Molecular and Metabolomic Mechanisms Affecting Growth in Children Born Small

A thesis submitted to The University of Manchester for the degree of Doctor of Philosophy in the Faculty of Medical and Human Sciences

2013

Imogen Butcher

School of Medicine
Chapter 1  Introduction ..................................................................................................................13
  1.1 Introduction .........................................................................................................................14
  1.2 Causes of SGA ....................................................................................................................14
    1.2.1 Nutritional status of the mother ......................................................................................16
    1.2.2 Smoking, alcohol and drug use during pregnancy .........................................................17
    1.2.3 Placental issues ................................................................................................................18
    1.2.4 Fetal factors .....................................................................................................................19
  1.3 Catch-up growth ...................................................................................................................20
  1.4 Post-natal growth ................................................................................................................20
    1.4.1 Growth Hormone ...........................................................................................................22
    1.4.2 Growth hormone signalling ............................................................................................24
  1.5 Growth hormone signalling regulation ..............................................................................26
  1.6 IGF-I, Insulin-like growth factor I .......................................................................................27
    1.6.1 The IGF-I receptor ..........................................................................................................27
    1.6.2 The phosphatidylinositol 3-kinase (PI3-K) pathway .......................................................28
    1.6.3 The MAPK pathway ........................................................................................................30
  1.7 Aberrations in GH and IGF-I signalling ..............................................................................30
    1.7.1 Growth hormone signalling aberrations ..........................................................................31
    1.7.2 Aberrations in IGF-I signalling ......................................................................................33
  1.8 Treatment of short stature ..................................................................................................33
  1.9 Metabolic consequences of being born SGA .....................................................................34
  1.10 Metabolomics ...................................................................................................................36
  1.11 Summary ............................................................................................................................39

Chapter 2  Materials and methods ..............................................................................................40
  2.1 Participants ..........................................................................................................................41
2.2 Biochemical assays ........................................................................................................42
  2.2.1 Serum IGF-I measurements .....................................................................................44
  2.2.2 Fasting Glucose measurements ..............................................................................45
  2.2.3 Insulin measurements ............................................................................................45
  2.2.4 Adiponectin measurements ....................................................................................45
2.3 Cell culture ....................................................................................................................46
  2.3.1 Establishing fibroblasts from skin biopsies .............................................................46
  2.3.2 Establishing a fibroblast cell line ............................................................................46
  2.3.3 Characterising fibroblasts .......................................................................................47
  2.3.4 Maintaining fibroblasts in culture ...........................................................................49
  2.3.5 Cell counting ...........................................................................................................49
  2.3.6 Cell lysis experiments .............................................................................................49
  2.3.7 Protein estimation ..................................................................................................51
2.4 Characterisation of cell proliferation .............................................................................51
  2.4.1 Cell counting experiments .....................................................................................51
  2.4.2 Bromodeoxyuridine (BrdU) incorporation .............................................................51
  2.4.3 Growth factor stimulation of proliferation .............................................................52
2.5 Characterisation of apoptosis .........................................................................................53
  2.5.1 Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling (TUNEL) .................................................................................................53
  2.5.2 Cleaved caspase-3 assay .........................................................................................54
  2.5.3 Multi-target apoptosis ELISA ................................................................................54
  2.5.4 Western blotting of proteins ..................................................................................55
  2.5.5 Densitometry and analysis .....................................................................................55
  2.5.6 Western blotting of signalling molecules ..............................................................56
  2.5.7 Immunoprecipitation .............................................................................................56
  2.5.8 Phosphoarray .........................................................................................................57
2.6 Metabolomics ..................................................................................................................60
  2.6.1 Sample acquisition ................................................................................................60
  2.6.2 Analysis ..................................................................................................................61

Chapter 3 Growth and biochemical characteristics of small for gestational age infants, with and without post-natal catch-up growth .................................................................62
3.1 Participants .....................................................................................................................63
  3.1.1 Patient characteristics and measurements .............................................................63
3.2 Height ...............................................................................................................................65
Chapter 3

3.3 Weight .................................................................................................................................68
3.4 Body mass index (BMI) ......................................................................................................70
3.5 Waist and hip measurements .............................................................................................70
  3.5.1 Waist to hip ratio ............................................................................................................73
3.6 Blood pressure ......................................................................................................................73
3.7 Baseline biochemical tests ..................................................................................................76
  3.7.1 IGF-I ...............................................................................................................................76
  3.7.2 Glucose ............................................................................................................................76
  3.7.3 Insulin .............................................................................................................................79
  3.7.4 Adiponectin .....................................................................................................................79
3.8 Summary ..............................................................................................................................81
3.9 Discussion ..............................................................................................................................81

Chapter 4

4.1 Introduction .........................................................................................................................86
4.2 Basal proliferation ..................................................................................................................87
  4.2.1 Cell counting ....................................................................................................................88
  4.2.2. BrdU incorporation ........................................................................................................88
  4.2.3 The effect of GH on cell number ....................................................................................90
  4.2.4 The effect of IGF-I on cell number ................................................................................92
  4.2.5 The effect of GH and IGF-I in combination on cell number ............................................94
  4.2.6 Summary of investigations in basal and hormone stimulated proliferation .................96
4.3 Basal apoptosis .....................................................................................................................97
  4.3.1 TUNEL staining ..............................................................................................................97
  4.3.2 Caspase-3 activity ..........................................................................................................99
  4.3.3 The effect of GH on control and SGA cell on apoptosis .................................................99
  4.3.4 The effect of IGF-I in control and SGA cells on apoptosis .............................................101
  4.3.5 Summary of investigations into apoptosis ......................................................................102
4.4 ELISA of key molecules in apoptosis ..................................................................................103
  4.4.1 Cleaved caspase-3 and PARP .......................................................................................103
  4.4.2 p53 ....................................................................................................................................104
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.4.4 Tyrosine</td>
<td>160</td>
</tr>
<tr>
<td>6.4.5 Threonine and serine</td>
<td>160</td>
</tr>
<tr>
<td>6.4.6 The urea cycle</td>
<td>161</td>
</tr>
<tr>
<td>6.4.7 Valine</td>
<td>163</td>
</tr>
<tr>
<td>6.4.8 Glycine</td>
<td>163</td>
</tr>
<tr>
<td>6.4.9 Cysteine</td>
<td>163</td>
</tr>
<tr>
<td>6.4.10 Amino acid summary</td>
<td>165</td>
</tr>
<tr>
<td>6.5 Inositols</td>
<td>165</td>
</tr>
<tr>
<td>6.6 Acids</td>
<td>166</td>
</tr>
<tr>
<td>6.7 Summary</td>
<td>166</td>
</tr>
<tr>
<td>6.8 Discussion</td>
<td>166</td>
</tr>
<tr>
<td>Chapter 7 General discussion</td>
<td>170</td>
</tr>
<tr>
<td>Chapter 8 References</td>
<td>174</td>
</tr>
</tbody>
</table>
## Abbreviations list

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGA</td>
<td>Appropriate for gestational age</td>
</tr>
<tr>
<td>ALS</td>
<td>Acid labile subunit</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BP</td>
<td>Blood pressure</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>CST</td>
<td>Cell signalling technology</td>
</tr>
<tr>
<td>CU</td>
<td>Catch-up</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’6-diamino-2-phenylinadole</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immuno-substrate assay</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal related kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>GHR</td>
<td>Growth hormone receptor</td>
</tr>
<tr>
<td>GHRH</td>
<td>Growth hormone releasing hormone</td>
</tr>
<tr>
<td>GM</td>
<td>Growth medium</td>
</tr>
<tr>
<td>Grb2</td>
<td>Growth receptor binding protein 2</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HOMA IR</td>
<td>Homeostatic models assessment of insulin resistance</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>IGF-I</td>
<td>Insulin like growth factor I</td>
</tr>
<tr>
<td>IGF-IR</td>
<td>Insulin like growth factor I receptor</td>
</tr>
<tr>
<td>IGFBP</td>
<td>Insulin like growth factor binding protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin receptor</td>
</tr>
<tr>
<td>IRS-1</td>
<td>Insulin receptor substrate 1</td>
</tr>
<tr>
<td>ISS</td>
<td>Idiopathic short stature</td>
</tr>
<tr>
<td>IUGR</td>
<td>Intra-uterine growth restriction</td>
</tr>
<tr>
<td>JAK2</td>
<td>Janus associated kinase 2</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Daltons</td>
</tr>
<tr>
<td>KIGS</td>
<td>Kabi Pharmacia International Growth Study</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen activated protein kinase kinase</td>
</tr>
<tr>
<td>mTORc2</td>
<td>Mammalian target of rampamysin complex 2</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NCU</td>
<td>Non-catch-up</td>
</tr>
<tr>
<td>NICE</td>
<td>National Institute for health and care excellence</td>
</tr>
<tr>
<td>NS</td>
<td>Non significant</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OGTT</td>
<td>Oral glucose tolerance test</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly ADP ribose polymerase</td>
</tr>
<tr>
<td>PC</td>
<td>Principal component</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal component analysis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDKI</td>
<td>Phosphoinositide dependent kinase</td>
</tr>
<tr>
<td>pGH</td>
<td>Placental growth hormone</td>
</tr>
<tr>
<td>PH</td>
<td>Plekstrin homolgy</td>
</tr>
<tr>
<td>PHLPP</td>
<td>PH domain leucine rich repeat protein phosphatase</td>
</tr>
<tr>
<td>PI3-K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PIP₃</td>
<td>Phosphatidylinositol (3,4,5)-triphosphate</td>
</tr>
<tr>
<td>pMAPK</td>
<td>Phospho mitogen activated protein kinase</td>
</tr>
<tr>
<td>PTB</td>
<td>Phospho tyrosine binding</td>
</tr>
<tr>
<td>PTP</td>
<td>Protein tyrosine phosphatases</td>
</tr>
<tr>
<td>Raf</td>
<td>Rapidly accelerated fibrosarcoma</td>
</tr>
<tr>
<td>Ras</td>
<td>Rat sarcoma</td>
</tr>
<tr>
<td>rhGH</td>
<td>Recombinant human growth hormone</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>SDS</td>
<td>Standard deviation score</td>
</tr>
<tr>
<td>SodiumDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SGA</td>
<td>Small for gestational age</td>
</tr>
<tr>
<td>SGACU</td>
<td>Small for gestational age with catch-up growth</td>
</tr>
<tr>
<td>SGANCU</td>
<td>Small for gestational age non-catch-up growth</td>
</tr>
<tr>
<td>SH-2</td>
<td>Src homology domain 2</td>
</tr>
<tr>
<td>SH-3</td>
<td>Src homology domain 3</td>
</tr>
<tr>
<td>SHP</td>
<td>Src homology containing protein</td>
</tr>
<tr>
<td>SHP-2</td>
<td>Src homology containing protein 2</td>
</tr>
<tr>
<td>SHP-3</td>
<td>Src homology containing protein 3</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor of cytokine signalling</td>
</tr>
<tr>
<td>Stat5b</td>
<td>Signal transducer activator of transcription 5b</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetra methyl benzidine</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TSBT</td>
<td>Tris buffered saline tween</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labelling</td>
</tr>
</tbody>
</table>
Small for gestational age (SGA) is defined as a birth weight or crown to heel length of -2 or more standard deviation scores. It is associated with increased risk of mortality and morbidity in the neonatal period and also has long term effects including an increased risk of developing type II diabetes, high blood pressure and cardiovascular disease later in life. Infants born SGA usually show catch-up growth within the first few years of life. However in the UK, ~1500 SGA children each year remain small and the reason for this is not understood.

The aim of this work was to investigate the molecular mechanisms known to contribute to post-natal growth and also to develop a metabolomic profile in children born SGA. Skin biopsies were obtained with local ethical approval from prepubertal healthy children and children born SGA. Cell turnover (proliferation and apoptosis) and growth hormone (GH) and insulin like growth factor-I (IGF-I) signalling in fibroblasts was assessed and the metabolomic profile in these groups was determined. During this study, blood samples and auxological data were also obtained in children born SGA with catch-up growth and children born SGA without catch-up growth.

Cell counting and bromodeoxyuridine incorporation demonstrated that proliferation was comparable between SGA cells compared to control cells under basal, GH (200ng/ml) and a combination of GH (20ng/ml) plus IGF-I (10ng/ml) stimulated conditions. However, IGF-I (100ng/ml) stimulated proliferation was significantly reduced in the SGA cells compared to control cells (p<0.001). Basal apoptosis was significantly increased in SGA cells compared to control cells (p<0.005). GH and IGF-I reduced apoptosis in SGA cells; however, the level of apoptosis remained significantly higher in SGA cells compared to control cells (p<0.005).

GH and IGF-I signalling pathways in SGA cells and control cells were assessed by western blotting, immunoprecipitation and phosphoarray. GH (200ng/ml) induced phosphorylation of Stat5b was significantly reduced in SGA cells compared to control cells (p<0.001). IGF-I (100ng/ml) activation of Akt was altered, as Akt2 was activated in SGA cells but not in controls cells (p<0.001).

Metabolomic profiling of SGA cells revealed alterations in respiration, up-regulation of the urea cycle, altered fatty acid metabolism and altered cell signalling compared to control cells.

The data presented in this thesis increases the understanding of the molecular mechanisms that affect growth in children born small, and has identified a metabolomic profile of SGA children without catch-up growth. This work may lead to new, more specific therapy for these individuals, and metabolomics may enable the identification of infants who do not show post-natal catch-up growth much earlier, leading to earlier commencement of recombinant human GH.
Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.

Copyright statement

i. The author of this thesis (including any appendices and/or schedules to this thesis) owns certain copyright or related rights in it (the “copyright”) and s/he has given The University of Manchester (“The University”) certain rights to use such copyright, including for administrative purposes.

ii. Copies of this thesis, either in full or in extracts and whether in hard or electronic copy, may be made only in accordance with the copyright, designs and patents act 1988 (as amended) and regulations issued under it or, where appropriate, in accordance with licensing agreements which the University has from time to time. This page must form part of any such copies made.

iii. The ownership of certain copyright, patents, designs, trademarks and other intellectual property (the “Intellectual Property”) and any reproductions of copyright works in the thesis, for example graphs and tables (“Reproductions”), which may describe in this thesis may not be owned by the author and may be owned by third parties. Such Intellectual Property and Reproductions cannot and must not be made available for use without the prior written permission of the owner(s) of the relevant Intellectual Property and/or Reproductions.

iv. Further information on the conditions under which disclosure, publication and commercialisation of this thesis, the Copyright and any Intellectual Property and/or Reproduction described in it may take place is available in the University IP Policy (see http://www.campus.manchester.ac.uk/medialibrary/policies/intellectualproperty.pdf), in any relevant Thesis restriction declarations deposited in the University Library, The University Library’s regulations (see
http://www.manchester.ac.uk/library/aboutus/regulations) and in The University’s policy presentation of Theses.

Acknowledgments

I would like to sincerely thank my co-supervisors Professor Peter Clayton and Dr Melissa Westwood for their time, patience and encouragement over the course of my PhD studies.

I would like to acknowledge Dr Warwick Dunn and Dr Marie Brown for their work, running samples for the metabolomic part of this study, and the statistical analysis.

I would also like to thank all the past and present members of ‘Team Clayton’, whom on a daily basis have provided me with support and expertise.

Finally, I need to thank all my wonderful friends and family. I do not know how you managed to put up with me and support me the way you did, and I will be forever grateful.
Chapter 1 Introduction
1.1 Introduction

Approximately ten percent of live infants are born small for gestational age (SGA) in the United Kingdom (Figure 1.1). SGA is defined as a crown-to-heel length or weight at birth of ≤-2 standard deviation scores (SDS) below the mean for gestational age (Clayton et al 2007). The growth of an SGA fetus is consistently below the normal range, which is different to that of a fetus with intra-uterine growth restriction (IUGR) whose growth pattern is more erratic due to an insult or pathology affecting the growth potential of the fetus in utero (Bamberg et al 2004). Being born SGA is associated with an increased risk of mortality and morbidity in the neonatal period (Verlijsdonk et al 2011). Furthermore, low birth weight is associated with an increased risk of developing diseases, such as metabolic syndrome, later in life (Barker 1997). There are known contributors to low birth weight; however, for most babies born SGA, there is no clear cause.

1.2 Causes of SGA

Numerous maternal and fetal factors can contribute to being born SGA. In utero the fetus relies on an adequate supply of carbohydrates, fats, protein, vitamins and oxygen from the mother’s circulation to grow and survive. The nutritional status of the mother is therefore important in determining fetal growth and size (Ramakrishnar et al 2012).

