plasma renin activity, IGF-1 down-regulates AT1 and Aogen levels in cardiomyocytes (Leri *et al.*, 1999a;Leri *et al.*, 1999b). Therefore, IGF-1 can alter the RAS system and raises plasma levels of Ang II.

The process of nephrogenesis is influenced by the renal RAS. When rats are given the AT1 antagonist, losartan, during the period of nephrogenesis, fewer nephrons are formed and nephron deficient offspring develop hypertension in adulthood (Woods & Rasch, 1998). Protein restricted rat pups also develop fewer nephrons (Woods *et al.*,

2001a). The nephrons that are formed undergo hypertrophy and kidney function is maintained as GFR, effective renal plasma flow (ERPF), and FF are normal (Woods *et al.*, 2001a). Thus, hyperfiltration occurs in each glomerulus and this condition is associated with hypertension and renal insufficiency (Ingelfinger & Woods, 2002). The low nephron number with protein deprivation appears to have a similar effect as removing a single normal kidney during early rat development which halves nephron number, causes hyperfiltration, and leads to hypertension in adult rats (Woods *et al.*, 2001b).

The purpose of this study was to test the hypothesis that LR3 IGF-1 would up-regulate components of the RAS in the kidney. If LR3 IGF-1 behaved in similar fashion to endogenous IGF-1 in fetal sheep, we would predict a similar increase in PRA and, consequently, up-regulation of message levels for renin and RAS components because the intrarenal components of the RAS are up-regulated in response to increasing Ang II (Ingelfinger *et al.*, 1999). Up-regulation of the renal RAS may contribute to the increase in kidney mass in response to 7 days of LR3 IGF-1

**Specific Methods:**

*RNA Isolation*: At the end of the LR3 IGF-1 infusion protocol, whole kidneys were removed, weighed, and snap frozen in liquid nitrogen and stored at –80˚C until RNA extraction. Five left kidneys from experimental and five left kidneys from vehicle control animals were used for RNA isolation. Total RNA was isolated from 200-500mg chunks of frozen tissue using Trizol reagent (Life Technologies, Rockville, MD, USA) and Phase-Lock Gel Heavy™ columns (Eppendorf, Hamburg, Germany) according to manufacturers' protocols. Prior to reverse transcription, RNA samples were DNase treated with DNA Free (Ambion, Austin, TX, USA), according to the manufacturer's protocol.

*Primers*: The Primer Designer 4 program (Scientific & Educational Software, Durham, NC, USA) was used to design pairs of 20-22mers to amplify a 165bp region of sheep GAPDH (GenBank Accession # AF035421), a 181bp region of sheep AT1 (GenBank Accession # AF254119), a 342bp region of sheep AT2 (GenBank Accession # S81979), a 296bp region of sheep renin (GenBank Accession # L43524), and a 183bp region of sheep angiotensinogen (GenBank Accession # D17520). Primer Sequences are listed in table 5-1.

*Reverse Transcription (RT)*: For each sample, a reverse transcription reaction was performed on 500ng-3μg total RNA with SuperScript II RNase H- RT (Life Technologies). The RT reaction used 2pmol of each primer for reverse transcription. An RT- control reaction was also run for each sample, under identical conditions, omitting the RT enzyme and replacing it with sterile water. One RNA sample was used to generate a standard curve. Dilutions of 1:1, 1:5, 1:10, 1:20, and 1:40 were used for the standard

curve and RT+ reactions run for each of these samples. All 10 samples (5 experimental and 5 controls) were diluted 1:10 for RT reactions. Four replicates of one of the RT reactions were run, to give an estimate of the reproducibility of the results. Following reverse transcription, the cDNA samples were purified using QIAquick® PCR purification columns (Qiagen Inc., Valencia, CA, USA). Purified samples were eluted in 30µl sterile water.

*Quantitative PCR*: All PCR reactions were performed on a LightCycler Instrument (Roche Diagnostics, Indianapolis, IN, USA), using the LightCycler-FastStart DNA Master SYBR Green I Kit (Roche). The optimal cycling conditions for each primer pair were experimentally determined. Fluorescence readings were taken at the end of each amplification cycle to monitor the amount of double-stranded DNA present. During these cycles, fluorescence was plotted as a function of cycle number, to show when reactions were in the linear phase of amplification. During the melting curve, fluorescence was monitored continuously while the temperature was raised from 65˚C to 95˚C at a rate of 0.1˚C/s. Fluorescence was plotted as a function of temperature to allow the determination of the specific Tm for each product. During initial PCR reactions, the size of each PCR product was verified by running it on a 1.2% agarose gel with a 1Kb+ Ladder (Life Technologies). In subsequent runs the melting curve analysis was used to verify the correct product.

The concentration of the amplicon in a 1:10 dilution of the standard curve was defined as a value of 1. For each primer pair a separate PCR reaction was run for five standard curve samples, each unknown sample, the four RT replicate samples, and the unknown RT- samples. At the completion of the PCR reaction, a crossing line was set at

a level of fluorescence where all RT+ reactions had entered the log-linear phase of amplification. The cycle number at which a standard curve sample reached the fluorescence level of the crossing line was plotted versus the log of the concentration in that sample. The concentration of each amplicon was calculated based on the equation derived from the standard curve. Quantification of individual fluorescence intensity indicated expression levels of the gene being amplified relative to its expression level in the sample upon which the standard curve was based. The concentrations of mRNA for AT1, AT2, renin, and Aogen were determined and normalized to the concentration of the housekeeping gene, GAPDH.

*Statistics*: Student's unpaired t-test was used for comparisons between experimental and control tissue. $p<0.05$ was considered significant.

**Results:**

LR3 IGF-1 down-regulated renal AT1, AT2, and Aogen RNA levels, but did not alter renal renin RNA levels. AT1 RNA levels in experimental fetuses (0.36 ± 0.13, mean ± sd, n=5) were 30% of control RNA levels (1.22 ± 0.48 n=5). AT2 RNA levels in experimental fetuses (0.61 ± 0.30, n=5) were 46% of control RNA levels (1.32 ± 0.46, n=5). Aogen RNA levels in experimental fetuses (0.65 ± 0.14, n=5) were 38% of control RNA levels (1.71 ± 0.75, n=5). However, renin RNA levels were not significantly different between experimental (0.79 ± 0.53, n=5) and control (0.59 ± 0.36, n=5). Values are in arbitrary units and are not comparable across RNA species. Hence, we cannot compare the levels of renin message to Aogen, for instance.

**Discussion:**

I hypothesized that LR3 IGF-1 would up-regulate the fetal RAS, but I found an overall down-regulation of the renal RAS in response to seven days of LR3 IGF-1. Other work has shown that IGF-1 increases plasma renin activity and renin concentration out as far as 10 days (Marsh *et al.*, 2001a). In culture, IGF-1 directly stimulates renin gene expression, but interestingly, only in serum free medium conditions and not under serum enriched medium conditions (Liu & Rose, 2003). Our data tended to have higher renin RNA levels in LR3 IGF-1 infused fetal sheep, but this was far from significant (Fig 5-1). Perhaps renin message level was not up-regulated because of exposure to serum *in vivo* as is the case *in vitro*. We do not have comparable data for plasma renin activity from our experiments. It remains an open question whether LR3 IGF-1 increases plasma renin activity as does recombinant IGF-1. LR3 IGF-1 and endogenous IGF-1 both bind and activate IGF1R. However, LR3 IGF-1 does not bind IGF binding proteins (IGFBPs) and endogenous IGF-1 activity may be enhanced or inhibited by interactions with IGFBPs. But given the known role of IGF-1 in raising plasma renin activity, it is likely that LR3 IGF-1 raised plasma renin activity in my experiments also.

Assuming LR3 IGF-1 does increase plasma renin activity, it would be reasonable to suggest that AT1 and AT2 receptor levels were down-regulated in a negative feedback mechanism in response to increased Ang II. However, Ingelfinger *et al.* (1999) reported that renal AT1, AT2, and Aogen levels are up-regulated in response to Ang II in proximal tubular cells demonstrating a positive feedback mechanism. The decrease in renal Aogen also suggests that the decrease in substrate would limit the amount of intrarenal Ang II

produced whether or not renin is slightly up-regulated. Therefore, our RNA data is most consistent with a down-regulation of the intrarenal RAS.

LR3 IGF-1 does increase kidney weight (Fig 3-1). But what components of the kidney are increased is unknown. Fetal sheep nephrogenesis is complete by 130 days of gestation (Moritz *et al.*, 2003). Thus our experiments began after nephrogenesis was complete and it is unlikely that our infusion increased the number of nephrons in the kidneys. Perhaps our experiment increased individual nephron size – a known effect of IGF-1 in the adult kidney (Feld & Hirschberg, 1996). This question is worthy of further investigation.

There is an important difference between our current experiments and those from Dr. Lumbers' lab. The experiments from Dr. Lumbers' lab (Marsh *et al.*, 2001a;Marsh *et al.*, 2001b) were on fetal sheep of 120 days gestation, just before nephrogenesis is complete in the sheep. We do not have functional renal measures such as GFR, ERPF, or FF to compare. However the increases in GFR and FF in response to IGF-1 may be due to a structural change since the increases did not occur until 4 days and not after 4 hours even though renin activity was similar at both time points (Marsh *et al.*, 2001a). At this younger age, IGF-1 may have stimulated a late surge in nephrogenesis that may not have been possible in our experiments.