Nutrients and gases are transported to the fetus via the placenta; any utero-placental circulatory issues would therefore have an effect on this supply (Sibley et al 2005). Other placental abnormalities, such as structural or functional defects that result in reduced transfer capacity, can also result in an SGA baby (Zhou et al 2013). Genetic syndromes are another cause of SGA (Yaghoostkar et al 2012).
Figure 1.1 Photograph depicting an appropriate-for-gestational age (left) and small-for-gestational age (SGA) set of twins, and factors affecting fetal growth

A) A set of twins born at 38 weeks. Photograph courtesy of Dr Philip Murray.

B) A schematic of factors affecting fetal growth including maternal, placental and fetal.
1.2.1 Nutritional status of the mother

A mother’s nutritional status is often reflected in the size of her baby (Ramakrishnar et al. 2012). A recent review by Ramakrishnar et al. reviewed 45 articles to determine the effect of maternal nutrition before and during early pregnancy on infants, and a review by Cetin and Alvino (2009) looked at maternal nutritional status at the stage of organogenesis and its impact on the infant. Both reviews looked at macro/micronutrients, anaemia and calorie restriction. A few examples of the studies reviewed are discussed below. Their findings revealed that maternal nutrition was correlated to birth weight.

An interesting case study to discuss is from the Dutch famine of 1944–46. Women were consuming less than 1000 calories a day, and when the famine occurred in their third trimester (the time when fetal growth is at its fastest), the babies were significantly small at birth (Susser 1994).

In a separate study in India, Mahajan et al. (2004) investigated the size of babies in malnourished mothers (BMI of ≤17), and in normal weight mothers (BMI >17). The group documented a 16% increase in the incidence of SGA births in malnourished mothers compared to the normal weight control group.

The role of maternal nutrition in influencing birth weight has also been studied in animals. In rats, a 50% caloric under-nutrition in the later stages of pregnancy resulted in significantly smaller newborns compared to newborns from rats fed on a normal diet (Lesage et al. 2001). In pigs, an experiment comparing normal and low protein diets, also in the later stages of pregnancy, showed significantly reduced birth weights in piglets of sows fed the restricted protein diet (Schoknecht et al. 1993).

Poor fetal growth due to poor nutritional status can be rescued. A study in humans assessed the affect of micronutrient supplementation in the second and third trimester of pregnancy in comparison to supplementation with only folic acid and iron (Osrin et al. 2005). A significant increase in birth weight was demonstrated in the group who received the micronutrient supplementation,
suggesting that birth weight is dependent on a variety of vitamins and minerals, and that receiving this supplementation improves maternal nutrition and has a positive effect on birth weight (Osrin et al 2005).

The effect of maternal under-nutrition has been investigated in more detail, and it appears it can cause increased levels of cortisol in both the maternal and cord blood (Fowden et al 1995, Lesage et al 2001). Glucocorticoids increase vasoconstriction of the chorionic plate arteries, thus increased vascular resistance is an indicator of fetal growth restriction (Nugent et al 2013).

Obese mothers, or mothers with excessive gestational weight gain, have larger babies than normal weight mothers (Mamun et al 2011). This ‘over nutritional’ state produces the opposite outcome to under-nutrition; however, both significantly small and large babies carry higher risks.

1.2.2 Smoking, alcohol and drug use during pregnancy

It has been documented that both maternal and passive smoking increases the risk of an SGA birth (Lieberman et al 1994, Ward et al 2007, Aagaard-Tillery et al 2008, Agrawal et al 2010). Whether this is associated with reduced transport of oxygen to the fetus, a direct effect of nicotine and other chemicals in cigarettes, or a combination, is yet to be determined.

A model of reduced oxygen availability to the fetus is maternal anaemia. Hypochromic red blood cells found in iron deficiency anaemia have reduced haemoglobin and therefore transport less oxygen in the circulation. In a study of 207 third trimester pregnancies, 46.8% were defined as anaemic; however, no correlation was found between haemoglobin status and birth weight (Demmouche et al 2011). This suggests the level of haemoglobin in the third trimester of pregnancy does not affect birth weight, and that it may be the components in cigarettes crossing the placenta that are responsible for increased risk of an SGA birth compared to oxygen transport via haemoglobin, or both.
The effect of nicotine and cotinine on trophoblast and human umbilical vein endothelial cells has been suggested to cause the poor fetal growth associated with maternal smoking (Romani et al 2011). The presence of these two chemicals was found to alter the secretion of key vaso-active molecules from these cells (Romani et al 2011). This may then affect the vasculature of the placenta, and consequently placental transfer of nutrients and gases to the fetus.

In a study of 202 children, maternal pre-natal alcohol consumption was significantly associated with lower birth weight and length (Lumeng et al 2007). In an animal study, rats were exposed to alcohol during the last two weeks of gestation and the pups were significantly smaller than the newborns from the control group (Wilcoxon et al 2003). In this study, it was determined that alcohol exposure resulted in placental corticosterone excess (Wilcoxon et al 2003).

1.2.3 Placental issues

The placenta is a highly vascularised organ responsible for maintaining a growing fetus. It transfers amino acids, nutrients and oxygen to the developing fetus and removes waste products and carbon dioxide. The efficiency of this process relies on adequate maternal blood flow to the fetus. Reduced blood flow, as measured by Doppler velocimetry analysis in either the uterine or umbilical arteries, is associated with a SGA birth (Sibley et al 2005). Another example of a circulatory issue restricting blood flow to the placenta is preeclampsia (Christians and Gruslin 2010). In comparison to a normal control group, the incidence of SGA births in women with preeclampsia was increased by 34.9% (Grisaru-Granovsky et al 2007).

Structural abnormalities in the placenta also cause pre-natal growth restriction (Zhou et al 2013). Analysis of 50 placentas from pregnancies which produced SGA babies demonstrated a greater incidence of chorioamnionitis, infarction and reduced size in comparison to those from babies born appropriate for gestational age (Oliveira et al 2002).
Reduced activity of the system A amino acid transporter in the placenta was demonstrated in placentas from SGA babies compared to AGA babies (Shibata et al 2008). This particular amino acid transporter transports alanine, serine, glutamine and glycine, all of which are essential amino acids which make up ~40% of fetal energy requirement (Shibata et al 2008).

The placenta produces growth hormone and other hormones (e.g. lactogen and leptin) which are thought to contribute to the mobilisation of maternal metabolites to increase nutrients available for fetal use; serum placental GH (pGH) levels are positively correlated with birth weight (Shibata et al 2008). In a study involving placentas from 15 SGA births and 23 appropriate for gestational age (AGA) births, the level of mRNA for GH, and four other placentally-expressed genes on the GH locus, was found to be significantly lower in placentas from SGA babies (Mannik et al 2010).

1.2.4 Fetal factors

Several genetic conditions are associated with low birth weight, for example the chromosomal abnormalities of Down syndrome and Turner syndrome (Pihl et al 2008, Oliveira et al 2009). Abnormalities in the expression of the imprinted genes, IGF2 and H19, are associated with the pre- and post-natal growth restriction seen in Silver-Russell syndrome (Eggermann et al 2010). Rare causes of being born SGA include mutations in the genes for IGF-I; its receptor, IGF-IR, and the signalling molecule, Stat5b. These will be discussed in more detail in section 1.7.
1.3 Catch-up growth

Approximately 90% of all SGA infants will exhibit catch-up growth by the age of 2 years and be discharged from neonatal clinics (Clayton et al 2007). Catch-up growth is defined as the one taking place in an infant that crosses over the third percentile in height before the age of two years. Another definition of catch-up growth is a growth velocity (centimetres per year) greater than the median for chronological age and gender (Wit et al 2008). In the UK, around 1500 of the infants born SGA each year do not demonstrate catch-up growth (Figure 1.2).

It is not clear why some infants catch up and others do not; however, there are some rare genetic causes associated with post-natal growth restriction, for example, mutations in genes encoding for key regulators of post-natal growth, such as IGF1, IGF1R and STAT5B (Wit et al 2007).

1.4 Post-natal growth

Post-natal growth is governed by the growth hormone/insulin-like growth factor-I (GH/IGF-I) axis (Le Roith et al 2001). This has been demonstrated in animal models with aberrations in key parts of the axis. Mice null for the GH receptor (Floyd et al 2007) are of a normal size at birth; however, they fail to demonstrate normal post-natal growth and are 50% of the size of wild type mice at 40 days of age (Lupu et al 2001). Igf1−/− mice are 30% of the size of wild type mice at 8 weeks (Lupu et al 2001). Humans with mutations in the GHR gene (Laron syndrome) also demonstrate severe post-natal growth failure with final heights in men falling between -4 and -10 SDS or 116-142cm (Laron 2001). Individuals with IGF1 mutations are rare; however, a case reported in the literature also demonstrates post-natal growth failure with a height at age 15.8yr of -6.9 SDS (Woods et al 1996).
Figure 1.2 Growth chart depicting the length/height measurements during the first 2 years of life of two male SGA infants. The blue line depicts the growth of a SGA infant with post-natal catch-up growth and the red line depicts a SGA infant without post-natal catch-up growth.
1.4.1 Growth Hormone

GH has growth promoting and metabolic roles. In a rat model, GH induces proliferation of germinal cells in the growth plate and it increases osteoblast proliferation in vitro (Ohlsson et al 1992, Olney 2003).

GH has an anabolic role and stimulates the release of free fatty acids from adipose tissue into the circulation (Vijayakumar et al 2010). GH also stimulates the production of hepatic glucose by gluconeogenesis and glycogenesis (Vijayakumar et al 2010). GH is also a key part of liver lipid metabolism, by promoting the uptake of triglycerides (Vijayakumar et al 2010). GH levels are also negatively correlated with adiposity (Berryman et al 2011). This anabolic effect is absent in individuals with Laron syndrome, who present with marked obesity (Laron 2001).

The hypothalamus produces growth hormone releasing hormone (GHRH), which stimulates the production of GH by the somatotroph cells in the anterior pituitary (Scanes 1984) (Figure1.3). Ghrelin also positively controls the production of growth hormone. Ghrelin is primarily synthesized in the stomach and is regulated negatively by calorie intake (Inui et al 2004).

GH stimulates IGF-I production mainly in the liver but also in other tissues. GH negatively affects its own production, as well as IGF-I and GHRH, which also negatively feedback to the pituitary and hypothalamus (Le Roith et al 2001). Somatostatin inhibits GH production by reducing hepatic GHR expression and IGF-I expression, maintaining circulating GH levels which negatively feedback to the hypothalamus (Hagemeister and Sheridan 2008). IGF-I can bind to the IGF-IR on both the pituitary and hypothalamus which inhibits the production of GH and GHRH respectively by reducing the levels of the signalling molecule cyclic AMP (Scanlon et al 1996).
**Figure 1.3 GH/IGF-I Axis.** Diagram showing the growth hormone (GH) and insulin-like growth factor I (IGF-I) axis. Red lines represent the negative feedback mechanisms and the green lines represent the stimulatory effects. The hypothalamus produces growth hormone releasing hormone (GHRH). GHRH then stimulates the somatotroph cells of the anterior pituitary to produce and secrete GH. GH then acts directly on target tissues such as adipose tissue and chondrocytes and works indirectly by promoting transcription of the IGF-I gene in the liver. GHRH feeds back negatively to the hypothalamus, GH feeds back negatively to the pituitary and the hypothalamus as does IGF-I. Somatostatin acts on the hypothalamus as a negative signal with Ghrelin produced in response to hypoglycaemia by the stomach as a positive regulator on the hypothalamus.
1.4.2 Growth hormone signalling

The growth hormone receptor, which belongs to the class I cytokine receptor family, exists as a dimer when bound to GH (Floyd et al 2007). The GHR has no intrinsic tyrosine kinase activity and recruits janus associated kinase 2 (JAK2) molecules which associate with the intracellular domains of the GHR (Waters et al 2006). GH binding results in a torsional movement of the receptor dimer resulting in the JAK2 molecules moving closer together and in the process, transphosphorylating themselves and the intracellular domain of the GHR (Waters et al 2006). The phosphorylation of JAK2 and the GHR generates docking sites for signalling molecules, including signal transducer activators of transcription 5b (Stat5b), Src homology domain 2 (SH-2)-containing proteins and other phosphotyrosine binding domain-containing signalling molecules (Shaonin et al 2002) (Figure 1.4).

Upon the binding of Stat5b to the GHR, Stat5b is phosphorylated and dimerises in a head-to-tail fashion before moving to the nucleus and initiating gene transcription (Smit et al 1997). Stat5b interacts directly with the IGF-I gene; chromatin immunoprecipitation assays have shown that Stat5b binds to 700 bp DNA region 75Kb upstream of exon 1 of the IGF-I gene and that this is associated with increased IGF-I mRNA expression (Wang and Jiang 2005). This is the main pathway involved in IGF-I production by GH. Consequently, patients with a mutation in the Stat5b gene have below average IGF-I production when stimulated with GH in an IGF-I stimulation test and very low circulating IGF-I levels (Hwa et al 2005).

Mitogen activated protein kinase (MAPK) is activated by GH via the growth factor receptor binding protein 2 (Grb2) adaptor protein (Figure 1.4). Grb2 associates with the phosphorylated GHR via its SH-2 domain and once activated, Grb2 associates with the Ras guanine nucleotide exchange factor, SOS, by its SH-3 domain (Lanning and Carter-Su 2006). GHR phosphorylation recruits the Grb2/SOS complex to the membrane. At the membrane, the Grb2/SOS complex associates with the small G protein, Ras, where it initiates...
Figure 1.4 Diagram depicting the GHR and the intracellular signalling pathways stimulated when GH binds to the GHR. Following GH binding to the GHR, Janus associate kinase 2 (JAK2) is recruited as a result of a conformational change in the GH/GHR complex, which causes transphosphorylation of the intracellular domain of the GHR. Signal transducer activator of transcription (Stat5b) then binds to docking sites on the receptor, dimerises and then binds to the promoter region of the IGF-I gene in the nucleus. JAK2 also stimulates SHP2 (Src homology containing protein 2), which activates the membrane associated proteins growth hormone receptor binding protein 2 (Grb2), SOS and RAS which in turn activate the mitogen activated kinase (MEK/MAPK) pathway.
an exchange of GDP to GTP invoking a conformational change in Ras. Ras then activates Raf at the membrane through an unknown mechanism which then goes on to activate MEK. MEK then activates ERK/MAPK (Waters et al 2006). Upon activation, MAPK translocates to the nucleus. MAPK activates a number of transcription factors including elk-1 and AP1, both of which promote the transition of G1 to S phase in the cell (Cargnello and Roux 2011).

Inhibition of MAPK in haemopoetic cells results in an 84% decrease in viable cells compared to a 38% decrease in non MAPK inhibited cells when cultured with IGF-I (Shelton et al 2004). In an oesophageal carcinoma cell line, expression of pMAPK was significantly increased compared to surrounding non cancerous tissue, 85.5% compared to 31.6% respectively (Zheng et al 2012). While MAPK is key in normal cell turnover, when MAPK activity is abnormal proliferation is also abnormal.

1.5 Growth hormone signalling regulation

GH signalling is tightly regulated, as alterations in GH signalling can lead to severe growth and metabolic disorders, which will be discussed in section 1.7.1 (Waters et al 2006). The suppressors of cytokine signalling (SOCS) family are involved in the negative regulation of GH signalling (Ahmed and Farquharson 2010). SOCS-1 binds to the activating tyrosine in the kinase domain of JAK2, which inhibits JAK2 activity. SOCS-2 binds to the phosphorylated GHR and competes for binding sites with Stat5b. SOCS-3 is thought to bind to the phosphorylated GHR and inhibit JAK2 activity (Lanning et al 2006).

Another mechanism of GHR signalling regulation is through protein tyrosine phosphatases (PTPs), for example SHP-1, SHP-2 and PTP1. SHP- 1 and 2 negatively regulate GH signalling by dephosphorylating phosphotyrosines on the GHR, JAK2, and associated proteins (Pilecka et al 2007). PTP1 knockout mice demonstrate an increase in JAK2/Stat5b phosphorylation upon stimulation with GH, suggesting a role for PTP1 in the negative regulation of GH signalling (Pasquali et al 2003).
1.6 IGF-I, Insulin-like growth factor I

IGF-I belongs to a family of structurally homologous molecules including IGF-II (involved in early fetal growth) and insulin (involved in glucose metabolism) (Pollak 2012). Both molecules have corresponding receptors although IGF-II is thought to elicit the majority of its effects through the type I IGF receptor. It is also thought that its associated receptor, the type II IGF receptor, serves to clear IGF-II from the circulation. Although the insulin receptor shares 60% homology with the IGF-I receptor, insulin elicits its metabolic actions only through the insulin receptor (Pavelic et al 2007). The IGF family also includes binding proteins (IGFBPs) that are involved in transporting the IGFs to different tissues and increasing their half-life in the circulation. IGFBPs also regulate IGF-I and IGF-IR interaction and, therefore, the function of IGFs (Buckway et al 2002).