Our data show that LR3 IGF-1 decreases RNA levels of AT1, AT2, and Aogen in fetal sheep kidneys. This is consistent with a down-regulation of the intrarenal RAS in response to LR3 IGF-1. Blockade of the RAS reduces nephron number (Woods & Rasch, 1998). However, it is unclear what a down-regulation in the RAS in response to LR3 IGF-1 means for kidney structure or function, especially given after the time

nephrogenesis is supposed to be complete. Further work needs to address the structural changes in the kidney and the plasma renin activity in response to LR3 IGF-1.

**Table 5-1: Primer sequences for Quantitative RT-PCR**

| | FWD primer | REV primer | REV B primer |
|---|---|---|---|
| GAPDH | gtcaccagggctgcttttaat | ctttccattgatgacgagcttc | |
| AT1 | ctcttccaccgaagatggta | actggccacagtcttcagtt | aggtagcggtcaatgcttag |
| AT2 | tggttggtgtatggcttgtc | agcatccaggaaggtcagaa | |
| Renin | tggtgcatcgtacatctcag | ccgtgtagaacttgcggata | |
| Aogen | ttccacctcctcgtccacag | cagccggtcttcagcctcta | ggcctgctctccatctgctt |

Sequences are written 5' to 3' for all primers. FWD and REV primer were used for PCR amplification. REV primer was used for reverse transcription except for AT1 and Aogen where REV B primer was used. REV B primers were designed downstream of intended PCR product.

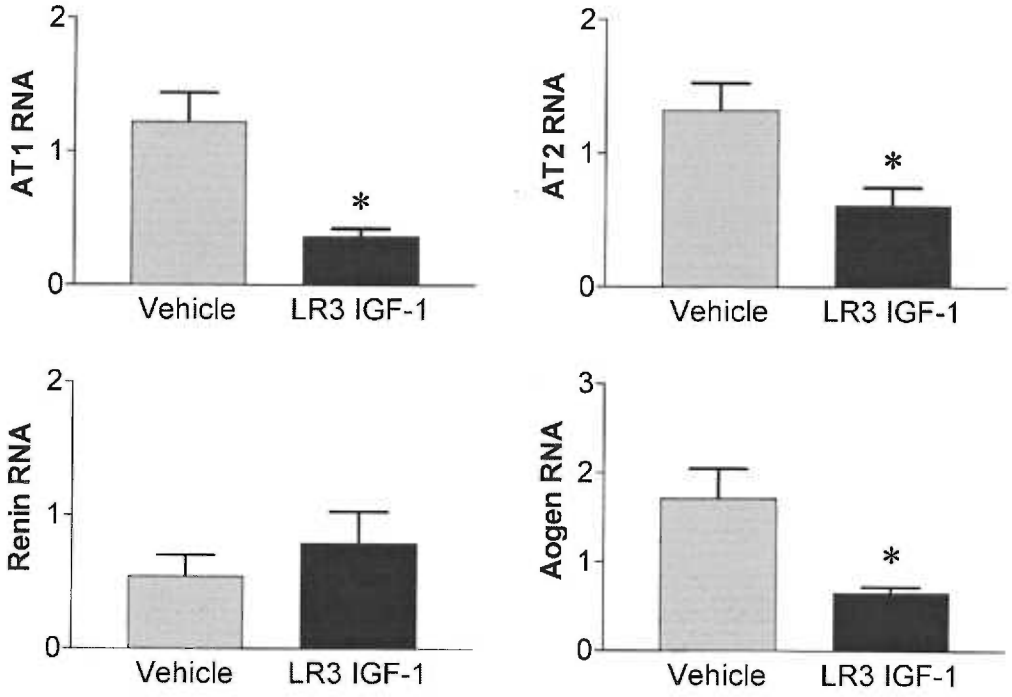


**Figure 5-1: Quantitative RT-PCR of Kidney RNA**

Kidney RNA levels of AT1, AT2, Renin, and Aogen are plotted as mean ± se. Fetuses received LR3 IGF-1 (black bars) or vehicle (gray bars) for seven days. RNA levels are expressed in arbitrary units normalized to GAPDH levels. Student's t-test, *$p<0.05$, $n=5$.

# Chapter 6:

# Conclusions

I originally hypothesized that IGF-1, through IGF1R signaling, would increase fetal cardiomyocyte proliferation, maturation, and size as does a systolic pressure load (Barbera *et al.*, 2000). I found evidence that IGF-1 stimulates proliferation, but I found no evidence that it increases maturation or size of fetal sheep cardiomyocytes. I also hypothesized that Ang II would increase fetal sheep cardiomyocyte cell size as has been well established in rat cardiomyocytes. But I discovered that Ang II stimulates a hyperplastic response in fetal ovine cardiomyocytes and not a hypertrophic response. IGF-1, Ang II, and PE all cause hypertrophy in rat cardiomyocytes, but only PE causes hypertrophy and then only for the more mature binucleated cardiomyocytes in fetal ovine cardiomyocytes.

**ERK signaling:**

Perhaps the most intriguing discovery of my work is that ERK signaling is required for both IGF-1 and Ang II induced hyperplasia and PE induced hypertrophy. It is not all that surprising that ERK signaling is implicated in hypertrophy or hyperplasia. Both types of cell growth have been previously demonstrated to be outcomes of ERK signaling. The novel discovery here is that ERK stimulation has different actions based on maturation level of the cell *within the same cell type*. I propose two possible explanations. The first is that the outcome of ERK signaling is maturation dependent. Thus, IGF-1 and Ang II both stimulate ERK but only in mononucleated cells where it leads to activation of the cell cycle. Furthermore, PE also stimulates ERK but only in binucleated cells where it stimulates hypertrophy. This theory says that there is a maturation dependent uncoupling or coupling of the receptors to ERK signaling

machinery. Since the cardiomyocytes are roughly 50% mononucleated and 50% binucleated in late gestation when I studied them, ERK stimulation with hyperplastic or hypertrophic stimuli could appear the same on a western blot (for instance, Fig 4-8). The second possibility is that ERK is stimulated in both mono- and bi-nucleate cells and is necessary but not sufficient for either hyperplasia or hypertrophy. The difference between the signaling outcomes is additional signaling cascades required for each process. This argument suggests that all three agonists studied (IGF-1, Ang II, and PE) stimulate ERK and lead to either hyperplasia or hypertrophy but not both depending upon the action of other signaling cascades. Unfortunately, I do not yet have the data to prove whether either of these two theories is correct.

I have shown that IGF-1 induced hyperplasia requires both ERK and PI3K (Fig 3-4). Blocking either signaling cascade alone is sufficient to block all increase in BrdU uptake. I hypothesized that Ang II would stimulate PI3K like IGF-1 and induce hyperplasia in combination with ERK signaling while PE would not stimulate PI3K. However, the same sets of stimulated cardiomyocytes shown in Fig 4-8B were probed for phospho-AKT by western blot analysis and failed to show any stimulation of AKT by Ang II or PE (data not shown). While BrdU uptake experiments were not conducted, there is still the possibility that Ang II stimulates PI3K in fetal sheep cardiomyocytes. The p-AKT antibody we used had weak binding and was difficult to get good signal even for LR3 IGF-1 stimulated cells. Since finishing with my experiments, it has been reported that Ang II induced hyperplasia of rat aortic smooth muscle cells (RASM) is dependent on both ERK and PI3K (Dugourd *et al.*, 2003). The Ang II induced hyperplasia in RASMs reported in that paper is remarkably similar to the IGF-1 induced

hyperplasia in our fetal ovine cardiomyocytes (Chapter 3). Further work is necessary to elucidate any role for PI3K in Ang II induced hyperplasia in our system.

**Hypertrophy of Cardiomyocytes:**

These data show for the first time a change in hypertrophic response dependent on maturation level. It has long been believed that binucleation is a marker for "terminal differentiation" when cardiomyocytes change from a hyperplastic to hypertrophic mode of growth (Clubb & Bishop, 1984;Oparil *et al.*, 1984). Fetal heart myocytes transition from predominantly mononucleated to predominantly binucleated during late gestation in the sheep. During this transition, only binucleated cells increased cell size in response to PE in culture (Figs 3-5, 4-2, & 4-3). The situation may be different for rodents.

Rat cardiomyocytes undergo binucleation that occurs between postnatal days 4 and 12 - not before birth as in sheep (Li *et al.*, 1996). Neonatal rat cardiomyocyte cultures are normally taken from hearts of less than 2-day-old rats. At this time most of the cardiomyocytes would be mononucleated. In fact, neonatal rat cardiomyocyte primary cultures are predominantly mononucleated cells, yet they are capable of hypertrophy in response to PE, Ang II, and IGF-1. Differences between rats and sheep would not be surprising in light of the fact that sheep cardiomyocytes – mononucleated or binucleated – do not increase cell size in response to IGF-1 or Ang II (Figs 3-5 & 4-2) while rat cardiomyocytes do. But the situation may be more complicated. RV cardiomyocytes in the fetus are larger than LV cardiomyocytes; the RV is responsible for the majority of systemic blood flow and fetal RV cardiomyocytes see a greater systolic load. An examination of Fig 4-2B reveals no statistical increase in RV binucleated cell

size in response to Ang II. However, of the four sets of cell size data from RV cardiomyocytes, two sets appeared to have an increase in the size of binucleated cells in response to Ang II (2806.70 ± 310.47$\mu m^2$, n=2) that exceeded the response to PE by RV cells (2456.99 ± 121.80$\mu m^2$, n=4). The other two RV sets did not show an increase in binucleated cell size in response to Ang II (1877.42 ± 160.24$\mu m^2$, n=2) above serum free conditions (2006.38 ± 172.19 $\mu m^2$, n=4). Therefore, the average of the four data sets has a large standard error and indicates no significant increase in size compared to basal binucleated cell size. Binucleated cells from the LV never showed any indication of increased cell size above serum free conditions (Fig 4-2A). It is possible, then, that there is a further maturation stage beyond binucleation that transitions cardiomyocytes from responding to Ang II with hyperplasia to responding with hypertrophy. Perhaps the rat has a maturational event for mononucleated cardiomyocytes that enables them to respond to Ang II by hypertrophy that is absent in the sheep.

The original load data from our lab did not report separate mononucleated and binucleated cell sizes (Barbera *et al.*, 2000). There was an overall increase in cell size along with an increase in binucleation in response to RV pressure loading. We do not know whether mononucleated myocytes increased cell size under these load conditions. Current preliminary work using a volume overload model of cardiac hypertrophy appears to increase both mononucleated and binucleated cell sizes (Karamlou T, Giraud GD, Jonker SS, Thornburg KL, 2003). The fact that mononucleated cells may enlarge in response to a load stimulus *in vivo* would be a unique finding and could be a difference between *in vivo* and *in vitro* experiments. It could also be a difference in the type of stimulus, i.e. a systolic load response vs. a diastolic load response vs. an endocrine

response. Under conditions of excess load to the working myocardium, which includes mononucleated and binucleated myocytes, the myocardium responds by increasing wall mass, decreasing its radius of curvature to wall thickness ratio and improving mechanical advantage. Under culture conditions PE cannot exert an increase in systolic or diastolic load. It could be argued that other hypertrophic endocrine factors may increase both mono- and bi-nucleate cardiomyocytes. In my experiments, PE did not increase the size of mononucleated cardiomyocytes. I also have preliminary data indicating that endothelin-1 (ET-1) increases binucleated but not mononucleated cell size in an ERK dependent manner *in vitro*, behaving just like PE (Fig 6-1).

**Fetal IGF-1 and Adult Heart Disease:**

IGF-1 is implicated in programming the fetus for adult onset cardiac disease. One mechanism by which low birth weight might increase risk for adult heart disease is the link between low plasma levels of IGF-1 and fetal under-growth. My experiments have demonstrated that IGF-1 is a hyperplastic factor in fetal heart growth. Therefore, fetal development in the presence of low IGF-1 levels may lead to a low number of cardiomyocytes at birth, and development in the presence of high IGF-1 levels may lead to an above average number of cardiomyocytes at birth. One could postulate that the number of cardiomyocytes one is born with determines risk for or sensitivity to heart disease during adulthood. Adult cardiomyocytes may indeed retain some ability to proliferate (Anversa & Kajstura, 1998;Nadal-Ginard *et al.*, 2003), but any proliferative ability does not appear to be adequate to replace myocardium lost during infarction. Fetal rats exposed to prenatal hypoxia have identical birth weights and heart weights

compared to control rats exposed to normoxia, but fewer cardiomyocytes (Zhang, 2003). When previously hypoxic hearts were challenged in adulthood with an ischemia-reperfusion insult, the hearts had much greater infarct sizes and greater degrees of apoptosis compared to normoxic offspring (Zhang, 2003). This work suggests that the number of cardiomyocytes one has during adulthood can determine susceptibility to a heart attack and possibly other heart diseases. This makes sense because in the mature myocardium there is about one capillary formed for each muscle fiber. Therefore, a myocardium with fewer cells may have both a mechanical and a perfusion disadvantage.

IGF-1 can induce hyperplasia in fetal myocardium not only directly, but perhaps also indirectly through Ang II. IGF-1 is known to increase plasma renin activity (Marsh *et al.*, 2001a;Marsh *et al.*, 2001b). An increase in PRA raises the plasma Ang II levels and, presumably, the amount of Ang II the heart is exposed to. Since we have demonstrated that Ang II stimulates fetal sheep cardiomyocyte proliferation, an increase in Ang II would also serve to increase cardiomyocyte number. However, LR3 IGF-1 appears to down-regulate the intrarenal RAS and lower Ang II levels in the fetal kidney (Chapter 5). This could lead to hypertension, based upon rat findings, if the RAS was down-regulated during nephrogenesis (Woods & Rasch, 1998;Woods *et al.*, 2001a). But the direct effect of IGF-1 and Ang II on the heart would appear to be protective as high IGF-1 during development should increase number of cardiomyocytes and would have a further protective effect in the adult (Juul *et al.*, 2002).

**Future Directions:**

*IGF-1 and Programming*: Further experiments are needed to test whether an increase in IGF-1 plasma levels correlate with susceptibility to adult heart disease. After 7 days of LR3 IGF-1 given *in vivo*, fetal sheep have an increase in heart weight that is not due to an increase in myocyte cell size (Fig 3-1 & Table 3-2). This along with *in vitro* data showing an increase in proliferation in response to LR3 IGF-1 (Fig 3-3) points to an increase in cardiomyocyte number. It is now important to know if increased IGF-1 exposure *in utero* decreases susceptibility to an ischemic insult in adulthood. Fewer cardiomyocytes appears to increase susceptibility to an ischemic insult (Zhang, 2003).

The susceptibility question could be addressed using the model I chose for my thesis. For example, after 7 days of LR3 IGF-1 infusion, fetal sheep could be allowed to deliver and grow to adulthood. They could be brought back in to the lab as adults for study. I would hypothesize that plasma IGF-1 levels would not be different between experimental and control sheep as adults. If true, any difference in response to acute or chronic cardiac ischemia would suggest a permanent structural or physiological alteration brought about by the fetal IGF-1 exposure.

*Hypertrophy In Vivo*: It will also be important to know if the hypertrophic effect of PE is restricted to binucleated myocytes under *in vivo* conditions as it is under *in vitro* conditions. The experiments will be technically difficult to do without changing the load conditions of the heart as $\alpha_1$-agonists cause systemic vasoconstriction. The key question is whether endocrine mediators of hypertrophy in the absence of altered load are capable of increasing mononucleated myocyte size *in vivo*. Blood pressure would need to be

clamped in the presence of PE stimulation, perhaps by co-administration of nitric oxide to counteract the PE-induced vasoconstriction.

*Maturation and Hypertrophy*: Several questions remain regarding the maturation level required for hypertrophy and what changes in the cell are required for hypertrophy. Binucleation itself is thought to be the marker of maturation that identifies terminally differentiated myocytes. However, it is possible that binucleation does not signify the final maturation state of a cardiomyocyte. We need to determine whether Ang II or IGF-1 can cause hypertrophy of cardiomyocytes obtained from neonatal and adult sheep in comparison to fetal sheep; preliminary experiments are already underway in our laboratory. If Ang II and IGF-1 can cause hypertrophy in neonatal sheep as they do in neonatal rat cardiomyocytes, but not in fetal sheep cardiomyocytes, it would suggest that binucleated cells undergo a further maturation event that has not been reported. This would leave many avenues left open for investigating the maturation of signaling pathways.

The hypertrophic actions of Ang II in neonatal rat cardiomyocytes are mediated by the AT1 but not the AT2 receptor (Sadoshima & Izumo, 1993b). We do not know at this time if Ang II induced hyperplasia of fetal sheep cardiomyocytes is via AT1 or AT2 signaling. Throughout development the AT2 receptor is the predominant Ang II receptor subtype in the sheep heart and most other organs (Burrell *et al.*, 2001). The membrane bound AT2 density declines on the cardiomyocyte throughout gestation and is replaced during early postnatal life by the AT1 receptor as the predominant receptor subtype (Burrell *et al.*, 2001). It will be also be important to know if the switch in receptor subtype mediates a switch between hyperplasia and hypertrophy in response to Ang II.

*Cell Signaling*: ERK signaling is necessary for both hyperplasia and hypertrophy in fetal sheep cardiomyocytes in response to all the agonists I have studied. But it is not sufficient for either hyperplasia or hypertrophy. Further investigation is needed to distinguish between maturation-dependent signaling vs. additional signaling cascades that may be necessary for receptor-mediated hyperplasia or hypertrophy. First, PI3K must be definitively ruled in or out as a mediator of Ang II induced hyperplasia by blocking PI3K signaling in the presence of Ang II and BrdU. Second, a search must be made for signaling cascades that are activated by IGF-1 and Ang II but not PE and vice versa. Searching one cascade at a time by western blot would be a laborious method. A technique more akin to a proteomics screen of cell signaling proteins is needed.

It is also curious, and a bit disconcerting, that ERK has been required for all the actions of every agonist that I have studied. It would be important to know if ERK has these same actions *in vivo*. In a current Thornburg grant, the investigators propose to study a pressure load in the presence of ERK signaling inhibitors (UO126 or PD98059). If ERK behaves *in vivo* as it does *in vitro*, pressure load induced hyperplasia and hypertrophy would both be attenuated with an unknown effect on binucleation.

*Mononucleated and Binucleated Myocytes*: My current work gives the field of fetal cardiac physiology a much clearer understanding for the vast differences between mononucleated and binucleated cardiomyocytes. Yet we still know astonishingly little about how the maturation process is regulated. I hypothesized that IGF-1 would be an important stimulant of binucleation but my results have shown otherwise (Table 3-2).