IGF-I exists in the circulation in a ternary complex with IGFBP-3 and the acid labile subunit (ALS). This complex is essential to the efficacy of IGF-I as patients with a mutation in the gene coding for ALS have a clinical phenotype similar to that of patients with IGF1R mutations and IGF1 mutations (discussed later) (Hwa et al 2006).

IGFBP-3 levels correlate with IGF-I levels, and in short children treated with GH, levels of IGFBP-3 are GH-dose dependent and correlate positively with growth (Buckway et al 2002). A review in 2003 also made the observation that serum IGFBP-3 levels correlate with post-natal growth velocity in babies born SGA (Kajantie 2003).

1.6.1 The IGF-I receptor

IGF-I binds to the type I IGF-I receptor, which is a member of the tyrosine kinase family of receptors and is made up of two extra cellular α subunits and two intracellular β subunits (Le Roith et al 1995). The formation of the IGF-I receptor relies on two pro-receptor molecules (single α and β molecules) forming disulphide bonds between their respective subunits and associating to form a
tetramer. IGF-I and insulin pro- receptor molecules have been known to form hybrid receptors by the same mechanism (Le Roith et al 1995).

Upon stimulation of the IGF-I receptor, a conformational change occurs and the intrinsic tyrosine kinase domains of the β subunits auto-phosphorylate and recruit insulin receptor substrate-1 and 2, Shc and Gab1 molecules which bind to docking sites on the β subunit (Baserga et al 1997) (Figure 1.5). Mapping of the tyrosine kinase domain via site directed mutagenesis has identified specific regions involved in eliciting the diverse effect of IGF-I; for example, IRS-1 binds to, and is phosphorylated by, S1131-1136 residues of the β subunit (Dews et al 2000).

1.6.2 The phosphatidylinositol 3-kinase (PI3-K) pathway

IRS-1 has an N-terminal plekstrin homology (PH) domain followed by a phosphotyrosine binding (PTB) domain and 18 potential tyrosine phosphorylation sites (Vainshtein et al 2001). It is the PH and PTB domains that direct IRS-1 to the membrane resulting in tyrosine phosphorylation of IRS-1 by the IGF-I receptor. IRS-1 then binds to Gab1, a docking protein which recruits phosphatidylinositol 3-kinase (PI3-K) upon activation of the receptor. Upon phosphorylation, IRS-1 activates the p85 regulatory subunit of PI3K, leading to the activation of Akt. Akt is phosphorylated on its threonine 308 residue by PI3-K, and subsequently, on its serine 473 residue by mTORc2. Both phosphorylation events have to occur for full activation of Akt (Siddle 2011).

Akt exists as three isoforms, each of which has a distinct role. Akt1 knockout mice are born small and demonstrate post-natal growth restriction (Garrofalo et al 2003). Once activated at the cellular level, Akt1 regulates apoptotic machinery by inhibitory phosphorylation of Bad and initiating the transcription of pro-survival genes (Peruzzi et al 1999). Recently, Akt1 activation rates have been associated with response to GH therapy in children with idiopathic short stature and children born SGA. Taking peripheral blood mononuclear cells, and measuring Akt activation upon stimulation with IGF-I, the higher the activation of Akt the greater the height improvement in response to GH (Lotton et al 2008).
Figure 1.5 Diagram to show the post receptor signalling pathways of IGF-I and the IGF-I receptor. Upon IGF-I binding to the IGF-I tyrosine kinase receptor, the beta subunits phosphorylate themselves creating docking sites for signalling molecules. On the right hand side of the diagram, the cascade is initiated by SHC (src homology domain containing protein) activating Grb2 which is bound to IRS-1 (insulin receptor substrate 1) and which then activates SOS (substrate of signalling) and initiates the MEK/ERK pathway to gene transcription. On the left hand side of the diagram, the IRS-1 activated pathway leading to Akt activation via PI3K (phosphatidylinositol 3-kinase) pathway.
Total Akt levels are reduced in placentas from IUGR pregnancies (Laviola et al 2005), and placental Akt1− demonstrated placental insufficiency and fetal growth restriction (Yang et al 2003).

Akt2 knockout mice have a diabetic phenotype (Garrofalo et al 2003). In zebra fish with Akt2 ablation, uptake of fluorescently-labelled glucose injected into the egg sack is reduced to 54%. (Jensen et al 2007). In humans, insulin-induced activation of Akt2 is reduced in muscle cells from patients with type II diabetes (Cozzone et al 2008). It is interesting to note that in a study of individuals born small but who had not developed type II diabetes, the activation of Akt2 in muscle biopsies obtained before and after hyperinsulinaemic euglycaemic clamp was reduced in comparison to that observed in control subjects (Friedrichsen et al 2010). This supports the hypothesis that SGA individuals are at an increased risk of developing type II diabetes later in life (Barker 1997).

Akt3 is thought to be involved in the central nervous system and the Akt3−/− knockout mouse has a 20% decrease in brain size (Cho et al 2005); the cells in the brain are both smaller and fewer in number, and the myelin content is also reduced, by 20% (Easton et al 2005). Cardiac-specific Akt3 transgenic mice show cardiac hypertrophy and down regulation of Akt1 and Akt2 expression (Taniyama et al 2005). This suggests that when Akt1 and Akt2 are inhibited Akt3 is able to stimulate proliferation.

1.6.3 The MAPK pathway

IGF-I activation of the IGF-I receptor activates the MAPK pathway. SHP-2, once activated by the tyrosine phosphorylated residues on the IGF-I receptor, activates Grb2 and initiates the MAPK pathway as described in detail in section 1.4.2.

1.7 Aberrations in GH and IGF-I signalling

In a minority of cases of post-natal growth failure, aberrations in the GH and IGF-I signalling pathways have been documented (Rosenbloom 2000).
1.7.1 Growth hormone signalling aberrations

Aberrations in this pathway include rare diseases such as Laron syndrome, which is a mutation in the gene encoding for the GHR, and also mutations in the Stat5b gene (Laron 2004, Hwa et al 2005).

Patients with Laron syndrome present with short stature, obesity and hypoglycaemia, with normal or raised levels of GH combined with low levels of IGF-I. The majority of mutations responsible for Laron syndrome are found in exons 2–7 of the extracellular domain of the GHR, and even a single amino acid substitution in this domain affects GH-GHR binding (Laron 2004). Experiments in fibroblasts from Laron patients have shown that the GHR mutation (first described by Laron et al in 1966) leads to a decrease, or total lack, of post receptor signalling following stimulation with GH (Silva et al 2002). This suggests the lack of GH binding due to the GHR mutation reduces downstream GH signalling which links this to high GH levels due to reduced feedback by the consequential low circulating IGF-I levels.

A mouse model to demonstrate the small phenotype associated with a GHR mutation also supported the effect of these mutations on post-natal growth (Rowland et al 2005). Femoral and tibial lengths were significantly reduced in GHR<sup>-/-</sup> mice at day 42, by 81% and 78% respectively (Rowland et al 2005). Hepatic IGF-I mRNA expression and serum IGF-I levels were also significantly reduced (16-20% and 36-67% respectively).

Mutations in the STAT5B gene have been identified in ten patients worldwide (Hwa et al 2011). The clinical phenotype is short stature (height SDS -5.3 to -9.9), and in all the female cases a degree of immunodeficiency has been noted. A typical example is the female presented by Rosenfeld et al (2004) who at age 16.5 years, was 117.8cm (-7.5 SDS) tall. Experiments performed on lysates of fibroblast cells obtained from this patient demonstrated a total lack Stat5b activation upon stimulation with GH as there was no detectable phospho Stat5b on immunoblot (Rosenfeld et al 2004). As well as severe growth failure the
patient also had severe immune dysfunction, ranging from generalised eczema to lymphoid interstitial pneumonia (Peng Fang et al 2006).

The \textit{STAT5B} knockout mouse demonstrates a loss of sexually dimorphic growth and a 30%-50% reduction in IGF-I levels compared to wild type littermates (Snow et al 2003).

As Stat5b is a key molecule in the GH pathway, and aberrations at this point in the pathway are associated with post-natal growth disorders, investigating the activation of this molecule in SGA infants with catch-up growth failure may help to identify the mechanism behind this growth restriction. It may also allow further molecular characterisation of this pathway in these infants.

Mutations in the gene encoding for SHP-2 are associated with Noonan syndrome. Noonan syndrome is characterised by short stature, facial dysmorphia, and heart defects (Limal et al 2006); 50% of these patients have a mutation in the gene encoding SHP-2 (Stein 2007). This mutation is gain-of-function (Limal et al 2006). SHP-2 is phosphorylated by JAK2 and is part of the MAPK pathway post GH-stimulation of the GHR. SHP-2 is known to be both a positive and negative regulator of GH signalling and in this case, as the phenotype involves growth retardation, this gain-of-function has a negative effect on growth. Both gain-of-function and loss-of-function mutations have been found in humans, with similar characteristics and short stature (Wang et al 2011). Proliferative and tumour suppressor functions of MAPK have been demonstrated in cell models also. For example, hepatocyte-specific SHP-2 ablation, results in tumour formation in aged mice (Bard-Chapeau et al 2011), and SHP-2 ablation in myeloid cells results in a rapid increase in cell number compared to normal cells (Jack et al 2009). A recent review suggested that alternative roles of SHP-2 may be tissue-specific and developmental stage-specific (Wang et al 2011).
1.7.2 Aberrations in IGF-I signalling

Aberrations in the IGF-I signalling pathways can also cause postnatal growth failure. Mutations in the gene coding for IGF-I have been identified in two patients to date. A guanine-to-adenosine substitution was identified in a male patient with intra-uterine and post-natal growth failure, and functional analysis of IGF-I binding demonstrated a 90-fold reduction in the affinity for the IGF-IR when compared to wild type IGF-I (Walenkamp et al 2005). At birth the patient had a weight of -3.9 SDS and length of -4.3 SDS, and a final adult height is -8.5 SDS. The phenotype is associated with deafness and mental impairment (Walenkamp et al 2005).

Mutations in the IGF-IR gene are more common. An example of a mutation in the IGF-IR gene is the arginine to glutamine substitution at amino acid 481 in exon 7, located near to the first disulphide bond between the two alpha subunits of the receptor. The patient with this mutation had a height of -5.0 SDS and a raised IGF-I level (Inagaki et al 2007). Another mutation in the IGF-IR gene has been located in the intracellular tyrosine kinase domain and in this case, the patient’s final height was -4.1 SDS.

PDK1 activates a group of kinases including Akt. Mutations in the PH domain of PDK1, which binds Akt, result in small size and insulin resistance in mice (Bayascas et al 2008). A mutation in PDK1 in humans may result in a similar phenotype, though none are documented to date.

1.8 Treatment of short stature

Currently, the only available treatment for post-natal growth disorders is recombinant human growth hormone (rhGH). rhGH is effective in treating many growth disorders (Bergada 2013); however, in SGA infants, the growth response is varied, even when high doses of GH are used (Ranke et al 2003). This study used the Kabi Pharmacia International Growth study (KIGS) data base to analyse data from 68 children born SGA started on rhGH. After the first year of
treatment, positive correlations were demonstrated between rhGH dose and age at start. Together these accounted for 52% of the variability in growth response in this study. The remaining variability could not be accounted for by the parameters the study used. These were comprehensive auxological data, genetic background (i.e. midparental height SDS), rhGH dose and patient variable including weight, bone age and BMI. Looking at this study, the remaining variability in growth response to rhGH requires investigation, such as the molecular mechanisms of GH and IGF-I signalling, which this thesis hopes to explore.

The dosing regime for SGA infants is 35-70µg/kg/day and the average height gains after 3 years of therapy are between 1.2 SDS and 2.0 SDS (Clayton et al 2007). rhGH is not a perfect treatment for these infants as, the mechanism behind their growth disorder is usually unknown. Despite this, it is important to treat these SGA infants as there exist numerous health problems associated with post-natal catch-up growth failure.

1.9 Metabolic consequences of being born SGA

Being born SGA increases the risk of developing metabolic syndrome later in life (Barker 1997). Signs of this can be detected in childhood, including insulin resistance, altered adiposity and changes in blood pressure. In this section the effect of rhGH will be discussed.

Short SGA children who are treated with rhGH show increased insulin insensitivity after initiating this treatment (Sas et al 2001). Pre-treatment, the oral glucose tolerance test (OGTT) results in this group were 7.8-11.1 mmol/L, which suggested impaired fasting tolerance. After 6 years of treatment, this had reduced to only 4% of the group having impaired glucose tolerance. Insulin levels and glucose stimulated insulin levels were significantly increased at the end of the study; however, glucose levels stayed within the normal range (Sas et al 2001). An interesting finding was that participants who remained prepubertal compared to ones who entered puberty showed no significant difference in
OGTT, insulin levels and glucose stimulated insulin levels. A point for future consideration was made, of the effect of long term hyperinsulinaemia on children born SGA treated with rhGH.

A more recent paper comparing data from 37 adults born SGA treated with GH, with 25 short untreated SGA controls found insulin insensitivity, glucose and insulin levels to be comparable (van Dijk et al 2007). The mean discontinuation time was 6.5 years, suggesting the adverse effects of GH to carbohydrate metabolism do not continue after GH treatment has stopped.

Chatelain et al (2009) performed a retrospective analysis of glucose metabolism by oral glucose tolerance test, and insulin secretion in children born SGA without catch-up growth after 1 year on rhGH. A negative correlation was found compared to measurements taken before rhGH. Insulin sensitivity was significantly reduced, and glucose levels significantly increased. Glucose levels remained within the normal range.

Bozzola et al (2005) also investigated carbohydrate metabolism in 24 children born SGA without catch-up growth, after 1 and 2 years rhGH. In their cohort, insulin sensitivity fell significantly, but remained within the normal range. This study was performed using varied doses of rhGH (assigned by clinicians to optimise growth), and it is interesting to note that the reduced insulin sensitivity demonstrated did not correlate with rhGH dose.

In addition, Cutfield et al (2003) demonstrated, with 12 children born SGA without catch-up growth, that after 1 year rhGH insulin sensitivity fell by 44% (p=0.018) and interestingly did not recover 3 months post termination of rhGH.

In a longitudinal study including 29 AGA children and 22 SGA children, significantly increased abdominal fat was found at baseline in the SGA group despite comparable weight and BMI (Ibanez et al 2007). Insulin resistance was also present at the age of 4 in the SGA group; (homeostatic models assessment of insulin resistance) HOMA IR 1.23±0.13 in the SGA group compared to 0.68±0.08 in the AGA group (Ibanez et al 2007). In another study, comparing 35
SGA and 35 AGA children, increased HOMA IR was demonstrated in the SGA group, $1.30\pm0.8$ versus $0.92\pm0.3$. SGA children who demonstrated catch-up growth appeared to have increased insulin resistance compared to short SGA children without rhGH treatment (Veening et al 2002).

Systolic blood pressure is increased at the age of rhGH commencement in individuals born small, with the greatest increase seen in individuals born small with post-natal catch-up growth (Huxley et al 2000). GH treatment reduces systolic BP in individuals born SGA to levels comparable with those observed in untreated short SGA controls (van Dijk et al 2007).

GH treatment in SGA infants who do not catch up may be metabolically beneficial; however, the long term effects of this treatment are yet to be documented, and there are concerns regarding cancer risk in this group.

1.10 Metabolomics

Predicting catch-up growth is difficult; however a few studies have found relationships between adipocytokines, and the steroid metabolism with catch-up growth.

Adiponectin levels are inversely associated with catch-up growth in serum samples from children born SGA (Cianfarani et al 2004). Adiponectin is an adipocytokine that is also associated with obesity, type II diabetes and heart disease (Lihn et al 2005, Hernandez-Morante et al 2008).

Metabolomics is a popular technique for non-invasive diagnostics. It is the measurement and analysis of metabolic intermediates in a biological sample to give a profile of a particular state. To date is has been used to profile diseases and also as a prognostic device. Using metabolomics will enable the metabolic status of children born SGA to be investigated further, and may enable a growth profile in children born SGA to be developed and potentially used as a growth marker or risk of post-natal growth failure risk assessment.
Clinical and laboratory predictors, such as growth velocity and IGF-I levels, are helpful in gauging risk but provide inconsistent insight into the mechanism of post-natal growth failure in these individuals.

A recent study from Wilson et al (2013) has been undertaken to determine the impact prematurity has on metabolism. Metabolomic profiling was undertaken on babies born at various stages of gestation: >36 weeks (373,819), 33–36 weeks (26,483), 28–32 weeks (4,354), and <28 weeks (1,146). Across all prematurity ranges, a significant difference in the levels of three amino acids (arginine, leucine and valine) was found compared to babies born at term (Wilson et al 2013).

A study using piglets by Nissen et al (2011), used metabolomics to investigate potential differences in metabolites in IUGR piglets (n=7) and normal birth weight piglets (n=7). Umbilical vein plasma was analysed in both groups. Several molecules were found to be significantly different between IUGR piglets and normal birth weight piglets. Arginine glutamine, branched chain amino acids, tryptophan and aromatic amino acids were all reduced in the IUGR group. Pyroglutamine acid, carnitine and creatinine were found to be increased in the IUGR group. These findings suggest a potential way to elucidate the mechanism, the implications, prevention, diagnosis and treatment of IUGR (Nissen et al 2011).

Favretto et al (2012) performed liquid chromatography mass spectroscopy on cord blood samples from 22 IUGR pregnancies and 21 AGA. Partial component analysis (PCA) was used to determine differences between the two groups. Phenylyalanine, tryptophan and methionine were present in significantly different levels between the two groups; again it was felt these findings were promising in studying metabolic alterations in these two groups.

Metabolomics is being used to predict the risk of developing type II diabetes (Wang et al 2011). Serum samples taken from the Framingham heart study of individuals, diabetic and non diabetic attendees who developed diabetes after a 12 year follow up period were analysed using metabolomics. Correlation with
disease was found to be associated with urea cycle metabolites, amino acids, nucleotide metabolism metabolites and methyl transfer metabolites. Paired analysis enabled the identification of five metabolites significantly associated with type II diabetes. Conditional logistic regression analysis identified a 3 amino acid combination resulted in a 5–7 fold increase in diabetes risk.

Tissue metabolomics is also used as a way to understand normal and pathological mechanisms. In a recent study by Imperiale et al (2013) 66 adrenal glands were analysed by high resolution magic angle nuclear magnetic resonance, with a view to investigate the metabolomic profile of healthy glands, and adrenocorticotocarcinoma glands. The results of their investigation showed a striking difference in metabolomic profiling between the two groups. The adrenocorticotocarcinoma profile contained biomarkers of anaerobic respiration; for example, increased glycolysis and increased levels of lactate and acetate (Imperiale et al 2013).

Another example of metabolomics performed on a tissue sample is in breast tumours (Brauer et al 2013). The metabolomic profile of 33 breast tumours and 6 normal breast tissues were compared. Upon analysis of the results, the more aggressive the tumour, the higher the levels of nucleic and amino acids, and glycolysis biproducts observed. Discovering the metabolomics profile of breast tumours increases the understanding of their metabolism.

Metabolomics is also performed on cells in culture, the cells themselves and the media they have been cultured in. An example of metabolomics performed on cells is in malarial infected erythrocytes (Sana et al 2013).

Metabolomics is already proving to be a useful tool; predicting disease and disease severity, treatment outcome, risk of metabolic disease and the metabolic implications of being born small.
1.11 Summary

Molecular and metabolomic studies to investigate pre- and post-natal growth failure have not been undertaken in SGA children without post-natal catch-up growth.

The metabolic disadvantages of being born small, although well documented, require further and more detailed analysis to fully understand the role being born small and post-natal growth have on an individual’s future health. Understanding and ‘predicting’ catch-up growth in SGA individuals is also important when taking into account again the affects that being born small and post-natal growth has on future health.

The aim of this study is to determine if children born SGA without post-natal catch-up growth have alterations in cell turnover, GH and IGF-I signalling and metabolomic profiling.

In order to address this, the aims are:

1) To assess cellular proliferation and apoptosis, and the affect of GH and IGF-I and a combination of GH and IGF-I on these processes in cells from children born SGA without catch-up growth

2) To identify the activation of GH and IGF-I signalling pathways

3) To investigate the metabolic status of SGA individuals and identify a metabolomic profile that is characteristic of post-natal growth restriction
Chapter 2  Materials and methods
2.1 Participants

Children born small for gestational age without post-natal catch-up growth were recruited at the Royal Manchester Children’s hospital with local ethical approval and informed consent from the parents. All children were between the ages of 3 and 8 years and had not yet commenced growth hormone therapy. Children were excluded from the study if they had chromosomal abnormalities, were growth hormone deficient, had a chronic illness, were on steroids or had an endocrine abnormality or disease. A series of anthropometric measurements and fasting blood samples were taken at the initial visit. These patients then returned 1, 4, 8, and 12 months after their initial consultation and commencement on growth hormone. The anthropometric measurements taken were height, weight, waist and hip circumference, sub scapular triceps and bicep skin fold thickness and blood pressure. Blood samples were taken to enable measurement of fasting glucose, insulin, adiponectin and insulin like growth factor I (IGF-I).

Punch hole skin biopsies were taken from three of the above patients in this study and cultured to produce primary fibroblasts. Parents gave informed consent and the biopsy was taken under approval by the local ethics committee. Fibroblasts from one small-for-gestational age infant with post-natal growth failure were already established and available for use in this study. Fibroblasts from an individual with pre- and post-natal growth restriction due to a deletion of one copy of the IGF-I gene (CAM1) were the kind gift of Professor DB Dunger, University of Cambridge. Cell lines from four children between 3 and 8 years of age who were born appropriate for gestational age had also been previously established and were used as controls in the experiments described below.

Consequently, four cell lines from SGA individuals without post-natal catch-up growth, one cell line from an individual with a deletion of one copy of the IGF-IR gene (CAM1) and four control cell lines were used in this study. The samples obtained from patients during this study are summarised in Tables 2.1 and 2.2.
### SGA non-catch up group

<table>
<thead>
<tr>
<th></th>
<th>Baseline Visit</th>
<th>1 Month Visit</th>
<th>4 Month Visit</th>
<th>8 Month Visit</th>
<th>12 Month Visit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthropometric</td>
<td>9</td>
<td>6</td>
<td>7</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Measurements</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood samples</td>
<td>7</td>
<td>2</td>
<td>6</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Skin biopsies</td>
<td>5</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

### SGA with catch up group

<table>
<thead>
<tr>
<th></th>
<th>20</th>
<th>N/A</th>
<th>N/A</th>
<th>N/A</th>
<th>N/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthropometric</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Measurements</td>
<td>13</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Blood samples</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.1 Patient recruitment and sample acquisition over the course of the study.** At the initial visit anthropometric measurements were obtained on 9 children born small-for-gestational age (SGA) without post-natal catch-up growth and 20 from SGA children with post-natal catch-up growth. The number of subsequent measurements and samples obtained at each visit from these children is documented above. N/A – not available.
Table 2.2. Characteristics of cells from SGA children without post-natal catch-up growth, and the control cells from appropriate-for-gestational age children with no post-natal growth restriction. These cells are both sex- and age-matched as pre-pubertal. *No age at time of biopsy was available from the AGA control cells, but were pre-pubertal.

<table>
<thead>
<tr>
<th>Cells from SGA children without post-natal catch up growth</th>
<th>Sex</th>
<th>Age at time of biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP</td>
<td>Female</td>
<td>6.4</td>
</tr>
<tr>
<td>SGA001</td>
<td>Male</td>
<td>4.2</td>
</tr>
<tr>
<td>SGA003</td>
<td>Female</td>
<td>4.6</td>
</tr>
<tr>
<td>SGA009</td>
<td>Male</td>
<td>7.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cells from AGA children with no post-natal growth restriction</th>
<th>Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>C6</td>
<td>Male</td>
</tr>
<tr>
<td>C10</td>
<td>Male</td>
</tr>
<tr>
<td>C54</td>
<td>Female</td>
</tr>
<tr>
<td>C56</td>
<td>Female</td>
</tr>
<tr>
<td></td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>*</td>
</tr>
</tbody>
</table>
2.2 Biochemical assays

Fasting blood samples were obtained from children born SGA with and without catch-up growth at study entry. Samples for IGF-I and adiponectin measurements were collected in plain tubes and immediately put on ice. Samples for insulin analysis were collected in citrate tubes whereas samples for glucose analysis were collected in fluoride oxalate tubes (BISS, Bradford, UK). All samples were spun at 5,000g for 10 minutes and the serum removed and stored at -80°C until analysis. All calculations were performed in Microsoft Excel 2003 and statistical analysis was undertaken using Mann Whitney testing in SPSS (SPSS Chicago, USA).

2.2.1 Serum IGF-I measurements

Serum IGF-I levels were measured in 13 participants from the small-for-gestational age catch-up (SGACU) group and 7 participants from the small-for-gestational age non catch-up (SGANCU) group.

Concentrations of total IGF-I were determined using an ELISA from Beckman Coulter (Beckman Coulter Ltd, High Wycombe, UK). IGF-I is dissociated from its binding partners using an acidic buffer, then once neutralised, an excess of IGF-II is added to each sample; this IGF-II binds to the binding proteins instead of IGF-I, thereby ensuring that IGF-I is available for detection by the specific anti-IGF-I antibody. The intra- and inter-assay CV was <4.2% and <8.1%, respectively. Kit standards of known concentrations were measured alongside the 20 study samples which were run in duplicate.

The mean absorbance of the standards was then plotted against concentration, and a linear trend line was applied. The line equation was then used to determine the IGF-I concentrations from the mean absorbance of each study sample.
2.2.2 Fasting Glucose Measurements

Fasting glucose levels were measured in 13 samples from the SGACU group and 6 from the SGANCU group. Glucose was measured using an ELISA kit from Mercodia (Mercodia, Sweden) according to the manufacturer’s protocol. The intra- and inter-assay CVs were <5%. Kit standards of known values were run alongside the study samples, which were measured in duplicate. The concentration of glucose in the study samples was determined using the method described in section 2.2.1.

2.2.3 Serum Insulin Measurements

Insulin levels were measured in 11 samples from the SGACU group and 6 from the SGANCU group. Insulin was measured using an ELISA kit from Mercodia, according to the manufacturer’s guidelines. The intra- and inter-assay CV was <2.1% and <10% respectively. Standard samples were run with the study samples and absorbance readings used to calculate concentration by a cubic spline regression of the standard samples.

2.2.4 Serum Adiponectin Measurements

Total adiponectin was measured in 13 samples from the SGACU group and 6 from the SGANCU group. Adiponectin was measured using an ELISA kit from R & D systems (Abingdon, UK). The intra- and inter-assay CV were both <6%. Standard samples were provided of known concentrations, which were read at 450nm with the study samples and their absorbance plotted against concentration on a log graph. A linear trend line was added and the line equation used to calculate the study sample concentrations.
2.3 Cell culture

2.3.1 Establishing fibroblasts from skin biopsies

Punch hole skin biopsies roughly 5mm³ in size were taken (by Dr Philip Murray) from the forearm of three SGA children without post-natal catch-up growth (Table 2.2) under sterile conditions. Each biopsy was firstly cut into four pieces with a scalpel and then attached to the bottom of a 25cm² or ‘T25’ (Corning) vented cell culture flask using a thin layer of cell culture media (GM; Dulbecco’s modified eagles serum (DMEM) low glucose, 10% fetal bovine serum (FBS), 2mM glutamine, 50IU penicillin, 50IU streptomycin, 2.5ug/ml amphotericin B; Sigma Aldridge, Dorset, UK). The flask was then turned upside down (segmented skin biopsy remained attached to the top of the flask) and placed in an incubator at 37°C, 5% CO₂. After 2 weeks, the skin biopsy had a thin layer of cells growing out from the segments and the flasks were turned the correct way up. These cells and biopsies were then maintained in 5mls of GM at 37°C, 5% CO₂. Cells were also maintained in 75cm² flasks (T75) and 225cm² flasks (T225).

2.3.2 Establishing a fibroblast cell line

Cells grown from the biopsy were maintained in GM at 37°C, 5% CO₂ and the media was changed every 2–3 days. When the cells reached 80-90% confluence, GM was removed from the flask and the cells were washed twice in sterile phosphate buffered saline (PBS). 1ml (for a T25) or 2mls (for a T75) of 0.25%trypsin/0.02%EDTA (Sigma, Dorset, UK) was added and left to incubate for 5 minutes at 37°C. This reaction was then neutralised by the addition of 10mls of GM. Cells were no longer adherent, and were removed from the tissue culture flask along with the GM. The suspension was spun at 4000g for 5 minutes. The supernatant of media and trypsin was then removed and the cell pellet was re-suspended in 1ml of freezing media containing 700µl GM, 200µl FBS and 100µl of dimethyl sulphoxide (DMSO; Sigma, Dorset, UK). After re-suspension this was then transferred to cryovials and stored at -80°C overnight. After 24 hours, the cryovials were then placed in liquid nitrogen for long term
storage. A total of 20 vials in liquid nitrogen were maintained per cell line throughout this study.

2.3.3 Characterising fibroblasts

The fibroblasts were characterised to ensure that no other cell types, for example keratinocytes and endothelial cells, were present in the culture. Fibroblasts were identified by their morphology as they are distinct, long striated adherent cells (Dickersin and Scully 1993). However, they were also characterised by immunocytochemical assessment of the cell surface marker, thymus epithelia-7 (TE-7) (Pilling et al 2009), which is known to be specific for cells from mesodermally-derive connective tissue.

Cells grown from the biopsies were trypsinised and plated at $1 \times 10^4$ onto cover slips in 12 well plates with 1.5mls of GM. 24 hours later, the GM was removed and the cover slips were washed 3 times in sterile PBS for 5 minutes. Cells were then fixed in 3% paraformaldehyde in PBS for 15 minutes at room temperature and washed in PBS as previously described. Cells were then permeabilised using 0.1% Triton x-100 in PBS for 10 minutes at room temperature and the wash step was repeated. Non-specific binding sites were then blocked in 10% bovine serum albumin (BSA) for 30 minutes at room temperature before incubating with an anti-TE-7 antibody (Abcam, Cambridge) at a 1:50 concentration in blocking buffer for 1 hour at room temperature (manufacturer’s guidelines). The antibody solution was then removed and the wash step repeated. 4’,6-diamidino-2-phenylindole (DAPI)-containing mounting media was used to mount the cover slips onto slides (DAPI is a blue nuclear stain) and staining was visualised using fluorescent microscopy (see Figure 2.1).
Figure 2.1 TE-7-stained cells grown from skin biopsies from children born small-for-gestational age without post-natal catch-up growth. Image is representative of four SGA cell lines staining positively for the fibroblast marker TE-7 (green). Nuclei are stained blue using DAPI. Cells were fixed in 3% paraformaldehyde in PBS for 15 minutes at room temperature and then washed in PBS. Cells were then permeabilised using 0.1% Triton x-100 in PBS for 10 minutes at room temperature and the wash step repeated. The cells were then blocked in 10% BSA PBS for 30 minutes at room temperature before incubating with a TE-7 antibody (Abcam, Cambridge) at a 1:50 concentration in blocking buffer for 1 hour at RT (manufacturer’s guidelines). The antibody solution was then removed and the wash step repeated. 4',6-diamidino-2-phenylindole (DAPI) containing mounting media was used for mounting the stained cells under a cover slip. These experiments were performed on four separate occasions using cells from both control and SGA participants.
2.3.4 Maintaining fibroblasts in culture

Cells were maintained in GM at 37°C, 5%CO₂ and the media was changed every 2–3 days. Upon reaching 80-90% confluence, cells were split by trypsinisation as described earlier (section 2.2.3) with 10mls of GM added post-centrifugation and seeded into new flasks in a 1:4 ratio. GM volume was maintained at 5mls per T25 flask, 10mls per T75 flask and 30mls per T225 (225cm³) flask.

2.3.5 Cell counting

Cells were trypsinised as described previously (section 2.2.3) and the cell pellet was re-suspended in 1ml of GM. 10µl of the suspension was added to a hycor glastic™ slide and cells were counted in 10 grids using a light microscope (x10 objective) and averaged. Following the manufacturer’s guidelines on the slide insert, the number was then multiplied by a given factor and the number of cells per µl of the suspension determined.

2.3.6 Cell lysis experiments

Cells were maintained as described in section 2.2.4 and upon reaching 80-90% confluence, GM was removed and cells were then incubated in serum- free GM for 24 hours. Cells were then stimulated with either 200ng/ml of GH (Novo Nordisk) or 100ng/ml of IGF-I (Sigma Aldrich). At baseline, 15 or 30 minutes post-stimulation, the growth factor-containing medium was removed and the cells were washed in sterile PBS. 800µl of PBS was then used as a suspension fluid and the cells were scraped from the flask, removed and spun for 15 seconds at 13,000 rpm. The supernatant was then removed and 100µl of lysis buffer (100nm NaCl, 5mM EDTA, 50nM TRIS, 1% Triton, pH 7.4) was added and the sample was vortexed. These lysates were then left rocking at 4°C for 30 minutes before spinning at 13,000rpm for 5 minutes. The supernatant, containing protein from the fibroblasts, was then removed and stored at -80°C. Figure 2.2 gives an overview of this process.
Figure 2.2 A schematic to demonstrate the process of creating a fibroblast cell lysate. Fibroblasts at 80-90% confluence were starved in serum-free growth media (Dulbecco’s modified eagle’s serum (DMEM) low glucose, 2mM glutamine, 50IU penicillin, 50IU streptomycin, 2.5ug/ml amphotericin B) for 24 hours. Growth factor (GH or IGF-I) was added for 15 or 30 minutes, then cells were washed in sterile PBS before being scraped into a PBS suspension. This suspension was centrifuged for 60 seconds at 13,000 rpm and the supernatant removed. 100µl of lysis buffer (100nm NaCl, 5mM EDTA, 50nM TRIS, 1% Triton pH 7.4) was added to the pellet and the tube vortexed to resuspend the pellet.
2.3.7 Protein estimation

Protein levels from fibroblast lysates were determined using the Biorad Bradford Assay (Biorad, UK). Standards were made using bovine serum albumin (BSA) (Invitrogen) of 5mg/ml, 2mg/ml, 1mg/ml, 0.5mg/ml, 0.2mg/ml and 0.1mg/ml to create a standard curve. The absorbance of the standards and lysates (measured at 450nm in triplicate) was recorded and the protein concentration calculated using the curve equation determined from analysis of the standards.