I believe that a large step forward in understanding the differences between these two myocyte maturation levels will be forthcoming once they can be sorted and studied

separately. I have made several attempts to sort fetal cardiomyocytes according to the number of nuclei, principally using flow cytometry. They have a wide overlap of length (forward scatter) and are unable to be sorted that way. Sorting by DNA fluorescence is complicated by the fact that mononucleated cells can be 4N in the nuclei after S-phase as can binucleated cells by virtue of being 2N in 2 nuclei. Thus, measuring a simple intensity of DNA fluorescence is not enough to distinguish the two categories. I used a combination of DNA signal intensity and length of signal to distinguish between mononucleated cells in $G_0$ or $G_1$ (DNA intensity signal equal to 2N in short, thin peak), mononucleated cell in $G_2$ (DNA intensity equal to 4N in taller, thin peak), and binucleated cells (DNA intensity equal to 4N in two smaller, wide peaks). However, technical problems, not yet solved, made this method unsuccessful. First, staining for nuclear DNA also stains for mitochondrial DNA, and myocytes have a lot of mitochondria. This results in a high background fluorescence signal. Second, for the sorting to work each myocyte must line up along its long axis as it passes through the flow cytometer. Flow cytometry work is designed to work best on relatively round cells, but cardiomyocytes are cigar shaped making flow cytometry even more difficult to use.

Once successfully sorted, live cells could be cultured on separate plates. Starting with a more homogeneous population would reduce the variability in measures such as BrdU uptake. We have seen, but cannot explain, BrdU positive binucleated cells in culture. Experiments with a pure population of binucleated cardiomyocytes could be used to determine if they truly are "terminally differentiated." Our assumption is that BrdU positive binucleated cardiomyocytes represent mononucleated cells that have

become binucleated during the experiment, but we cannot rule out binucleated cells entering S-phase under our current culture conditions.

In sorted groups, it would be possible to study other signaling questions. Important but subtle differences in the way PE stimulates ERK in mononucleated cells compared to binucleated cells may be found. Or additional cell signaling cascades could be found that are only stimulated in one population but not the other. This could be true for many other agonists such as ET-1, Ang II, and IGF-1.

**The Conclusion's Conclusion:**

Programming of the fetal cardiovascular system may be a significant contributor to adult-onset heart disease; understanding the factors that contribute to programming may lead to novel therapies for heart disease long before symptoms appear. Because of the central role played by IGF-1 in the programming process, the effect IGF-1 has on fetal heart development was investigated in this thesis. The link between IGF-1 and programming is multi-layered. Low IGF-1 levels are associated with low birth weights and I have shown that increased IGF-1 plasma levels cause an increase in cardiomyocyte proliferation. Thus, I would hypothesize that babies born small at term with lower IGF-1 levels may be at an increased risk for heart disease due to a smaller cardiomyocyte endowment and fewer coronary capillaries from birth throughout life. After birth, IGF-1 may switch from a hyperplastic to hypertrophic agent in the heart after further maturation of existing cardiomyocytes. Thus, later increases of IGF-1 levels in childhood due to IGF-1 resistance (Fall *et al.*, 1995) may further exacerbate the problem. The process of "catch up" growth may lead to a pathological hypertrophy of the fewer cardiomyocytes

that are present. The studies presented in this thesis may offer a foundation for work that will further our understanding of the role of the prenatal environment and cardiovascular disease in adults.

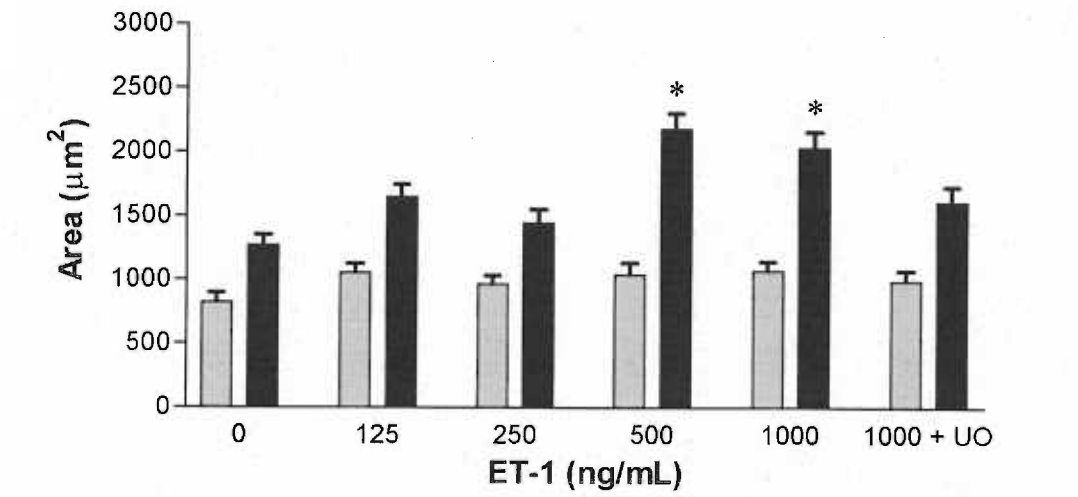


**Figure 6-1: ET-1 Induced Hypertrophy**

Increasing doses of ET-1 increase binucleated LV cardiomyocyte size (black bars) but not LV mononucleated cardiomyocytes (gray bars). ET-1 induced hypertrophy is blocked by 10μM UO126 at the highest dose given. Values are shown as mean ± standard error for 50 myocytes from a single culture experiment. *$p<0.05$ compared to serum free conditions (0ng/mL ET-1), n=50, ANOVA.

# References

Adi S, Bin-Abbas B, Wu NY & Rosenthal SM. (2002). Early stimulation and late inhibition of extracellular signal-regulated kinase 1/2 phosphorylation by IGF-I: a potential mechanism mediating the switch in IGF-I action on skeletal muscle cell differentiation. *Endocrinology* 143, 511-516.

Anversa P & Kajstura J. (1998). Ventricular myocytes are not terminally differentiated in the adult mammalian heart. *Circ.Res.* 83, 1-14.

Aoki H, Richmond M, Izumo S & Sadoshima J. (2000). Specific role of the extracellular signal-regulated kinase pathway in angiotensin II-induced cardiac hypertrophy in vitro. *Biochem.J.* 347 Pt 1, 275-284.

Baker J, Liu JP, Robertson EJ & Efstratiadis A. (1993). Role of insulin-like growth factors in embryonic and postnatal growth. *Cell* 75, 73-82.

Baker KM, Chernin MI, Wixson SK & Aceto JF. (1990). Renin-angiotensin system involvement in pressure-overload cardiac hypertrophy in rats. *Am.J.Physiol* 259, H324-H332.

Barbera A, Giraud GD, Reller MD, Maylie J, Morton MJ & Thornburg KL. (2000). Right ventricular systolic pressure load alters myocyte maturation in fetal sheep. *Am.J.Physiol Regul.Integr.Comp Physiol* 279, R1157-R1164.

Barker DJ. (2002). Fetal programming of coronary heart disease. *Trends Endocrinol.Metab* 13, 364-368.

Barker DJP. (1998). *Mothers, Babies and Health in Later Life*, 2nd ed. Churchill Livingstone, New York.

Barker DJ, Osmond C, Golding J, Kuh D & Wadsworth ME. (1989a). Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. *BMJ* 298, 564-567.

Barker DJ, Osmond C & Law CM. (1989b). The intrauterine and early postnatal origins of cardiovascular disease and chronic bronchitis. *J.Epidemiol.Community Health* 43, 237-240.

Barker DJ, Winter PD, Osmond C, Margetts B & Simmonds SJ. (1989c). Weight in infancy and death from ischaemic heart disease. *Lancet* 2, 577-580.

Barlucchi L, Leri A, Dostal DE, Fiordaliso F, Tada H, Hintze TH, Kajstura J, Nadal-Ginard B & Anversa P. (2001). Canine ventricular myocytes possess a renin-angiotensin system that is upregulated with heart failure. *Circ.Res.* 88, 298-304.

Baserga R & Rubin R. (1993). Cell cycle and growth control. *Crit Rev.Eukaryot.Gene Expr.* 3, 47-61.

Bassett NS, Breier BH, Hodgkinson SC, Davis SR, Henderson HV & Gluckman PD. (1990). Plasma clearance of radiolabelled IGF-1 in the late gestation ovine fetus. *J.Dev.Physiol* 14, 73-79.

Bastian SE, Walton PE, Wallace JC & Ballard FJ. (1993). Plasma clearance and tissue distribution of labelled insulin-like growth factor-I (IGF-I) and an analogue LR3IGF-I in pregnant rats. *J.Endocrinol.* 138, 327-336.

Belcheva MM & Coscia CJ. (2002). Diversity of G protein-coupled receptor signaling pathways to ERK/MAP kinase. *Neurosignals.* 11, 34-44.

Beltrami AP, Urbanek K, Kajstura J, Yan SM, Finato N, Bussani R, Nadal-Ginard B, Silvestri F, Leri A, Beltrami CA & Anversa P. (2001). Evidence that human cardiac myocytes divide after myocardial infarction. *N.Engl.J.Med.* 344, 1750-1757.

Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, Zlotchenko E, Scrimgeour A, Lawrence JC, Glass DJ & Yancopoulos GD. (2001). Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nat.Cell Biol.* 3, 1014-1019.

Booz GW & Baker KM (1996). Role of type 1 and type 2 angiotensin receptors in angiotensin II-induced cardiomyocyte hypertrophy. *Hypertension* 28, 635-640.