2.4 Characterisation of cell proliferation

Basal proliferation was characterised by cell counting and by assessing bromodeoxyuridine (BrdU) incorporation. Three separate experiments were undertaken in triplicate for each method of characterising proliferation; the three technical repeats were used to determine a mean value for each of the three experiments. Cells from four SGA participants, along with CAM1 and 3 control cell lines, were analysed. Statistical analysis was undertaken using a two-way ANOVA in SPSS (SPSS, Chicago).

2.4.1 Cell counting experiments

Cells were seeded into 6 well plates at $1 \times 10^4$ cells / well and maintained in GM at 37°C, 5%CO$_2$ for 24 hours before starting the experiment. Cells were then trypsinised after 24 hours, and at each subsequent 24 hours for 10 days, and then counted as described in section 2.3.5. A total of 3 experiments were performed, with each experiment containing 3 technical repeats per cell line, per time point. Growth factor-stimulated proliferation was measured over a 5 day period.

2.4.2 Bromodeoxyuridine (BrdU) incorporation

BrdU is a thymidine analogue and is incorporated into the DNA of dividing cells (Beisker et al 1999). BrdU incorporation was measured using a kit from Roche (Roche, Sussex, UK) which provides BrdU for incorporation and an anti-BrdU
antibody for detecting incorporated BrdU by immunocytochemistry and light microscopy. Cells were seeded onto cover slips in 12 well plates at 1 x 10^4 cells/well and maintained in GM at 37°C, 5%CO₂. Media containing 10mol/l BrdU was placed in the well plates for 24 hours, then removed and replaced with normal GM. Cells were analysed each day for ten days and BrdU incorporation was identified and measured according to the Roche protocol. First, cells were fixed in ethanol for 2 minutes at -20°C then washed three times in washing buffer. Second, the cells were covered with anti-BrdU working solution, incubated for 30 minutes at 37°C and afterwards washed 3 times as previously described. Third, the cells were covered in an anti-mouse IgG antibody linked to alkaline phosphatase, incubated for 30 minutes at 37°C and then washed as previously described. Fourth, the cells were covered with the colour substrate solution for 15-20 minutes at room temperature. Five, the cells were counterstained with Gills Haematoxylin solution (Sigma, Dorset, UK) and examined under a light microscope. Three fields of 100 cells were counted for percentage BrdU incorporation and an average obtained. Three cover slips per time point were examined and three independent experiments were performed in total.

2.4.3 Growth factor stimulation of proliferation

Cells were maintained in serum-free media for 24 hours and then vehicle (PBS), 200ng/ml or 20ng/ml GH, 100ng/ml or 10ng/ml IGF-I, or a combination of GH and IGF-I (at 100ng/ml plus 50ng/ml respectively or 10ng/ml plus 5ng/ml respectively) was added on day zero. After 24 hours, and at each subsequent 24 hours for 5 days, cells were counted as previously described in section 2.3.5. This timescale was informed by the experiments performed to investigate basal cell proliferation over 10 days. Control cells were run alongside SGA and CAM1 cells in each experiment. Statistical analysis of data was performed using a two-way ANOVA to determine the effect of time, cell line and the combination of time with cell line on cellular proliferation.
2.5 Characterisation of apoptosis

Apoptosis (stimulated by maintaining cells in serum-free media) was assessed using two assays: terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) staining and a cleaved caspase 3 assay.

Cells from four SGA participants were measured, along with CAM1 and 3 control cell lines. Statistical analysis was undertaken using SPSS (SPSS, Chicago). Three experiments (performed in triplicate) were undertaken for each method of characterising apoptosis.

2.5.1 Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labelling (TUNEL)

Cell were seeded onto cover slips in 12 well plates at $1 \times 10^4$ cells / well and maintained in GM for 24 hours. GM was removed the next day and serum-free GM was added to induce apoptosis. Cover slips were harvested every 24 hours for 10 days. A total of three experiments were performed with three technical repeats per experiment, per cell line per time point. At each time point, the serum-free GM was removed and the cells were washed twice with PBS. The cells were then fixed in 4% paraformaldehyde for 1 hour at room temperature and permeabolised using 0.1% Triton X 100 in 0.1% sodium citrate for 20 minutes. The cover slips were then incubated with TUNEL staining reagents as described in the Roche protocol. DAPI containing mounting media was then used to mount the cover slips onto slides and TUNEL staining was determined by fluorescence microscopy. Three fields per cover slip were assessed, using DAPI-stained nuclei to count 100 cells and then counting these using a different fluorescent channel to score the TUNEL-stained nuclei, therefore determining the percentage of cells staining positive for TUNEL. The average of three fields for each technical repeat was calculated and then the average of the three technical repeats was used to give an $n = 3$ per cell line per time point over the three experiments.
2.5.2 Cleaved caspase-3 assay

Caspase-3 cleavage is a feature of the majority of pathways which results in apoptosis and is therefore used as an indicator of the apoptotic process (Karran and Hennighausen 2001). Cells were incubated in serum-free GM for 10 days and cleaved caspase-3 activity was determined every 24 hours by ELISA (cell signalling technology), following the manufacturers protocol. Cell lysates containing 20µg protein were diluted 1:1 in sample diluent. 100µl of the diluted sample was then added to a well containing an anti-body to cleaved caspase-3 (in triplicate). Cleaved caspase-3 activity was calculated by the sample’s absorbance at 450nm, compared to the kit standards using a linear trend line equation. This experiment was performed on three separate occasions.

2.5.3 Multi-target apoptosis ELISA

The multi-target ELISA (cell signalling technology) detects levels of Bad, phospho Bad, cleaved caspase-3, p53, phospho p53 and p38. Using no serum, apoptosis was induced starving the SGA and control cells for 5 days, and then the cells were lysed according to the manufacturer’s protocol. Lysates containing a total protein value of 25µg per well were diluted 1:1 with sample diluent and 100µl of each diluted cell lysate was added to a well containing antibody to the aforementioned molecules, sealed with tape and incubated overnight at 4°C. The wells were then washed by discarding the contents of each well and adding 200µl of wash buffer and striking plates onto paper towels, repeating four times. 100µl of detection antibody was added to each well and incubated for 1 hour at 37°C. The wash step was then repeated as described previously, and 100µl of horse radish peroxidase (HRP)-linked secondary antibody was added to each well and incubated for 30 minutes at 37°C. The wash step was then repeated and 100µl of TMB substrate was added to each well and the plate was incubated for 10 minutes at 37°C. 100µl of STOP solution was then added to each well and the absorbance was read at 450nm.
2.5.4 Western blotting of proteins

20µg of total protein (determined as described in section 2.3.7) per sample (approximately 10µl of lysate) was mixed 1:1 (v:v) with loading buffer (62.4 mM TRIS-HCL, 2.5% SodiumDS, 0.002% bromophenol blue, 10% glycerol, 1mM mercaptoethanol) then samples were boiled at 100°C for 5 minutes and put on ice until loading.

All investigations were undertaken using a Bio-Rad Protean III gel system using an 8 or 10% acrylamide, 0.375M TRIS-HCL pH 8.8, 0.1% SodiumDS, 0.01% APS and 0.001% TEMED resolving gel with a 3% acrylamide, 0.125M Tris Cl pH 6.8, 0.1% SDS, 0.01% APS, 0.001 TEMED stacking gel. All gels were run at 40V for 30 minutes, then 100V for 1 hour 30 minutes in running buffer (48mM Tris Base, 30mM Glycine, 0.1% SodiumDS). Proteins within the gels were then transferred onto nitrocellulose in 1mM TRIS, 1mM glycine, 10% methanol buffer at 70 V for 1 hour at room temperature. Upon completion of the transfer, membranes were washed for 5 minutes in Tris buffered saline, 0.02% Tween 20 pH 8.8 (TBST) and incubated overnight at 4ºC in 5% Marvel TBST solution.

Membranes were then incubated in primary antibodies for one hour at room temperature with gentle rocking. Each individual primary antibody was used at an optimised concentration in either TBST alone, or TBST containing 5% BSA (see Table 2.3). Membranes were then washed 3 times for ten minutes in TBST and incubated in the appropriate HRP-conjugated secondary antibody for one hour at room temperature with gentle rocking. The wash step was repeated and proteins were visualised using enhanced chemiluminscence and exposure to autoradiography film.

2.5.5 Densitometry and analysis

Western blots were scanned and the resulting images were saved as JPEG files in Adobe Photoshop 3.2. Densitometric analysis was undertaken using Image J software (NIH, USA). A set box size was used to analyse the intensity of each of the bands on the western blot and the mean value per experiment was
calculated using Microsoft Excel 2003. Measurements from the back ground of each Western blot were also taken and averaged for each blot and subtracted from the band intensity to control for varying background intensity on the photographic film. Statistical analysis (Mann Whitney U test) was undertaken in SPSS (Chicago).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Diluent</th>
<th>Species</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Stat5</td>
<td>1/1000</td>
<td>5% BSA TBST</td>
<td>Mouse</td>
<td>CST</td>
</tr>
<tr>
<td>Stat5b</td>
<td>1/1000</td>
<td>5% BSA TBST</td>
<td>Mouse</td>
<td>Zymed</td>
</tr>
<tr>
<td>pMAPK</td>
<td>1/1000</td>
<td>TBST</td>
<td>Rabbit</td>
<td>CST</td>
</tr>
<tr>
<td>MAPK</td>
<td>1/1000</td>
<td>TBST</td>
<td>Rabbit</td>
<td>CST</td>
</tr>
<tr>
<td>pIRS-1</td>
<td>1/1000</td>
<td>TBST</td>
<td>Rabbit</td>
<td>CST</td>
</tr>
<tr>
<td>IRS-1</td>
<td>1 in 2000</td>
<td>5% BSA TBST</td>
<td>Mouse</td>
<td>CST</td>
</tr>
<tr>
<td>pAkt(ser473)</td>
<td>1 in 1000</td>
<td>5% BSA TBST</td>
<td>Rabbit</td>
<td>CST</td>
</tr>
<tr>
<td>pAkt(Thr308)</td>
<td>1 in 1000</td>
<td>5% BSA TBST</td>
<td>Rabbit</td>
<td>CST</td>
</tr>
<tr>
<td>Akt</td>
<td>1 in 1000</td>
<td>5% BSA TBST</td>
<td>Rabbit</td>
<td>CST</td>
</tr>
<tr>
<td>pJNK</td>
<td>1 in 1000</td>
<td>5% BSA TBST</td>
<td>Rabbit</td>
<td>CST</td>
</tr>
<tr>
<td>pP38</td>
<td>1 in 500</td>
<td>TBST</td>
<td>Rabbit</td>
<td>CST</td>
</tr>
<tr>
<td>Akt1</td>
<td>1 in 500</td>
<td>5% BSA TBST</td>
<td>Rabbit</td>
<td>CST</td>
</tr>
<tr>
<td>Akt2</td>
<td>1 in 1000</td>
<td>5% BSA TBST</td>
<td>Rabbit</td>
<td>CST</td>
</tr>
<tr>
<td>Akt3</td>
<td>1 in 1000</td>
<td>5% BSA TBST</td>
<td>Rabbit</td>
<td>CST</td>
</tr>
<tr>
<td>IGF-IR</td>
<td>1 in 1000</td>
<td>5% BSA TBST</td>
<td>Rabbit</td>
<td>CST</td>
</tr>
<tr>
<td>IR</td>
<td>1 in 1000</td>
<td>5% BSA TBST</td>
<td>Rabbit</td>
<td>CST</td>
</tr>
</tbody>
</table>

Table 2.3 Information on primary antibodies used for Western blotting

2.5.6 Western blotting of signalling molecules

Western blots were performed as described in section 2.5.4. Information on the antibodies used in this study is given in Table 2.3. Secondary antibodies used were all from CST 1/1000 dilution.

2.5.7 Immunoprecipitation

500µg of total protein were required for immunoprecipitation (IP) of the Akt isoforms, thus cells in a T225 flask were incubated in serum-free GM for 24 hours and then either vehicle (PBS) or 100ng/ml IGF-I was added to the media and cells were lysed 15 minutes later. Samples were pre-cleared with protein A
dynabeads (silicone beads that bind to antibodies with high affinity; Invitrogen) by adding 20µl of a 50% bead slurry and incubating at 4°C, with rotation, for 1 hour to ensure that any proteins that would interact with the dynabeads were removed prior to sample processing. The samples were then spun at 13,000rpm at 4°C for 10 minutes and the supernatant transferred to another tube. Figure 2.3 presents a schematic of the immunoprecipitation process.

2.5.8 Phospho-array

SGA and control cells were serum-starved for 24 hours (baseline) then cells were stimulated with 100ng/ml IGF-I for 15 minutes. Lysates were prepared as described in section 2.2.6 then analysed using a phospho-array (R & D Systems) in accordance with the kit protocol (Figure 2.4).

Four arrays per SGA cell line were used in this experiment: one for each of the lysates from SGA and control cells at baseline (unstimulated) lysate and one for each of the lysates from SGA and control cells stimulated with IGF-I. Each SGA and control sample consisted of a pool of lysates obtained from four separate experiments. Primary antibody (diluted 1:50 in accordance with the manufacturer’s guidelines) was incubated overnight in the supernatant at 4°C with gentle rocking. Protein A beads were added (20µl of a 50% bead slurry) and incubated with gentle rocking for 3 hours at 4°C and then samples were spun at 10,000rpm at 4°C for 30 seconds and the supernatant removed. The bead pellet containing the adhered antibody and protein from the cell lysates was washed five times in 200µl of ice-cold PBS, being careful to keep the samples on ice at all times. The pellet was then re suspended in 5µl of SDS loading buffer, vortexed and spun at 10,000rpm for 1 minute. Samples were then heated at 100°C for 5 minutes to disassociate the protein from the dynabeads and spun at 10,000rpm for 1 minute. The supernatant was then analysed by Western blotting. Experimental control samples included a total protein lysate from control cells stimulated with IGF-I for 15 minutes, which was
Figure 2.3 A schematic of the immunoprecipitation process. Antibodies specific to Akt isoforms are incubated in a 1:50 dilution with pre-cleared lysate for one hour at room temperature. Dynabeads are then added to the solution and incubated for 3 hours at room temperature. The sample is centrifuged at 13,000rpm for 30 seconds and the beads with antibody and Akt isoform attached are pellet-precipitated from the cell lysate. The supernatant is removed and then the precipitate is boiled in sample buffer to dissociate the Akt isoform from the bead-antibody complex. The sample is then spun and the supernatant containing the immunoprecipitated Akt isoform is removed and stored at -80°c until use.
**Figure 2.4 Analysis of lysates from SGA and control cells by phosphoarray.** A) Protein lysates are incubated with a membrane embedded with antibodies to phospho proteins. After washing, the bound phospho proteins are visualised using a pan phosphor-tyrosine antibody conjugated to HRP followed by chemiluminescence and exposure to X-ray film. B) A representative phosphoarray. Each dot represents a different embedded phospho antibody (in duplicate). Intensity in variable due to different levels of the phospho proteins present in a cell lysate.
used as a positive control for Akt activation, and a sample that went through the IP process but with the omission of primary antibody which was used as a control for IP specificity. Analysis was undertaken by densitometry as described in section 2.5.5. Arrays were scanned and analysed using Image J software as described in section 2.5.5. The mean baseline array measurements were subtracted from the IGF-I stimulated array, after both being corrected for background levels and negative controls. A SDS of +/- 1 was used to define proteins of significant increased or decreased activation in these experiments.

2.6 Metabolomics

2.6.1 Sample acquisition

All cell lines were at the same passage when processed and each line was assessed in sextuplicate. Cells were grown in T225 vented cell culture flasks and maintained in GM until 80–90% confluence, then the media was removed (stored immediately at -80°C) and the cells were washed in 5mls of PBS at 4°C and the PBS wash stored at -80°C. The cells were then quenched using 5mls of an 80% methanol solution kept at -40°C, scraped off the flask and then aliquoted into a 15ml falcon tube. The aliquot then underwent three freeze-thaw cycles, which involved 30 second incubation in liquid nitrogen followed by thawing on wet ice. Samples were then spun at -10°C for 2 minutes at 5000rpm. The supernatant was then removed and stored immediately at -80°C.

The samples were then transferred to Dr Warwick Dunn in the Manchester Innovation Building, where the samples were evaporated to dryness, and then, upon analysis, reconstituted in 90% dH2O 0.1% formic acid. Samples were next analysed by gas chromatography tandem mass spectroscopy by the Agilent Q time of flight analyser.
2.6.2 Analysis

Analysis was kindly performed by Dr Marie Brown, University of Manchester, using Matlab® (http://www.mathworks.com). Exploratory multivariate analysis was performed using principal components analysis (PCA), which is an unsupervised approach that transforms a large set of related variables into a new, smaller set of independent variables, termed principal components (PCs) (Joliffe and Morgan 1992). Each PC represents an axis in multidimensional space and corresponds to the direction of maximum variation of the original data. PCA was performed on data normalised to zero mean and unit variance, so that results were not dominated by a small number of high intensity peaks but gave equal weighting to peaks of low intensity.

Univariate statistical analysis was performed, using the non-parametric Mann Whitney U-test to determine those metabolites showing a statistically significant difference (p-value <0.05) between classes under observation. All missing values were replaced by NaN for univariate analysis and 0 for PCA.
Chapter 3  Growth and biochemical characteristics of small for gestational age infants, with and without post-natal catch-up growth
3.1 Participants

Children who are born small for gestational age (SGA) are routinely followed by their neonatal consultant to assess whether catch-up growth will occur. 90% of such children will catch-up within 2–3 years, and in the absence of other health problems are discharged. In the 10% who fail to catch-up, longer-term follow-up is indicated with referral to a growth clinic for consideration for recombinant human growth hormone (rhGH) treatment. In fact only a proportion of such children find their way to growth clinics. If the following criteria are fulfilled – Current Height SDS <-2.5, Parent-adjusted Height SDS <-1, Age >4 years – then an SGA child who has failed to catch-up is eligible for rhGH treatment.

The work in this thesis has focused on comparing *in vitro* functions in cell lines from SGA children who have not caught up compared to controls, and comparing metabolic status in those that catch-up compared to those that do not. In this chapter the aims were:

- To compare the auxological and biochemical parameters of SGA children with and without catch-up growth at baseline, and

- To determine the effect of 1-year rhGH treatment on post-natal growth, and verify that the response in these patients is representative of that previously reported in short SGA children treated with rhGH

3.1.1 Patient characteristics and measurements

Over the course of this research project, 28 children born small for gestational age were identified, 19 of whom demonstrated postnatal catch-up growth, and 9 who did not (current height <-2SDS and parental adjusted height SDS <-1) (Table 3.1). There were 13 male and 6 female participants in the small for gestational age group with catch-up growth (SGACU group) and 6 male and 3 female participants in the small for gestational age group without catch-up.
Table 3.1 Birth parameters of participants in the SGA non-catch up group (NCU) and of the SGA catch up group (CU). Parameters include sex (M = male, F=female), ethnicity (WE = white European, MR = mixed race and SA = south Asian), mid-parental height SDS, birth weight, gestation (weeks) and birth weight SDS.
growth (SGANCU group). The median age of the SGANCU group was 5.1 years compared to 6.6 years in the SGACU group; p=ns.

The height, weight and body mass index of all children were measured at the start of the study in the two SGA groups, and are shown in Table 3.1. In the SGANCU group these measurements were also recorded at 1, 4, 8 and 12 months after starting rhGH therapy. All measurements were undertaken by paediatric research nurses by validated methods, taken twice and the average recorded for this study.

Serum IGF-I, insulin, glucose and adiponectin were measured in both SGA groups at the start of the study, and at the aforementioned time points post rhGH in the SGANCU group. Not all participants were available at all time points for measurements and testing.

Dosing of rhGH was undertaken by the clinical paediatric endocrinology team at the Royal Manchester Children’s hospital according to the NICE guidelines for treating growth disturbance in children born small for gestational age. Median rhGH dose for the SGANCU group was 1.0mg, and this remained the same throughout the course of this study.

### 3.2 Height

At the start of this study, as expected, the mean height SDS in the SGANCU group was -2.9 ±0.38 (Table 3.2), compared to -0.58±0.9 in the SGACU group (p<0.001).The mid-parental adjusted height for the SGANCU group were all <-1 SDS, in line with the National Institute for Health and Care Excellence (NICE) guidelines indicating use of rhGH in short children born small for gestational age (Table 3.1).

Figure 3.1A illustrates height, using the measurements from the SGANCU group, during the course of 1 year of rhGH treatment in this study. On average, our SGANCU group improved in height by 6.09cm. Figure 3.1B illustrates the
<table>
<thead>
<tr>
<th>Patient Number</th>
<th>group</th>
<th>DOS</th>
<th>Age at sampling (yrs)</th>
<th>Ht (cm)</th>
<th>Ht SDS</th>
<th>Wt (Kg)</th>
<th>Wt SDS</th>
<th>BMI</th>
<th>BMI SDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGA001</td>
<td>NCU</td>
<td>28/03/2008</td>
<td>4.62</td>
<td>95.9</td>
<td>-2.50</td>
<td>10.80</td>
<td>-4.85</td>
<td>11.74</td>
<td>-4.80</td>
</tr>
<tr>
<td>SGA003</td>
<td>NCU</td>
<td>07/05/2008</td>
<td>4.24</td>
<td>94.8</td>
<td>-1.89</td>
<td>10.50</td>
<td>-4.16</td>
<td>11.68</td>
<td>-4.17</td>
</tr>
<tr>
<td>SGA004</td>
<td>NCU</td>
<td>08/05/2008</td>
<td>5.09</td>
<td>94.2</td>
<td>-3.50</td>
<td>11.22</td>
<td>-5.04</td>
<td>12.64</td>
<td>-3.18</td>
</tr>
<tr>
<td>SGA010</td>
<td>NCU</td>
<td>15/09/2008</td>
<td>4.68</td>
<td>90.0</td>
<td>-3.94</td>
<td>14.88</td>
<td>-1.64</td>
<td>18.37</td>
<td>2.01</td>
</tr>
<tr>
<td>SGA015</td>
<td>NCU</td>
<td>31/10/2008</td>
<td>6.83</td>
<td>108.7</td>
<td>-2.21</td>
<td>15.60</td>
<td>-2.76</td>
<td>13.20</td>
<td>-1.77</td>
</tr>
<tr>
<td>SGA019</td>
<td>NCU</td>
<td>09/04/2009</td>
<td>7.67</td>
<td>114.2</td>
<td>-2.15</td>
<td>18.46</td>
<td>-2.35</td>
<td>14.15</td>
<td>-1.16</td>
</tr>
<tr>
<td>SGA020</td>
<td>NCU</td>
<td>18/06/2009</td>
<td>4.09</td>
<td>92.4</td>
<td>-2.35</td>
<td>12.36</td>
<td>-2.46</td>
<td>14.48</td>
<td>-0.99</td>
</tr>
<tr>
<td>SGA022</td>
<td>NCU</td>
<td>06/08/2009</td>
<td>4.74</td>
<td>94.3</td>
<td>-2.97</td>
<td>11.80</td>
<td>-3.98</td>
<td>13.27</td>
<td>-2.39</td>
</tr>
<tr>
<td>SGA024</td>
<td>NCU</td>
<td>13/08/2009</td>
<td>4.01</td>
<td>84.3</td>
<td>-4.48</td>
<td>10.45</td>
<td>-4.47</td>
<td>14.70</td>
<td>-0.81</td>
</tr>
</tbody>
</table>

**Mean**

- **Ht (cm):** 96.53
- **Wt (Kg):** 12.90
- **BMI:** 13.81

**SD**

- **Ht (cm):** 9.24
- **Wt (Kg):** 2.80
- **BMI:** 2.03

**Number**

- **Ht (cm):** 9
- **Wt (Kg):** 9
- **BMI:** 9

**p value**

- **Ht (cm):** 0.019
- **Wt (Kg):** <0.001
- **BMI:** <0.001

---

Table 3.2 Baseline parameters of participants in the SGA non-catch up group (NCU) and from the SGA with catch up group (CU). Parameters include age (years), height (cm), height SDS, weight, weight SDS, BMI and BMI SDS.
A)

![Graph](A.png)

B)

![Graph](B.png)

Figure 3.1 Height in centimetres (A) and in standard deviation scores (B), over the first year of rhGH treatment in short children born small for gestational age. Height was measured at baseline, 1, 4, 8 and 12 months over the first year of GH treatment.
height SDS over 1 year of rhGH, and there was a mean change in height SDS of +0.47 (Table 3.2).

The average first year response to rhGH in short children born small for gestational age from the KIGS database on 613 patients, was 8.7 cm equating to a height increment of +0.7 SDS (Ranke et al 2003). In this study the mean growth velocity was greater at 11cm; however, the increase in height SDS was comparable at +0.58 (0.47 in this study). It is important to demonstrate a comparable height increment in SDS in this study compared to a much larger cohort as it validates this study group as a typical example of children born small for gestational age without post-natal catch-up growth.

As shown in Figure 3.1, there is considerable variation in response to rhGH within this group. This is a well-recognised phenomenon in any condition treated with rhGH, with significant variation in growth response being seen at any given dose of rhGH. It is possible to predict some of this variability based on baseline characteristics such as age at start of treatment, rhGH dose, weight SDS and mid-parental height SDS ($r^2=52\%$) (Ranke et al 2003), but the remainder is unexplained, hence the need to better understand underlying biological mechanisms in tissue from SGANCU children.

### 3.3 Weight

At the start of this study the SGANCU group had a mean weight SDS of -3.52 whereas the SGACU group were significantly heavier with a mean weight SDS of -0.78, p<0.001 (Table 3.2). 7 out of 8 in the SGANCU group have post-natal growth failure in both height and weight.

Figure 3.2A shows the weight and Figure 3.2B the weight SDS of the participants during the first year of rhGH. Mean weight increased by 3.52kg in the first year of GH therapy, which equates to an increase in SDS of +0.4.

The initial phase on GH therapy is associated with significant anabolic changes – including loss of subcutaneous fat and an increase in lean mass. Overall, this
Figure 3.2 Weight measurements (A. in kg, B. as SDS) in the first year of rhGH therapy in short children born small for gestational age. Weight was measured at baseline, 1, 4, 8 and 12 months in the first year of rhGH.
usually results in an increase in weight as seen in our children. Data on BMI are presented to better capture changes in body composition.

3.4 Body mass index (BMI)

At baseline the average BMI in the SGANCU group was 13.9 ± 2.2 kg/m². This was significantly lower than the SGACU group whose BMI was 16.3 (p=0.001). The BMI SDS in the SGANCU group was -2 and 1.1 in the SGACU group.

After one year on rhGH, the BMI of the SGANCU group increased to 15.8 kg/m². This equated to an increase in BMI SDS, from -1.24 to -0.33.

This increase in BMI in the SGANCU group during the first year of rhGH implies a relative gain of weight greater than the gain in height, and will reflect the greater mass of the lean mass accrued.

3.5 Waist and hip measurements

At baseline, waist circumference in the SGANCU was smaller than that of the SGACU group (44 and 52cm, respectively) (p=0.001) (Table 3.3).

Figure 3.3 illustrates waist measurements taken at 0, 1, 4, 8 and 12 months during the first year of rhGH. In comparison to other auxological measurements fewer measurements of waist circumference were obtained. Only one measurement was taken at the 12-month point, and the most measurements were taken at one month. The mean waist circumference for the latter was 46.5 ± 0.6cm. At the 8 month visit (in which 3 participant measurements were taken) the mean waist circumference was 52.5 ±6.7cm, equating to an increase of 6.8cm.
<table>
<thead>
<tr>
<th>Patient Number</th>
<th>group</th>
<th>waist (cm)</th>
<th>hip (cm)</th>
<th>waist:hip ratio</th>
<th>Systolic BP</th>
<th>Diastolic BP</th>
<th>IGF-I (g/L)</th>
<th>Insulin (mU/L)</th>
<th>Adiponectin (mg/L)</th>
<th>Glucose (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGA001</td>
<td>NCU</td>
<td>43.6</td>
<td>45.2</td>
<td>0.96</td>
<td>116</td>
<td>80</td>
<td>70</td>
<td>1.24</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>SGA003</td>
<td>NCU</td>
<td>40</td>
<td>46</td>
<td>0.87</td>
<td>107</td>
<td>65</td>
<td>42</td>
<td>0.26</td>
<td>15.17</td>
<td></td>
</tr>
<tr>
<td>SGA004</td>
<td>NCU</td>
<td>39.5</td>
<td>45</td>
<td>0.88</td>
<td>83</td>
<td>35</td>
<td>48</td>
<td>3.36</td>
<td>4.57</td>
<td></td>
</tr>
<tr>
<td>SGA010</td>
<td>NCU</td>
<td>48.8</td>
<td>55</td>
<td>0.89</td>
<td>118</td>
<td>71</td>
<td>34</td>
<td>6.10</td>
<td>15.62</td>
<td></td>
</tr>
<tr>
<td>SGA015</td>
<td>NCU</td>
<td>48</td>
<td>49</td>
<td>0.96</td>
<td>93</td>
<td>45</td>
<td>92</td>
<td>4.40</td>
<td>6.16</td>
<td></td>
</tr>
<tr>
<td>SGA020</td>
<td>NCU</td>
<td>47</td>
<td>49</td>
<td>0.96</td>
<td>87</td>
<td></td>
<td></td>
<td></td>
<td>10.39</td>
<td></td>
</tr>
<tr>
<td>SGA022</td>
<td>NCU</td>
<td>44.5</td>
<td>48</td>
<td>0.93</td>
<td>102</td>
<td>57</td>
<td>79</td>
<td>11.33</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>mean</strong></td>
<td></td>
<td>43.90</td>
<td>48.03</td>
<td>0.91</td>
<td>106</td>
<td>61</td>
<td>69.0</td>
<td>4.1</td>
<td>10.4</td>
<td>4.1</td>
</tr>
<tr>
<td><strong>SD</strong></td>
<td></td>
<td>3.71</td>
<td>3.76</td>
<td>0.04</td>
<td>15</td>
<td>16</td>
<td>24.8</td>
<td>3.8</td>
<td>5.0</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Table 3.3 Baseline metabolic parameters from the SGA non-catch up group (NCU) and the SGA with catch up group (CU). Parameters include waist (cm), hip (cm), waist: hip ratio, systolic blood pressure (mmHg), diastolic blood pressure (mmHg), fasting IGF-I, Insulin (mU/L), adiponectin (mg/L) and glucose (mmol/L).
Figure 3.3 Waist circumference measurements in short children born small for gestational during 1 year of rhGH (n=6). Waist circumference (cm) was measured at baseline, 1, 4, 8 and 12 months during the first year of rhGH in this study.
Hip circumference at baseline was significantly reduced in the SGANCU group compared to SGACU, with a mean measurement of 48.5 cm and 59 cm respectively (p=0.001) (Figure 3.4A). At one month into this study – the time point at which the most measurements were taken – the mean hip circumference was 53cm; an increase of 5.5cm. It appears that within one month of rhGH the SGANCU group have a similar hip circumference to the SGACU group at baseline (Figure 3.4B). Over the first year of rhGH, only one measurement of hip circumference was obtained at the 12 month point. At the 8 month visit 3 were taken, with a mean of 53 ± 2.5 cm, an average increase of 5.5cm in hip circumference had occurred.

Unfortunately these data are inadequate for any major analysis, but do indicate that SGANCU have less truncal fat, and show an anabolic response to rhGH with an increase in these measurements.

3.5.1 Waist to hip ratio

Waist to hip ratio is an important determinant of cardiovascular risk factors, with a high ratio being favourable. At baseline the SGANCU and SGACU group have a comparable hip:waist (0.91 compared to 0.88, respectively; p=0.088). At one month into the study the hip to waist ratio had increased to 1.14, which suggests that after a month of rhGH the hip to waist ratio became more favourable. At 8 months post rhGH treatment, the hip to waist ratio had decreased to 1.01, which was not significant; however, it appears the one-month improvement in waist to hip ratio was an initial change that did not continue to the 8 month point in this study.