Brodsky S, Gurbanov K, Abassi Z, Hoffman A, Ruffolo RR, Feuerstein GZ & Winaver J. (1998). Effects of eprosartan on renal function and cardiac hypertrophy in rats with experimental heart failure. *Hypertension* 32, 746-752.

Buday L & Downward J. (1993a). Epidermal growth factor regulates p21ras through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor. *Cell* 73, 611-620.

Buday L & Downward J. (1993b). Epidermal growth factor regulates the exchange rate of guanine nucleotides on p21ras in fibroblasts. *Mol.Cell Biol.* 13, 1903-1910.

Bueno OF, De Windt LJ, Lim HW, Tymitz KM, Witt SA, Kimball TR & Molkentin JD. (2001). The dual-specificity phosphatase MKP-1 limits the cardiac hypertrophic response in vitro and in vivo. *Circ.Res.* 88, 88-96.

Bueno OF, De Windt LJ, Tymitz KM, Witt SA, Kimball TR, Klevitsky R, Hewett TE, Jones SP, Lefer DJ, Peng CF, Kitsis RN & Molkentin JD. (2000). The MEK1-ERK1/2 signaling pathway promotes compensated cardiac hypertrophy in transgenic mice. *EMBO J.* 19, 6341-6350.

Burrell JH, Hegarty BD, McMullen JR & Lumbers ER. (2001). Effects of gestation on ovine fetal and maternal angiotensin receptor subtypes in the heart and major blood vessels. *Exp.Physiol* 86, 71-82.

Castoria G, Migliaccio A, Bilancio A, Di Domenico M, de Falco A, Lombardi M, Fiorentino R, Varricchio L, Barone MV & Auricchio F. (2001). PI3-kinase in concert with Src promotes the S-phase entry of oestradiol-stimulated MCF-7 cells. *EMBO J.* 20, 6050-6059.

Clerk A, Pham FH, Fuller SJ, Sahai E, Aktories K, Marais R, Marshall C & Sugden PH. (2001). Regulation of mitogen-activated protein kinases in cardiac myocytes through the small G protein Rac1. *Mol.Cell Biol.* 21, 1173-1184.

Clubb FJ & Bishop SP. (1984). Formation of binucleated myocardial cells in the neonatal rat. An index for growth hypertrophy. *Lab Invest* 50, 571-577.

Clubb FJ, Penney DG, Baylerian MS & Bishop SP. (1986). Cardiomegaly due to myocyte hyperplasia in perinatal rats exposed to 200 ppm carbon monoxide. *J.Mol.Cell Cardiol.* 18, 477-486.

Condorelli G, Drusco A, Stassi G, Bellacosa A, Roncarati R, Iaccarino G, Russo MA, Gu Y, Dalton N, Chung C, Latronico MV, Napoli C, Sadoshima J, Croce CM & Ross J. (2002). Akt induces enhanced myocardial contractility and cell size in vivo in transgenic mice. *Proc Natl.Acad.Sci.U.S.A* 99, 12333-12338.

Coolican SA, Samuel DS, Ewton DZ, McWade FJ & Florini JR. (1997). The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signaling pathways. *J.Biol.Chem.* 272, 6653-6662.

Crackower MA, Oudit GY, Kozieradzki I, Sarao R, Sun H, Sasaki T, Hirsch E, Suzuki A, Shioi T, Irie-Sasaki J, Sah R, Cheng HY, Rybin VO, Lembo G, Fratta L, Oliveira-dos-Santos AJ, Benovic JL, Kahn CR, Izumo S, Steinberg SF, Wymann MP, Backx PH & Penninger JM. (2002). Regulation of myocardial contractility and cell size by distinct PI3K-PTEN signaling pathways. *Cell* 110, 737-749.

Delafontaine P. (1995). Insulin-like growth factor I and its binding proteins in the cardiovascular system. *Cardiovasc.Res.* 30, 825-834.

Dell'Italia LJ, Balcells E, Meng QC, Su X, Schultz D, Bishop SP, Machida N, Straeter-Knowlen IM, Hankes GH, Dillon R, Cartee RE & Oparil S. (1997). Volume-overload cardiac hypertrophy is unaffected by ACE inhibitor treatment in dogs. *Am.J.Physiol* 273, H961-H970.

Dell'Italia LJ, Meng QC, Balcells E, Straeter-Knowlen IM, Hankes GH, Dillon R, Cartee RE, Orr R, Bishop SP & Oparil S. (1995). Increased ACE and chymase-like activity in cardiac tissue of dogs with chronic mitral regurgitation. *Am.J.Physiol* 269, H2065-H2073.

Diehl JA, Cheng M, Roussel MF, & Sherr CJ. (1998). Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev.* 12, 3499-3511.

Dostal DE & Baker KM. (1992). Angiotensin II stimulation of left ventricular hypertrophy in adult rat heart. Mediation by the AT1 receptor. *Am.J.Hypertens.* 5, 276-280.

Dugourd C, Gervais M, Corvol P & Monnot C. (2003). Akt is a major downstream target of PI3-kinase involved in angiotensin II-induced proliferation. *Hypertension* 41, 882-890.

Dussaillant GR, Gonzalez H, Cespedes C & Jalil JE. (1996). Regression of left ventricular hypertrophy in experimental renovascular hypertension: diastolic dysfunction depends more on myocardial collagen than it does on myocardial mass. *J.Hypertens.* 14, 1117-1123.

Edwards LJ & McMillen IC. (2001). Maternal undernutrition increases arterial blood pressure in the sheep fetus during late gestation. *J.Physiol* 533, 561-570.

Egan SE, Giddings BW, Brooks MW, Buday L, Sizeland AM & Weinberg RA. (1993). Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. *Nature* 363, 45-51.

English J, Pearson G, Wilsbacher J, Swantek J, Karandikar M, Xu S & Cobb MH. (1999). New insights into the control of MAP kinase pathways. *Exp.Cell Res.* 253, 255-270.

Everett AD, Tufro-McReddie A, Fisher A & Gomez RA. (1994). Angiotensin receptor regulates cardiac hypertrophy and transforming growth factor-beta 1 expression. *Hypertension* 23, 587-592.

Fall CH, Pandit AN, Law CM, Yajnik CS, Clark PM, Breier B, Osmond C, Shiell AW, Gluckman PD & Barker DJ. (1995). Size at birth and plasma insulin-like growth factor-1 concentrations. *Arch.Dis.Child* 73, 287-293.

Feld S & Hirschberg R. (1996). Growth hormone, the insulin-like growth factor system, and the kidney. *Endocr.Rev.* 17, 423-480.

Fischer TA, Singh K, O'Hara DS, Kaye DM & Kelly RA. (1998). Role of AT1 and AT2 receptors in regulation of MAPKs and MKP-1 by ANG II in adult cardiac myocytes. *Am.J.Physiol* 275, H906-H916.

Fukuda K & Izumo S. (1998). Angiotensin II potentiates DNA synthesis in AT-1 transformed cardiomyocytes. *J.Mol.Cell Cardiol.* 30, 2069-2080.

Gallaher BW, Breier BH, Keven CL, Harding JE & Gluckman PD. (1998). Fetal programming of insulin-like growth factor (IGF)-I and IGF-binding protein-3: evidence for an altered response to undernutrition in late gestation following exposure to periconceptual undernutrition in the sheep. *J.Endocrinol.* 159, 501-508.

Geenen DL, Malhotra A & Scheuer J. (1993). Angiotensin II increases cardiac protein synthesis in adult rat heart. *Am.J.Physiol* 265, H238-H243.

Giussani DA, Forhead AJ, Gardner DS, Fletcher AJ, Allen WR & Fowden AL. (2003). Postnatal cardiovascular function after manipulation of fetal growth by embryo transfer in the horse. *J.Physiol* 547, 67-76.

Graff JR, Konicek BW, McNulty AM, Wang Z, Houck K, Allen S, Paul JD, Hbaiu A, Goode RG, Sandusky GE, Vessella RL & Neubauer BL. (2000). Increased AKT activity contributes to prostate cancer progression by dramatically accelerating prostate tumor growth and diminishing p27Kip1 expression. *J.Biol.Chem.* 275, 24500-24505.

Gray MO, Long CS, Kalinyak JE, Li HT & Karliner JS. (1998). Angiotensin II stimulates cardiac myocyte hypertrophy via paracrine release of TGF-beta 1 and endothelin-1 from fibroblasts. *Cardiovasc.Res.* 40, 352-363.

Green LR, Kawagoe Y, Hill DJ, Richardson BS & Han VK. (2000). The effect of intermittent umbilical cord occlusion on insulin-like growth factors and their binding proteins in preterm and near-term ovine fetuses. *J.Endocrinol.* 166, 565-577.

Grimm D, Kromer EP, Bocker W, Bruckschlegel G, Holmer SR, Riegger GA & Schunkert H. (1998). Regulation of extracellular matrix proteins in pressure-overload cardiac hypertrophy: effects of angiotensin converting enzyme inhibition. *J.Hypertens.* 16, 1345-1355.

Han VK & Hill DJ. (1994). Growth factors in fetal growth. In *Textbook of Fetal Physiology*, eds. Thorburn GD & Harding R, pp. 48-69. Oxford University Press Inc., New York.

Harding J. (2001). The nutritional basis of the fetal origins of adult disease. *Int.J.Epidemiol.* 30, 15-23.

Harding JE, Liu L, Evans PC & Gluckman PD. (1994). Insulin-like growth factor 1 alters feto-placental protein and carbohydrate metabolism in fetal sheep. *Endocrinology* 134, 1509-1514.

Hirsch E, Katanaev VL, Garlanda C, Azzolino O, Pirola L, Silengo L, Sozzani S, Mantovani A, Altruda F & Wymann MP. (2000). Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. *Science* 287, 1049-1053.

Holm S. (1979). A simple sequentially rejective multiple test procedure. *Scandinavian Journal of Statistics* 6, 65-70.

Holt RI. (2002). Fetal programming of the growth hormone-insulin-like growth factor axis. *Trends Endocrinol.Metab* 13, 392-397.

Hong F, Kwon SJ, Jhun BS, Kim SS, Ha J, Kim SJ, Sohn NW, Kang C & Kang I. (2001). Insulin-like growth factor-1 protects H9c2 cardiac myoblasts from oxidative stress-induced apoptosis via phosphatidylinositol 3-kinase and extracellular signal-regulated kinase pathways. *Life Sci.* 68, 1095-1105.

Ihaka R & Gentleman R. (1999). R: A language for data analysis and graphics. *Journal of Computational and Graphical Statistics* 5, 229-314.

Ingelfinger JR, Jung F, Diamant D, Haveran L, Lee E, Brem A & Tang SS. (1999). Rat proximal tubule cell line transformed with origin-defective SV40 DNA: autocrine ANG II feedback. *Am.J.Physiol* 276, F218-F227.

Ingelfinger JR & Woods LL. (2002). Perinatal programming, renal development, and adult renal function. *Am.J.Hypertens.* 15, 46S-49S.

Ito H, Hiroe M, Hirata Y, Tsujino M, Adachi S, Shichiri M, Koike A, Nogami A & Marumo F. (1993). Insulin-like growth factor-I induces hypertrophy with enhanced expression of muscle specific genes in cultured rat cardiomyocytes. *Circulation* 87, 1715-1721.

Izumi Y, Kim S, Zhan Y, Namba M, Yasumoto H & Iwao H. (2000). Important role of angiotensin II-mediated c-Jun NH(2)-terminal kinase activation in cardiac hypertrophy in hypertensive rats. *Hypertension* 36, 511-516.

Jalil JE, Janicki JS, Pick R & Weber KT. (1991). Coronary vascular remodeling and myocardial fibrosis in the rat with renovascular hypertension. Response to captopril. *Am.J.Hypertens.* 4, 51-55.

Jensen EC, Harding JE, Bauer MK & Gluckman PD. (1999). Metabolic effects of IGF-I in the growth retarded fetal sheep. *J.Endocrinol.* 161, 485-494.

Juul A, Scheike T, Davidsen M, Gyllenborg J & Jorgensen T. (2002). Low serum insulin-like growth factor I is associated with increased risk of ischemic heart disease: a population-based case-control study. *Circulation* 106, 939-944.

Kajstura J, Leri A, Finato N, Di Loreto C, Beltrami CA & Anversa P. (1998). Myocyte proliferation in end-stage cardiac failure in humans. *Proc Natl.Acad.Sci.U.S.A* 95, 8801-8805.

Kaliman P, Canicio J, Shepherd PR, Beeton CA, Testar X, Palacin M & Zorzano A. (1998). Insulin-like growth factors require phosphatidylinositol 3-kinase to signal myogenesis: dominant negative p85 expression blocks differentiation of L6E9 muscle cells. *Mol.Endocrinol.* 12, 66-77.

Kaliman P, Vinals F, Testar X, Palacin M & Zorzano A. (1996). Phosphatidylinositol 3-kinase inhibitors block differentiation of skeletal muscle cells. *J.Biol.Chem.* 271, 19146-19151.

Kellerman S, Moore JA, Zierhut W, Zimmer HG, Campbell J & Gerdes AM. (1992). Nuclear DNA content and nucleation patterns in rat cardiac myocytes from different models of cardiac hypertrophy. *J.Mol.Cell Cardiol.* 24, 497-505.

Kim JM, Yoon MY, Kim J, Kim SS, Kang I, Ha J & Kim SS. (1999). Phosphatidylinositol 3-kinase regulates differentiation of H9c2 cardiomyoblasts mainly through the protein kinase B/Akt-independent pathway. *Arch.Biochem.Biophys.* 367, 67-73.

Kim NN, Villarreal FJ, Printz MP, Lee AA & Dillmann WH. (1995). Trophic effects of angiotensin II on neonatal rat cardiac myocytes are mediated by cardiac fibroblasts. *Am.J.Physiol* 269, E426-E437.

Kurihara S, Hakuno F & Takahashi S. (2000). Insulin-like growth factor-I-dependent signal transduction pathways leading to the induction of cell growth and differentiation of human neuroblastoma cell line SH-SY5Y: the roles of MAP kinase pathway and PI 3-kinase pathway. *Endocr.J.* 47, 739-751.

Langley SC & Jackson AA. (1994). Increased systolic blood pressure in adult rats induced by fetal exposure to maternal low protein diets. *Clin.Sci.(Lond)* 86, 217-222.

Lassarre C, Hardouin S, Daffos F, Forestier F, Frankenne F & Binoux M. (1991). Serum insulin-like growth factors and insulin-like growth factor binding proteins in the human fetus. Relationships with growth in normal subjects and in subjects with intrauterine growth retardation. *Pediatr.Res.* 29, 219-225.

Lavandero S, Foncea R, Perez V & Sapag-Hagar M. (1998). Effect of inhibitors of signal transduction on IGF-1-induced protein synthesis associated with hypertrophy in cultured neonatal rat ventricular myocytes. *FEBS Lett.* 422, 193-196.

Lazou A, Sugden PH & Clerk A. (1998). Activation of mitogen-activated protein kinases (p38-MAPKs, SAPKs/JNKs and ERKs) by the G-protein-coupled receptor agonist phenylephrine in the perfused rat heart. *Biochem.J.* 332 ( Pt 2), 459-465.

Leri A, Liu Y, Claudio PP, Kajstura J, Wang X, Wang S, Kang P, Malhotra A & Anversa P. (1999a). Insulin-like growth factor-1 induces Mdm2 and down-regulates p53, attenuating the myocyte renin-angiotensin system and stretch-mediated apoptosis. *Am.J.Pathol.* 154, 567-580.

Leri A, Liu Y, Wang X, Kajstura J, Malhotra A, Meggs LG & Anversa P. (1999b). Overexpression of insulin-like growth factor-1 attenuates the myocyte renin-angiotensin system in transgenic mice. *Circ.Res.* 84, 752-762.

Li F, Wang X, Bunger PC & Gerdes AM. (1997a). Formation of binucleated cardiac myocytes in rat heart: I. Role of actin-myosin contractile ring. *J.Mol.Cell Cardiol.* 29, 1541-1551.

Li F, Wang X & Gerdes AM. (1997b). Formation of binucleated cardiac myocytes in rat heart: II. Cytoskeletal organisation. *J.Mol.Cell Cardiol.* 29, 1553-1565.

Li F, Wang X, Capasso JM & Gerdes AM. (1996). Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development. *J.Mol.Cell Cardiol.* 28, 1737-1746.

Li Z, Jiang H, Xie W, Zhang Z, Smrcka AV & Wu D. (2000). Roles of PLC-beta2 and -beta3 and PI3Kgamma in chemoattractant-mediated signal transduction. *Science* 287, 1046-1049.

Linz W, Scholkens BA & Ganten D. (1989). Converting enzyme inhibition specifically prevents the development and induces regression of cardiac hypertrophy in rats. *Clin.Exp.Hypertens.A* 11, 1325-1350.

Liu J & Rose J. (2003). Effects of Insulin and Insulin-like Growth Factor-I on Renin Gene Expression in the Renal Cortical Cells of Ovine Fetuses. *Arch.Physiol Biochem.* 111, 70-76.

Liu JP, Baker J, Perkins AS, Robertson EJ & Efstratiadis A. (1993). Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). *Cell* 75, 59-72.

Liu T, Lai H, Wu W, Chinn S & Wang PH. (2001a). Developing a strategy to define the effects of insulin-like growth factor-1 on gene expression profile in cardiomyocytes. *Circ.Res.* 88, 1231-1238.

Liu W, Liu Y & Lowe JW. (2001b). The role of phosphatidylinositol 3-kinase and the mitogen-activated protein kinases in insulin-like growth factor-I-mediated effects in vascular endothelial cells. *Endocrinology* 142, 1710-1719.

Liu Y, Leri A, Li B, Wang X, Cheng W, Kajstura J & Anversa P. (1998). Angiotensin II stimulation in vitro induces hypertrophy of normal and postinfarcted ventricular myocytes. *Circ.Res.* 82, 1145-1159.

Lok F, Owens JA, Mundy L, Robinson JS & Owens PC. (1996). Insulin-like growth factor I promotes growth selectively in fetal sheep in late gestation. *Am.J.Physiol* 270, R1148-R1155.

Malhotra R, Sadoshima J, Brosius FC & Izumo S. (1999). Mechanical stretch and angiotensin II differentially upregulate the renin-angiotensin system in cardiac myocytes In vitro. *Circ.Res.* 85, 137-146.

Marsh AC, Gibson KJ, Wu J, Owens PC, Owens JA & Lumbers ER. (2001a). Chronic effect of insulin-like growth factor I on renin synthesis, secretion, and renal function in fetal sheep. *Am.J.Physiol Regul.Integr.Comp Physiol* 281, R318-R326.