3.6 Blood pressure

Figure 3.5A depicts blood pressure (BP) at baseline in the SGANCU compared to SGACU group. At baseline, the mean systolic BP of the SGANCU group was similar to that measured in the SGACU group (102 ± 16.1 mmHg compared to A)
Figure 3.4 Hip circumference in SGANCU compared to SGANCU at baseline (a), and hip measurements in short children born small for gestational age during 1 year of rhGH (B). * relates to significant difference in hip circumference at baseline in the study between SGANCU infants and SGACU infants, p=0.001.
Figure 3.5 Blood pressure in SGANCU and SGACU groups at baseline, and SGANCU over 1 year of rhGH A) Blood pressure was measured at baseline in 32 SGA infants, 18 with post-natal catch-up growth and 6 without, full colour block represents systolic blood pressure, and hatched blocks represent diastolic blood pressure. B) Blood pressure was measured in 6 SGANCU participants in five clinic visits over 1 year of rhGH. Full lines are systolic blood pressure values, and interrupted lines are diastolic values.
97.2 ± 13.7 mmHg, p=0.658). After 12 months rhGH treatment in the SGANCU group, systolic BP increased by 5 to 107 ±15.9 mmHg. Diastolic BP in the SGANCU group was 54.6 ± 14 mmHg compared to 58.8 ±3.8 mmHg in the SGACU group (p=0.609). After 12 months rhGH treatment the diastolic BP increased by 9 to 65 ±13 mmHg in the SGANCU group.

### 3.7 Baseline biochemical tests

#### 3.7.1 IGF-I

IGF-I is a key regulator of post-natal growth (Klover and Hennighausen 2007) and is also used as a measure of response to rhGH therapy. 12 participants from the SGACU group and 8 from SGANCU group had IGF-I levels measured at baseline. Lower mean levels of IGF-I in the SGANCU group were observed compared to the SGACU group, with values of 69µg/L compared to 122µg/L (p=0.059) (Table 3.4 and Figure 3.6).

<table>
<thead>
<tr>
<th></th>
<th>NCU mean</th>
<th>SD</th>
<th>N</th>
<th>CU mean</th>
<th>SD</th>
<th>N</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I (µg/L)</td>
<td>69.0</td>
<td>24.8</td>
<td>8</td>
<td>122.0</td>
<td>63.4</td>
<td>12</td>
<td>0.059</td>
</tr>
<tr>
<td>Insulin (mU/L)</td>
<td>4.1</td>
<td>3.8</td>
<td>7</td>
<td>5.4</td>
<td>4.0</td>
<td>10</td>
<td>NS</td>
</tr>
<tr>
<td>Adiponectin (mg/L)</td>
<td>10.4</td>
<td>5.0</td>
<td>5</td>
<td>4.3</td>
<td>2.7</td>
<td>7</td>
<td>0.042</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>4.1</td>
<td>0.7</td>
<td>5</td>
<td>6.5</td>
<td>0.9</td>
<td>12</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Table 3.4 Mean levels of IGF-I, adiponectin, insulin and glucose at baseline in the SGANCU and SGACU groups.

#### 3.7.2 Glucose

Fasting glucose levels were measured in 17 SGACU and 5 SGACU infants. The SGANCU group had a significantly lower glucose level than SGACU, 4.1 compared to 6.3 mmol/L respectively (p<0.001) (Figure 3.7) Fasting glucose of >6mmol/L represents impaired glucose fasting, 7 participants have this impaired glucose fasting. It is interesting to note the significantly higher level of glucose in the SGACU group compared to the SGANCU infants. Insulin resistance has been noted in children born small for gestational age with spontaneous catch-up growth. Raised fasting glucose levels are an indicator of insulin resistance, and
Figure 3.6 IGF-I levels in SGA children with (SGACU) and without (SGANCU) post-natal catch-up growth. Blood was taken under fasting conditions from 18 SGA children with postnatal catch-up growth and 9 without. Whole blood was centrifuged at 13,000rpm for 10 minutes and serum removed. Serum IGF-I levels were measured using ELISA according to the R&D kit protocol and are presented in µg/L (±SEM). * represents a significant difference in the level of IGF-I found at baseline between SGANCU and SGACU infants, p=0.05.
Figure 3.7 Serum Glucose and Insulin levels in SGA children with (SGACU) and without (SGANCU) post-natal catch-up growth. A) Glucose levels were measured in 18 SGACU and 8 SGANCU children at baseline. * represents a significant difference in fasting glucose levels between SGANCU and SGACU infants, p=0.002. B) Insulin levels were measured in 16 SGA infants with postnatal catch-up growth and 8 without. Insulin was measured in fasting samples on an ELISA from Mercodia according to the manufacturer’s protocol. P=NS.
it appears the SGACU group based on the fasting glucose levels are at a higher risk of insulin resistance compared to the SGANCU group. Values of blood glucose from the SGANCU group over their first year of rhGH would have provided further data to define whether rhGH and the attendant catch-up growth and changes in body composition would have changed fasting glucose levels. Studies have been undertaken describing the metabolic effects of rhGH on short children born small for gestational age, and the majority show that rhGH has a more favourable outcome with lower fasting glucose levels (Ibanez et al 2007, Sas et al 2000).

3.7.3 Insulin

Fasting insulin at baseline was measured in 15 SGA infants, 10 with post-natal catch-up growth and 7 without. It appears that the SGACU group have higher levels of insulin, 5.4 mU/L compared to the SGANCU group, 4.1 mU/L (p=non significant (NS)) (Figure 3.7B).

3.7.4 Adiponectin

Serum adiponectin was measured in 25 SGA infants, 7 with catch-up growth and 5 without. Adiponectin levels were increased in the SGANCU group at 10.4 mg/L compared to 4.3mg/L in the SGACU group (p=0.042) (Figure 3.8).
Figure 3.8 Adiponectin levels are increased in children born SGA without postnatal catch-up growth. Adiponectin levels were measured in 16 SGA infants with postnatal catch-up growth and 8 without. Adiponectin was measured on fasting samples using an ELISA kit from R&D according to the manufacturer’s protocol. * represents a significant difference in adiponectin levels at baseline in the SGANCU group compared to the SGACU group, p=0.042.
3.8 Summary

The key findings in this section are:

- Mean height after one year of rhGH increased by 6.09cm, mean height SDS by +0.47SDS.
- Mean weight increased by 3.52kgs, and mean weight SDS by +1.7 SDS.
- Waist measurements were comparable at baseline and did not significantly increase during treatment with rhGH.
- Hip measurements were significantly increased in the SGACU group compared to the SGANCU group at baseline and did not change significantly in the SGANCU group during treatment with rhGH.
- BP was comparable at baseline and did not significantly change upon treatment with rhGH.
- Glucose levels were significantly increased and adiponectin levels were significantly decreased in the SGACU group compared to the SGANCU group.
- IGF-I and insulin levels were comparable at baseline.

3.9 Discussion

The aims of this part of the study were to compare the basal auxological and biochemical data in children born small for gestational age with and without post-natal catch-up growth, and to see how these parameters change in the SGANCU group during 1 year of rhGH treatment.

Both the height and weight in the SGANCU group at baseline were significantly lower than the SGACU group (p=0.001), and corresponded with the entry requirements of the study. The mean height velocity in the SGANCU group after 1 year treatment with rhGH was 6.1 ± 1.5 cm. Response to rhGH in short children born small for gestational age is variable (Ranke et al 2003). Factors influencing growth response include birth weight, mid-parental height and gestation, as well as many unknown factors. In a study by Ranke et al (2003)
using data from KIGS to predict growth response in the short SGA child, 385 individuals demonstrated a mean height velocity of 8.7cm/yr, with a SD of 1.8. In this study, the mean height velocity was less but with a lower SD. In a review by Jung et al (2008), the mean height SDS increased on average by +1.3-1.4 after 2 years individualised rhGH.

It was well documented and reviewed by Johnston (2008) that younger, shorter and lighter children respond more favourably in the first year or rhGH treatment.

After 1 year’s rhGH treatment, weight increased in the SGANCU group by 3.52kg ± 0.56. 3.52kg is a moderate increase in weight over 1 year, and the SDS increased by +1.4. It is interesting to note weight gain increase is greater than height.

BMI was significantly reduced at baseline in the SGANCU group, 13.9 ± 2.15 kg/m² compared to 16.3 kg/m² in the SGACU group (p=0.001). After one year of rhGH treatment, mean BMI had increased to 15.75kg/m² ± 3.9 which represents a +0.9 change in BMI SDS. Rapid weight gain in infancy is known to increase the risk of obesity later in life (Monteiro and Victora 2005). As individuals born SGA are at higher risk of metabolic disease, weight gain or increase BMI in this group is important to monitor. The study group appears to have a greater increase in BMI in the first year of rhGH treatment than some previous studies suggest, which may reflect differences in study number, rhGH dosing and geography (Hediger et al 1998, Sas et al 2000, Ibanez et al 2010).

Numbers in the study were lower than planned; however, a similar pattern in terms of response to rhGH, as reported in the literature, is present in this cohort. SGANCU infants fail to grow in both height and weight, and when treated with rhGH mean height increases significantly, and mean weight even more significantly.

Both waist circumference and waist to hip ratio at baseline were comparable between the two groups at baseline; over the 1-year rhGH treatment, the former did not alter significantly and the latter did not change.
However, the number of measurements at each time point post-GH commencement was lower than expected so the conclusion as to whether waist circumference changes during rhGH treatment cannot be confirmed in this study. Abdominal fat mass is known to increase significantly in SGA infants with catch-up growth compared to AGA controls, and as the SGANCU group has comparable waist measurements one may suggest that they may share this unfavourable characteristic (Ibanez et al 2007, Ibanez et al 2010). Indeed, increased abdominal subcutaneous fat was found in a study by Ibanez et al (2010) in short children born small for gestational age over 6 years or rhGH treatment compared to controls.

Hip circumference was significantly increased in the SGACU group compared to the SGANCU group at baseline. The waist hip ratio was not significantly different between the two groups. Comparing the SGANCU to the SGACU group, it appears neither has a more beneficial body composition.

It is difficult to compare data from 8 SGANCU individuals to studies in the literature; however it appears the participants are comparable in baseline height, weight and BMI, and in first year response to rhGH treatment.

Blood pressure is comparable between our two groups of children at baseline, when looking at both the systolic and diastolic measurements. It was important to measure this in our two groups as it is well documented in the literature that SGACU individuals have higher blood pressure than AGA (Hemachandra et al 2007, de Kort et al 2010). Raised blood pressure can lead to metabolic syndrome later in life, which babies born SGA are at increased risk of developing (Barker 1997). Because the SGANCU group have comparable BP measurements, one may assume BP may also be high compared to the normal population. Children born SGA who cross weight percentiles are at a higher risk of raised BP (Hemachandra et al 2007). The weight of the SGANCU group increased by +0.9 SDS during the first year of rhGH treatment in this study, and because a significant rise in blood pressure has not been demonstrated this increase may now be seen as less important. However, we have not used an AGA population as a comparison in this study. It is important to note rhGH has
not significantly increased systolic or diastolic blood pressure measurements in our SGANCU group. Although the literature has demonstrated many positive metabolic affects of rhGH in SGA children, it is important to ensure this is true within this SGA cohort (Sas et al 2000).

IGF-I levels are variable in children born SGA; however, the trend tends to be towards higher levels in the SGACU group (Sas et al 2000, Polo Perucchin et al 2011). This is not reflected in our study; IGF-I levels are higher in the SGANCU group compared to SGACU group, however, this did not reach significance (p=0.47). Studies suggest IGF-I resistance may be a cause of non-catch-up growth, and there are short children born small for gestational age who have IGF-IR mutations and raised levels of IGF-I (Inagaki et al 2007). However, the levels in these individuals are above the normal range for IGF-I, which is not seen in our SGANCU group, and height SDS are typically lower in IGF-I resistance.

SGA infants with post-natal catch-up growth are more likely to demonstrate insulin resistance and develop type II diabetes than the general population (Barker 1997). In the literature, SGA infants have reduced fasting glucose levels compared to age matched controls, with levels similar to those found in our SGANCU group, 4.4mmol/L compared 4.1mmol/L, respectively (Cianfarani et al 2003). In the aforementioned study, however, there was no significant difference between SGA infants with or without catch-up growth, which differs from our study results, where glucose was significantly higher in the SGACU group, and they both were above the normal range for fasting glucose. High glucose levels can be an indication of insulin resistance.

The SGANCU group had lower levels of insulin than the SGACU group; however, this was not significant. This corresponds with findings in the literature whereby children born SGA who catch up have higher fasting insulin levels (Sancakli et al 2008). Significantly raised insulin levels and insulin resistance were also demonstrated in a SGACU compared to children born AGA (Ibanez et al 2007).
Adiponectin levels in the SGANCU group were significantly higher than in the SGACU group. Adiponectin levels are inversely related to catch-up growth (Cianfarani et al 2004). Low adiponectin levels are also associated with metabolic syndrome (Hernandez-Morante et al 2008). As the SGANCU group has higher levels of adiponectin compared to the SGACU group it appears they are at lower risk of metabolic syndrome later in life.

In summary, the SGANCU group has a more favourable biochemical profile compared to the SGACU group in this study. This reflects the negative effect catch-up growth has on the metabolic profile of these individuals according to the literature. It is interesting to note that we demonstrated similar waist circumferences in the two groups, as abdominal fat mass levels are known to be higher in SGACU infants than to AGA controls. Although the SGANCU group has a more favourable profile than the SGACU group, both groups are likely to have profiles less favourable than those in normal AGA controls.

In this study, our study numbers were not as high as anticipated. Despite this, we demonstrated similar findings to those in the literature in regards to baseline characteristics and longitudinal measurements in short children born small for gestational age. A few anomalies remained (e.g. increased levels of IGF-I in the SGANCU group compared to SGACU group); however, these anomalies may be due to an alteration in IGF-I signalling in these individuals. Further studies will need to be performed in order to explore the IGF-I response in these individuals.

Another limitation to this section is the lack of an appropriate for gestational age (AGA) control group for comparison. Although the aim was to compare SGA infants with and without catch-up growth it might have been beneficial to have – in the biochemical assays in particular – an AGA comparison. We were able to determine the SGANCU group had a more favourable profile, but we could not demonstrate how this compared to ‘normal’ children.
Chapter 4  Characterisation of proliferation and apoptosis in fibroblasts from children born small-for-gestational age without post-natal catch-up growth, and from children born appropriate-for-gestational age
4.1 Introduction

Most SGA children without post-natal catch-up growth appear to have no clearly defined cause for their failure to demonstrate said growth (Clayton et al 2007). Characterisation of the turnover of cells (e.g. fibroblast cell lines) from such individuals, in comparison to that of cells from AGA children, may help to identify the mechanism(s) involved in SGA post-natal growth restriction.

Proliferation and apoptosis are key cellular processes in growth and development as well as normal tissue or organ maintenance, and an imbalance in these processes may lead to disease. Consequently, there are many regulators to ensure ‘normal’ cell turnover and the association between cancer and cell cycle alterations are well documented with a large variety of gene mutations and abnormal protein function being associated with tumour formation (Favaloro et al 2012).

Undertaking investigations in SGA cells which focus on cell cycle will produce a profile of behaviour of these SGA cells compared to control cells. Both GH and IGF-I promote proliferation and cell survival; their roles as the main regulators of post-natal growth, suggests that an investigation of how GH and IGF-I affect cell turnover in SGA cells compared to control cells is warranted.

Aims

- To assess basal cell turnover (proliferation and apoptosis) in SGA cells compared to control cells.
- To elucidate how SGA cells respond to GH, IGF-I and a combination of GH and IGF-I.

4.2 Basal proliferation

Fibroblast cells from SGA children and control cells were cultured under basal conditions, in normal growth medium. Measuring proliferation under these conditions will enable the rate of proliferation of control cells from normal pre-
pubertal infants to be established, and will provide a view of how SGA cells divide in comparison. Proliferation was measured by two techniques – cell counting and BrdU incorporation. Cell counting allows the physical number of cells at a given time-point to be measured, whereas BrdU is incorporated into DNA during cell division and allows the rate of proliferation to be determined. Three separate experiments were performed in triplicate for each technique on four SGA and three control cell lines over ten days.

4.2.1 Cell counting

Cells were plated at $1 \times 10^5$ and by the end of the experiment (day 10) both SGA and control cells demonstrated approximately a two fold increase in cell number to $2.1 \times 10^5$ and $2.2 \times 10^5$ cells, respectively (Figure 4.1A). Analysis of cell number in relation to cell type and time using a two-way ANOVA demonstrated that there was a significant increase in the number of both cell types over time but no difference between the cell lines, or cell line and day when considered together (Figure 4.1A). These data suggest that under these basal conditions, SGA cell growth is similar to that of control cells.

CAM1 cells ($IGF1R^{+/-}$) showed a very small increase in cell number over the ten day experimental period (Figure 4.1A), from $8.4 \times 10^4$ cells on day one, and to $10.2 \times 10^4$ on day ten. Consequently, at day 10 there were fewer cells in comparison to control ($10.2 \times 10^4$ CAM1 cells versus $2.2 \times 10^5$ control cells). These data suggest that unlike SGA cells, CAM1 cells are unable to proliferate at the same rate as control cells. The growth restriction observed in SGA infants without post-natal catch-up growth may, therefore, be occurring via a different pathway to that responsible for the growth defect in CAM1.