Marsh AC, Gibson KJ, Wu J, Owens PC, Owens JA & Lumbers ER. (2001b). Insulin-like growth factor I alters renal function and stimulates renin secretion in late gestation fetal sheep. *J.Physiol* 530, 253-262.

Mehrhof FB, Muller FU, Bergmann MW, Li P, Wang Y, Schmitz W, Dietz R & von Harsdorf R. (2001). In cardiomyocyte hypoxia, insulin-like growth factor-I-induced antiapoptotic signaling requires phosphatidylinositol-3-OH-kinase-dependent and mitogen-activated protein kinase-dependent activation of the transcription factor cAMP response element-binding protein. *Circulation* 104, 2088-2094.

Miyata S & Haneda T. (1994). Hypertrophic growth of cultured neonatal rat heart cells mediated by type 1 angiotensin II receptor. *Am.J.Physiol* 266, H2443-H2451.

Modesti PA, Vanni S, Bertolozzi I, Cecioni I, Polidori G, Paniccia R, Bandinelli B, Perna A, Liguori P, Boddi M, Galanti G & Serneri GG. (2000). Early sequence of cardiac adaptations and growth factor formation in pressure- and volume-overload hypertrophy. *Am.J.Physiol Heart Circ.Physiol* 279, H976-H985.

Moelling K, Schad K, Bosse M, Zimmermann S & Schweneker M. (2002). Regulation of Raf-Akt Cross-talk. *J.Biol.Chem.* 277, 31099-31106.

Moritz KM, Dodic M & Wintour EM. (2003). Kidney development and the fetal programming of adult disease. *Bioessays* 25, 212-220.

Moritz KM, Johnson K, Douglas-Denton R, Wintour EM & Dodic M. (2002). Maternal glucocorticoid treatment programs alterations in the renin-angiotensin system of the ovine fetal kidney. *Endocrinology* 143, 4455-4463.

Mueller C, Baudler S, Welzel H, Bohm M & Nickenig G. (2002). Identification of a novel redox-sensitive gene, Id3, which mediates angiotensin II-induced cell growth. *Circulation* 105, 2423-2428.

Muscella A, Greco S, Elia MG, Storelli C & Marsigliante S. (2002). Angiotensin II stimulation of Na+/K+ATPase activity and cell growth by calcium-independent pathway in MCF-7 breast cancer cells. *J.Endocrinol.* 173, 315-323.

Nadal-Ginard B, Kajstura J, Leri A & Anversa P. (2003). Myocyte death, growth, and regeneration in cardiac hypertrophy and failure. *Circ.Res.* 92, 139-150.

Ogawa T, Linz W, Stevenson M, Bruneau BG, Kuroski de Bold ML, Chen JH, Eid H, Scholkens BA & de Bold AJ. (1996). Evidence for load-dependent and load-independent determinants of cardiac natriuretic peptide production. *Circulation* 93, 2059-2067.

Oliver MH, Breier BH, Gluckman PD & Harding JE. (2002). Birth Weight Rather Than Maternal Nutrition Influences Glucose Tolerance, Blood Pressure, and IGF-I Levels in Sheep. *Pediatr.Res.* 52, 516-524.

Oliver MH, Harding JE, Breier BH, Evans PC & Gluckman PD. (1993). Glucose but not a mixed amino acid infusion regulates plasma insulin-like growth factor-I concentrations in fetal sheep. *Pediatr.Res.* 34, 62-65.

Oliver MH, Harding JE, Breier BH & Gluckman PD. (1996). Fetal insulin-like growth factor (IGF)-I and IGF-II are regulated differently by glucose or insulin in the sheep fetus. *Reprod.Fertil.Dev.* 8, 167-172.

Oparil S, Bishop SP & Clubb FJ. (1984). Myocardial cell hypertrophy or hyperplasia. *Hypertension* 6, III38-III43.

Osgerby JC, Wathes DC, Howard D & Gadd TS. (2002). The effect of maternal undernutrition on ovine fetal growth. *J.Endocrinol.* 173, 131-141.

Pardee AB. (1989). G1 events and regulation of cell proliferation. *Science* 246, 603-608.

Pene F, Claessens YE, Muller O, Viguie F, Mayeux P, Dreyfus F, Lacombe C & Bouscary D. (2002). Role of the phosphatidylinositol 3-kinase/Akt and mTOR/P70S6-kinase pathways in the proliferation and apoptosis in multiple myeloma. *Oncogene* 21, 6587-6597.

Penheiro JC & Baudler S. (2000). *Mixed-effects models in S and S-Plus* Springer, New York.

Perry GJ, Wei CC, Hankes GH, Dillon SR, Rynders P, Mukherjee R, Spinale FG & Dell'Italia LJ. (2002). Angiotensin II receptor blockade does not improve left ventricular function and remodeling in subacute mitral regurgitation in the dog. *J.Am.Coll.Cardiol.* 39, 1374-1379.

Phillips D. (2002). Endocrine programming and fetal origins of adult disease. *Trends Endocrinol.Metab* 13, 363.

Quaini F, Urbanek K, Beltrami AP, Finato N, Beltrami CA, Nadal-Ginard B, Kajstura J, Leri A & Anversa P. (2002). Chimerism of the transplanted heart. *N.Engl.J.Med.* 346, 5-15.

Quinn LS, Steinmetz B, Maas A, Ong L & Kaleko M. (1994). Type-1 insulin-like growth factor receptor overexpression produces dual effects on myoblast proliferation and differentiation. *J.Cell Physiol* 159, 387-398.

Reik W, Constancia M, Fowden A, Anderson N, Dean W, Ferguson-Smith A, Tycko B & Sibley C. (2003). Regulation of supply and demand for maternal nutrients in mammals by imprinted genes. *J.Physiol* 547, 35-44.

Reiss K, Cheng W, Ferber A, Kajstura J, Li P, Li B, Olivetti G, Homcy CJ, Baserga R & Anversa P. (1996). Overexpression of insulin-like growth factor-1 in the heart is coupled with myocyte proliferation in transgenic mice. *Proc.Natl.Acad.Sci.U.S.A* 93, 8630-8635.

Rommel C, Bodine SC, Clarke BA, Rossman R, Nunez L, Stitt TN, Yancopoulos GD & Glass DJ. (2001). Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. *Nat.Cell Biol.* 3, 1009-1013.

Rommel C, Clarke BA, Zimmermann S, Nunez L, Rossman R, Reid K, Moelling K, Yancopoulos GD & Glass DJ. (1999). Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt. *Science* 286, 1738-1741.

Rossi F, Ferraresi A, Romagni P, Silvestroni L & Santiemma V. (2002). Angiotensin II stimulates contraction and growth of testicular peritubular myoid cells in vitro. *Endocrinology* 143, 3096-3104.

Rossig L, Jadidi AS, Urbich C, Badorff C, Zeiher AM & Dimmeler S. (2001). Akt-dependent phosphorylation of p21(Cip1) regulates PCNA binding and proliferation of endothelial cells. *Mol.Cell Biol.* 21, 5644-5657.

Ruzicka M, Yuan B & Leenen FH. (1994). Effects of enalapril versus losartan on regression of volume overload-induced cardiac hypertrophy in rats. *Circulation* 90, 484-491.

Sadoshima J, Aoki H & Izumo S. (1997). Angiotensin II and serum differentially regulate expression of cyclins, activity of cyclin-dependent kinases, and phosphorylation of retinoblastoma gene product in neonatal cardiac myocytes. *Circ.Res.* 80, 228-241.

Sadoshima J & Izumo S. (1996). The heterotrimeric G q protein-coupled angiotensin II receptor activates p21 ras via the tyrosine kinase-Shc-Grb2-Sos pathway in cardiac myocytes. *EMBO J.* 15, 775-787.

Sadoshima J, Qiu Z, Morgan JP & Izumo S. (1995). Angiotensin II and other hypertrophic stimuli mediated by G protein-coupled receptors activate tyrosine kinase, mitogen-activated protein kinase, and 90-kD S6 kinase in cardiac myocytes. The critical role of Ca(2+)-dependent signaling. *Circ.Res.* 76, 1-15.

Sadoshima J & Izumo S. (1993a). Mechanical stretch rapidly activates multiple signal transduction pathways in cardiac myocytes: potential involvement of an autocrine/paracrine mechanism. *EMBO J.* 12, 1681-1692.

Sadoshima J & Izumo S. (1993b). Molecular characterization of angiotensin II--induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts. Critical role of the AT1 receptor subtype. *Circ.Res.* 73, 413-423.

Sadoshima J, Takahashi T, Jahn L & Izumo S. (1992). Roles of mechano-sensitive ion channels, cytoskeleton, and contractile activity in stretch-induced immediate-early gene expression and hypertrophy of cardiac myocytes. *Proc Natl.Acad.Sci.U.S.A* 89, 9905-9909.

Sasaki T, Irie-Sasaki J, Jones RG, Oliveira-dos-Santos AJ, Stanford WL, Bolon B, Wakeham A, Itie A, Bouchard D, Kozieradzki I, Joza N, Mak TW, Ohashi PS, Suzuki A & Penninger JM. (2000). Function of PI3Kgamma in thymocyte development, T cell activation, and neutrophil migration. *Science* 287, 1040-1046.

Schoenfeld JR, Vasser M, Jhurani P, Ng P, Hunter JJ, Ross J, Chien KR & Lowe DG. (1998). Distinct molecular phenotypes in murine cardiac muscle development, growth, and hypertrophy. *J.Mol.Cell Cardiol.* 30, 2269-2280.

Scholkens BA, Linz W & Martorana PA. (1991). Experimental cardiovascular benefits of angiotensin-converting enzyme inhibitors: beyond blood pressure reduction. *J.Cardiovasc.Pharmacol.* 18 Suppl 2, S26-S30.

Schorb W, Booz GW, Dostal DE, Conrad KM, Chang KC & Baker KM. (1993). Angiotensin II is mitogenic in neonatal rat cardiac fibroblasts. *Circ.Res.* 72, 1245-1254.

Schunkert H, Sadoshima J, Cornelius T, Kagaya Y, Weinberg EO, Izumo S, Riegger G & Lorell BH. (1995). Angiotensin II-induced growth responses in isolated adult rat hearts. Evidence for load-independent induction of cardiac protein synthesis by angiotensin II. *Circ.Res.* 76, 489-497.

Segar JL, Dalshaug GB, Bedell KA, Smith OM & Scholz TD. (2001). Angiotensin II in cardiac pressure-overload hypertrophy in fetal sheep. *Am.J.Physiol Regul.Integr.Comp Physiol* 281, R2037-R2047.

Segar JL, Scholz TD, Bedell KA, Smith OM, Huss DJ & Guillery EN. (1997). Angiotensin AT1 receptor blockade fails to attenuate pressure-overload cardiac hypertrophy in fetal sheep. *Am.J.Physiol* 273, R1501-R1508.

Sekiguchi K, Yokoyama T, Kurabayashi M, Okajima F & Nagai R. (1999). Sphingosylphosphorylcholine induces a hypertrophic growth response through the mitogen-activated protein kinase signaling cascade in rat neonatal cardiac myocytes. *Circ.Res.* 85, 1000-1008.

Shioi T, Kang PM, Douglas PS, Hampe J, Yballe CM, Lawitts J, Cantley LC & Izumo S. (2000). The conserved phosphoinositide 3-kinase pathway determines heart size in mice. *EMBO J.* 19, 2537-2548.

Shioi T, McMullen JR, Kang PM, Douglas PS, Obata T, Franke TF, Cantley LC & Izumo S. (2002). Akt/protein kinase B promotes organ growth in transgenic mice. *Mol.Cell Biol.* 22, 2799-2809.

Silha JV & Murphy LJ. (2002). Insights from insulin-like growth factor binding protein transgenic mice. *Endocrinology* 143, 3711-3714.

Sleight P. (2000). The HOPE Study (Heart Outcomes Prevention Evaluation). *J.Renin.Angiotensin.Aldosterone.Syst.* 1, 18-20.

Smolich JJ, Walker AM, Campbell GR & Adamson TM. (1989). Left and right ventricular myocardial morphometry in fetal, neonatal, and adult sheep. *Am.J.Physiol* 257, H1-H9.

Soonpaa MH, Kim KK, Pajak L, Franklin M & Field LJ. (1996). Cardiomyocyte DNA synthesis and binucleation during murine development. *Am.J.Physiol* 271, H2183-H2189.

Stewart CE, James PL, Fant ME & Rotwein P. (1996). Overexpression of insulin-like growth factor-II induces accelerated myoblast differentiation. *J.Cell Physiol* 169, 23-32.

Stewart CE & Rotwein P. (1996). Growth, differentiation, and survival: multiple physiological functions for insulin-like growth factors. *Physiol Rev.* 76, 1005-1026.

Stork PJ & Schmitt JM. (2002). Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. *Trends Cell Biol.* 12, 258-266.

Sundgren NC, Giraud GD, Stork PJ, Maylie JG & Thornburg KL. (2003). Angiotensin II stimulates hyperplasia but not hypertrophy in immature ovine cardiomyocytes. *J.Physiol* 548, 881-891.

Susic D, Nunez E, Frohlich ED & Prakash O. (1996). Angiotensin II increases left ventricular mass without affecting myosin isoform mRNAs. *Hypertension* 28, 265-268.

Takahashi K, Azuma M, Taira K, Baba A, Yamamoto I, Schaffer SW & Azuma J. (1997). Effect of taurine on angiotensin II-induced hypertrophy of neonatal rat cardiac cells. *J.Cardiovasc.Pharmacol.* 30, 725-730.

Thienelt CD, Weinberg EO, Bartunek J & Lorell BH. (1997). Load-induced growth responses in isolated adult rat hearts. Role of the AT1 receptor. *Circulation* 95, 2677-2683.

Thornburg KL & Morton MJ. (1983). Filling and arterial pressures as determinants of RV stroke volume in the sheep fetus. *Am.J.Physiol* 244, H656-H663.

Thurmann PA, Kenedi P, Schmidt A, Harder S & Rietbrock N. (1998). Influence of the angiotensin II antagonist valsartan on left ventricular hypertrophy in patients with essential hypertension. *Circulation* 98, 2037-2042.

Tomas FM, Knowles SE, Chandler CS, Francis GL, Owens PC & Ballard FJ. (1993). Anabolic effects of insulin-like growth factor-I (IGF-I) and an IGF-I variant in normal female rats. *J.Endocrinol.* 137, 413-421.

Ueyama T, Kawashima S, Sakoda T, Rikitake Y, Ishida T, Kawai M, Yamashita T, Ishido S, Hotta H & Yokoyama M. (2000). Requirement of activation of the extracellular signal-regulated kinase cascade in myocardial cell hypertrophy. *J.Mol.Cell Cardiol.* 32, 947-960.

Vemuri GS & Rittenhouse SE. (1994). Wortmannin inhibits serum-induced activation of phosphoinositide 3-kinase and proliferation of CHRF-288 cells. *Biochem.Biophys.Res.Commun.* 202, 1619-1623.

Vickers MH, Breier BH, Cutfield WS, Hofman PL & Gluckman PD. (2000). Fetal origins of hyperphagia, obesity, and hypertension and postnatal amplification by hypercaloric nutrition. *Am.J.Physiol Endocrinol.Metab* 279, E83-E87.

Vickers MH, Ikenasio BA & Breier BH. (2001). IGF-I treatment reduces hyperphagia, obesity, and hypertension in metabolic disorders induced by fetal programming. *Endocrinology* 142, 3964-3973.

Vivanco I & Sawyers CL. (2002). The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat.Rev.Cancer* 2, 489-501.

Wang L & Proud CG. (2002). Ras/Erk signaling is essential for activation of protein synthesis by Gq protein-coupled receptor agonists in adult cardiomyocytes. *Circ.Res.* 91, 821-829.

Watanabe T, Pakala R, Katagiri T & Benedict CR. (2001). Serotonin potentiates angiotensin II--induced vascular smooth muscle cell proliferation. *Atherosclerosis* 159, 269-279.

Wei C, Cardarelli MG, Downing SW & McLaughlin JS. (2000). The effect of angiotensin II on mitogen-activated protein kinase in human cardiomyocytes. *J.Renin.Angiotensin.Aldosterone.Syst.* 1, 379-384.

Weyman CM & Wolfman A. (1998). Mitogen-activated protein kinase kinase (MEK) activity is required for inhibition of skeletal muscle differentiation by insulin-like growth factor 1 or fibroblast growth factor 2. *Endocrinology* 139, 1794-1800.

Whorwood CB, Firth KM, Budge H & Symonds ME. (2001). Maternal undernutrition during early to midgestation programs tissue-specific alterations in the expression of the glucocorticoid receptor, 11beta-hydroxysteroid dehydrogenase isoforms, and type 1 angiotensin ii receptor in neonatal sheep. *Endocrinology* 142, 2854-2864.

Wollert KC & Drexler H. (1999). The renin-angiotensin system and experimental heart failure. *Cardiovasc.Res.* 43, 838-849.

Woods LL, Ingelfinger JR, Nyengaard JR & Rasch R. (2001a). Maternal protein restriction suppresses the newborn renin-angiotensin system and programs adult hypertension in rats. *Pediatr.Res.* 49, 460-467.

Woods LL & Rasch R. (1998). Perinatal ANG II programs adult blood pressure, glomerular number, and renal function in rats. *Am.J.Physiol* 275, R1593-R1599.

Woods LL, Weeks DA & Rasch R. (2001b). Hypertension after neonatal uninephrectomy in rats precedes glomerular damage. *Hypertension* 38, 337-342.

Yu CF, Liu ZX & Cantley LG. (2002). ERK negatively regulates the epidermal growth factor-mediated interaction of Gab1 and the phosphatidylinositol 3-kinase. *J.Biol.Chem.* 277, 19382-19388.

Yue TL, Gu JL, Wang C, Reith AD, Lee JC, Mirabile RC, Kreutz R, Wang Y, Maleeff B, Parsons AA & Ohlstein EH. (2000). Extracellular signal-regulated kinase plays an essential role in hypertrophic agonists, endothelin-1 and phenylephrine-induced cardiomyocyte hypertrophy. *J.Biol.Chem.* 275, 37895-37901.

Zhang DY, Lumbers ER, Simonetta G, Wu JJ, Owens JA, Robinson JS & McMillen IC. (2000). Effects of placental insufficiency on the ovine fetal renin-angiotensin system. *Exp.Physiol* 85, 79-84.

Zhang L. (2003). Prenatal Hypoxia: Does the Adult Heart Care? *J.Soc.Gynecol.Investig.* 10, Society for Gynecologic Investigation 50th Annual Meeting.

Zimmermann S & Moelling K. (1999). Phosphorylation and regulation of Raf by Akt (protein kinase B). *Science* 286, 1741-1744.