4.2.2. BrdU incorporation

The percentage of BrdU positive control cells increased more than five-fold over ten days in culture (Figure 4.1B). BrdU staining under basal conditions over 10 days remained comparable between SGA cells and controls, with 59% incorporation in control cells at day 10 and 56% in SGA cells (Figure 4.1B).
Figure 4.1 Comparable basal cell proliferation between SGA and controls fibroblasts and reduced proliferation of CAM1 cells compared to control fibroblasts. A) Cells were plated at 1x10^5 in 12 well-tissue culture plates and harvested every 24 hours for ten days. Cells were then counted using a HYCOR slide, and cell number was determined using the manufacturer’s protocol. Data (mean ± SE) from four SGA fibroblast lines, CAM1 and three control fibroblast cell lines analysed in triplicate on 3 separate occasions are shown. B) Cells were plated at 1x10^5 in 12 well-tissue culture plates and harvested every 24 hours for ten days. Cells were stained for BrdU and haematoxylin using the Roche protocol and percentage BrdU incorporation was determined. Data (mean ± SE) of four SGA fibroblast lines, CAM1 and three control cells lines performed in triplicate are shown.
A 2-way ANOVA was performed and no significant difference was observed between SGA and control cells when comparing cell line and the interaction between cell line and day. The percentage of BrdU-positive CAM1 cells was lower than control cells (26% compared to 59% respectively at day 10), which demonstrates that these cells are capable of proliferation albeit at a slower rate than control cells (Figure 4.1B). These observations are likely due to the IGF-IR gene deletion in this individual and correspond well with the work undertaken on these cells, who demonstrated a significant reduction in thymidine incorporation in CAM1 cells compared to controls (Okubo et al 2003).

It appears that SGA cells proliferate normally under basal conditions, thus their ability to respond to key regulators of proliferation – GH and IGF-I – was next investigated by cell counting.

4.2.3 The effect of GH on cell number

Two concentrations of GH, 20ng/ml and 200ng/ml, were used in these experiments; data obtained using the former are shown in Figure 4.2. Both concentrations of GH stimulated control cells over five days in culture, demonstrated by a significant increase in cell number compared to untreated cells. 20ng/ml GH caused a 68% increase in cell number (p=0.02), whereas cell number more than doubled (147% increase) in response to 200ng/ml GH over five days (p=0.01). A 2-way ANOVA (time and treatment) was performed and indicated that both responses to GH in these control cells were significant compared to un-stimulated control cells (Figure 4.2).

SGA cells demonstrated an increase in cell number from $10.2 \times 10^4$ cells to $15.8 \times 10^4$ cells when treated with 20ng/ml GH, and from $9.6 \times 10^4$ cells to $22.2 \times 10^4$ cells following 200ng/ml GH over 5 days. These data demonstrate that GH can stimulate proliferation in both control and SGA cells. Comparing the response of control and SGA cells to either concentration of GH revealed no significant difference in cell line (p=0.069) or cell line and day (p=0.903) (Figure 4.2). Consequently, it appears that SGA cells respond to GH in a similar manner to control cells.
Figure 4.2 GH stimulated proliferation in SGA cells is comparable to control cells. Cells were plated out at 1 x 10^5 and incubated in serum-free growth media. Control cells were treated with vehicle (unstimulated control, UC) or 20ng/ml GH (stimulated control, SC), whereas SGA and CAM1 cells were stimulated with 20ng/ml GH. All cells were harvested every 24 hours for counting using a HYCOR slide, according to the manufacturer’s protocol. Data (mean ± SE) from four SGA lines, three control cell lines and CAM1 analysed in triplicate on 3 separate occasions are shown.
CAM1 cells demonstrated a small increase in cell number upon stimulation with GH over five days (from $8.3 \times 10^4$ to $12.6 \times 10^4$ at 20ng/ml GH, and from $9.7 \times 10^4$ to $11.6 \times 10^4$ at 200ng/ml) (Figure 4.2). It was interesting to note that the response of CAM1 cells to GH was diminished in comparison to control and SGA cells, and in fact the increase in cell number over this time period is comparable to that observed under basal conditions. The reduction in IGF-I R expression predicts reduced IGF-I signalling, and this has been proposed as a contributory factor in growth failure; however, it seems that the proliferative response to GH is also reduced and may therefore play a role. Whether this is a direct defect or a reflection of the cells' inability to respond to GH-stimulated IGF-I production remains to be investigated.

4.2.4 The effect of IGF-I on cell number

Two concentrations of IGF-I, 10ng/ml and 100ng/ml, were used in these experiments that were performed over 5 days. Control cell number increased from $9 \times 10^4$ to $31 \times 10^4$ with 10ng/ml IGF-I (Figure 4.3) and from $10.1 \times 10^4$ cells to $41 \times 10^4$ cells with 100ng/ml IGF-I over five days. A 2-way ANOVA (time and cell) was performed and both parameters were significant compared to unstimulated control cells (Figure 4.3). SGA cells demonstrated an increase in number from $10.2 \times 10^4$ cells to $15.8 \times 10^4$ cells at 10ng/ml IGF-I, and from $9.6 \times 10^4$ cells to $22.2 \times 10^4$ cells in response to 100ng/ml IGF-I over five days.

It appears that IGF-I stimulated the proliferation of both control and SGA cells. However, comparing the response between IGF-I-stimulated control and IGF-I-stimulated SGA cells, at both concentrations of IGF-I, demonstrated a significant difference in cell number ($p<0.001$, 2-way ANOVA) (Figure 4.3). Following IGF-I treatment, there were significantly fewer SGA cells than control cells. This suggests a degree of IGF-I insensitivity in the SGA cells despite a significant increase in cell number over five days.
Figure 4.3 IGF-I stimulated cell number is decreased in SGA and CAM1 cells compared to controls. Cells were plated out at $1 \times 10^5$ and incubated in serum free GM with 100ng/ml IGF-I for five days. Cells were harvested every 24 hours and counted using a hycor slide according to the manufacturer’s protocol. Data (mean ± SE) from four SGA lines, CAM1 and three control cell lines performed in triplicate are plotted. * represents significant difference ($p<0.05$) in unstimulated control cells (UC) compared to stimulated controls cells (SC), SC to stimulated SGA (SGA) by 2-way ANOVA.
CAM1 cells showed a small increase in cell number following treatment with 10ng/ml (from $11.3 \times 10^4$ to $13.6 \times 10^4$) and 100ng/ml (10.1 $\times 10^4$ to 15.2 $\times 10^4$) IGF-I over five days. However, this response was less than that observed in control and SGA cells (Figure 4.3). In fact, the increase in cell number was no different to that observed after 5 days in culture under basal conditions.

SGA cells increase in number upon treatment with IGF-I; however, their response was significantly less than that of the control cells. A degree of IGF-I insensitivity may be present in SGA cells. CAM1 cell number did not increase in the same manner upon treatment with IGF-I; the response was reduced compared to both control cells, and also to SGA cells.

4.2.5 The effect of GH and IGF-I in combination on cell number

GH and IGF-I are both key regulators of post-natal growth, and it is important to determine whether the addition of these hormones together would provide a different growth response to GH and IGF-I alone. Combinations of 10ng/ml GH and 5ng/ml IGF-I, and 100ng/ml GH and 50ng/ml IGF-I were used in these experiments.

Control cells responded similarly (approximately 4.5-fold increase in number) when treated with the 10ng/ml + 5ng/ml (from $9.67 \times 10^4$ to $47.2 \times 10^4$ cells) and 100ng/ml + 50ng/ml (from $10 \times 10^4$ to $46 \times 10^4$ cells) GH and IGF-I combination over five days (Figure 4.4). A 2-way ANOVA was performed and demonstrated a significant increase in the number of control cells upon the addition of each of the combination treatments ($p<0.001$ for both combination treatments), SGA cells increased in number from $10.4 \times 10^4$ to $47.9 \times 10^4$ at the 10ng/ml + 5ng/ml combination of hormones and from $10.3 \times 10^4$ to $42.3 \times 10^4$ at the 100ng/ml + 50ng/ml combination of GH and IGF-I over five days (Figure 4.4).
Figure 4.4 SGA and control cells respond similarly to the combined GH + IGF-I treatment whereas the response of CAM1 cells is reduced. Cells were plated at 1 x 10^5 and either cultured in basal condition, or incubated with a combination of 20ng/ml GH + 10ng/ml IGF-I. Cells were harvested every 24 hours and counted using a hycor slide, according to the manufacturer’s protocol. Data (mean ± SE) from four SGA lines, CAM1 and three control lines performed in triplicate on 3 separate occasions are shown. * represents significant difference (p<0.05) in unstimulated control cells (UC) compared to stimulated controls cells (SC), SC to stimulated SGA (SGA) by 2-way ANOVA.
Comparing the control cell response to the combination treatment with that of the SGA cells revealed no significant difference (Figure 4.4). SGA cells appear to respond in a similar manner to control cells when treated with a combination of GH and IGF-I. In fact, both control and SGA cells responded better to the combination treatments than to either GH or IGF-I stimulation alone (Figure 4.4).

CAM1 cells showed an increase from $10.25 \times 10^4$ to $18.25 \times 10^4$ following stimulation with the $10 + 5\text{ng/ml}$ combination of GH and IGF-I and from $9.8 \times 10^4$ to $17.1 \times 10^4$ after treatment with $100\text{ng/ml GH} + 50\text{ng/ml IGF-I}$ over five days (Figure 4.4). This increase was lower than that observed following GH + IGF-I treatment of control cells. Nonetheless, the combination of hormones caused a greater increase in CAM1 cell number than treatment with either hormone alone, reflecting the response of both the control and SGA cells to combined treatment.

It appears when GH and IGF-I are used together in these experiments lower concentrations demonstrate a comparable response in proliferation; however, when they are separate higher concentrations elicit a greater response. This suggests that GH and IGF-I combined work better to promote proliferation than the hormones alone.

4.2.6 Summary of investigations in basal and hormone stimulated proliferation

Basal proliferation is comparable between SGA and control cells, using two techniques. Analysis using a 2-way ANOVA confirmed this result as no significant difference in cell number or BrdU incorporation was determined.

The SGA cells appear to respond to GH in a similar manner to control cells, suggesting that the mechanisms by which GH promotes proliferation in fibroblasts are not altered in these SGA cells. However, SGA cells have a reduced response to IGF-I compared to control cells, which suggests that the pathways by which IGF-I promotes proliferation are altered or inhibited. Interestingly, GH in combination with IGF-I appears to correct or bypass this abnormality as after five days of culture with GH+IGF-I, the number of SGA cells was comparable to that of control cells maintained under the same conditions.
(Figures 4.3 and 4.4) and was higher than when SGA cells were treated with either hormone alone.

The proliferation of CAM1 cells is sub-optimal, under both basal and hormone-stimulated conditions as, after five days in culture; cell number was consistently lower than that of both control and SGA cells. The defect in CAM1 cells appears to allow only minimal proliferation, with little or no improvement upon ligand stimulation.

4.3 Basal apoptosis

Apoptosis was induced by culturing cells in serum-free media over 10 days and then assessed using two methods – TUNEL staining and measuring cleaved caspase-3 activity. Four SGA cell lines and three control cell lines were used, and all experiments were undertaken on three separate occasions and performed in triplicate.

4.3.1 TUNEL staining

The nuclei of all cells are stained by DAPI and the proportion of cells stained green gives the percentage (apoptotic index) (Figure 4.5). The apoptotic index was determined by calculating the percentage of total number nuclei (DAPI stained) and labelled green (using TUNEL).

There was a 24% increase in the number of TUNEL-positive cells after 10 days in culture under serum-free conditions, indicating the validity of serum starvation as a method of inducing apoptosis in fibroblasts. SGA cells also demonstrated an increase (30%) in TUNEL staining over the ten days in culture, and, interestingly, the proportion of SGA cells undergoing apoptosis was significantly increased compared to control cells (Figure 4.5). These data suggest that SGA cells may have an increased rate of apoptosis compared to control cells.
Figure 4.5 Increased apoptosis in SGA cell compared to control cells maintained under serum starved conditions. A) Serum-starved cells were harvested every 24 hours over 10 days, and then analysed using TUNEL according to the Roche protocol and counterstained with DAPI; the percentage of TUNEL-positive cells was then determined. Data (mean ± SE) from four SGA and three control lines performed on three separate occasions in triplicate are shown. * represents a significant (p<0.05), difference between control and SGA cells by 2-way ANOVA. B) Serum-starved cell were lysed every 24 hours over ten days and samples were analysed using an ELISA to measure caspase-3 activity. SGA cells showed an increase (4-fold) in OD (cleaved caspase-3 levels) over time and compared to controls had a significantly higher level of activity at day ten of the experiment. This indicated an increase in apoptosis in SGA cells compared to controls, as previously demonstrated in the TUNEL experiment.
4.3.2 Caspase-3 activity

In order to confirm whether SGA cells do have an increased rate of apoptosis compared to controls, the activity of caspase-3 in both cell types was analysed. The caspase-3 assay detects levels of cleaved caspase-3 which is the mid-point of many apoptotic pathways. After ten days under serum starved conditions, control cells showed a 7.75-fold increase in OD at λ 450nm, which is representative of an increase in the activation of apoptotic pathways (Figure 4.5B). In order to further investigate this increase in apoptosis/apoptotic pathway activation in SGA cells compared to control cells, the level of apoptosis following addition of GH and IGF-I, which are known survival factors, was determined.

The two assays used to assess apoptosis gave similar results; however, TUNEL staining produced more consistent results so this method of analysis was selected for subsequent experiments. These were performed over five days as the data presented in Figure 4.5 demonstrates that the difference between the level of apoptosis in control and SGA cells is greatest at this time point.

4.3.3 The effect of GH on control and SGA cell on apoptosis

GH is a key hormone involved in the regulation of post-natal growth (Le Roith et al 2001). Consequently, determining the effect of GH in an apoptotic environment, and the ability of SGA cells to respond to GH under these conditions, will enable the effect of GH in the SGA child to be further elucidated.

Comparing unstimulated control cells to GH-stimulated control cells using a 2-way ANOVA revealed significant differences between treatment (p<0.001), time (p<0.001) and treatment plus time (p<0.001). GH appears to reduce the number of TUNEL-positive cells over 5 days and there is an association between treatment and time. Figure 4.6 shows the results at day five between unstimulated control cells and GH-stimulated (20 and 200ng/ml) control cells (p=0.02).
Figure 4.6 Apoptosis is reduced by GH in a similar manner in both SGA and control cells. Cells were plated out at $1 \times 10^5$ in 12 well-tissue culture plates and incubated in serum free media with 20ng/ml or 200ng/ml GH and harvested every 24 hours for five days. Apoptotic cells were then identified by TUNEL according to the Roche protocol and counterstained with DAPI. Apoptotic index was calculated as the percentage of total (DAPI-stained) nuclei that were also TUNEL-positive. Data (mean ± SE) from four SGA fibroblast lines and three control cell lines performed on three separate occasions in triplicate are shown. * represents a significant (p<0.05) difference at day five between unstimulated controls (UC) and stimulated controls (SC), unstimulated SGA cells (USGA) and GH stimulated SGA cells (SSGA), and SC versus SSGA by Mann Whitney.
As previously observed, the level of apoptosis in unstimulated SGA cells (9%) was greater than that of unstimulated control cells (1%; Figure 4.6). With the addition of GH, at both 20 and 200 ng/ml, the number of TUNEL-positive SGA cells is significantly reduced (p=0.012), as indicated by the data obtained at day five (Figure 4.6). However, apoptosis remained significantly increased in the SGA cells compared to the control cells (p=0.00). The mode of action by which GH inhibits apoptosis appears unaltered in SGA cells compared to control cells.

4.3.4 The effect of IGF-I in control and SGA cells on apoptosis

IGF-I is known to play a vital role in cell survival and the inhibition of apoptosis (Weroha and Haluska 2012). Characterising the effect of IGF-I on SGA cells under apoptotic conditions will enable the effect of IGF-I in such children to be further understood.

A 2-way ANOVA demonstrated that there was a significant effect of IGF-I (p<0.001), time (p<0.01) and treatment plus time (p<0.001) on the level of apoptosis in control cells. Thus, IGF-I (10 and 100 ng/ml) appears to reduce the number of TUNEL-positive cells as illustrated by the data obtained at day 5 (Figure 4.7). The addition of 10 and 100 ng/ml IGF-I decreased the rate of apoptosis by 3% and 4% respectively in control cells over five days. Again, the level of apoptosis was significantly greater in unstimulated SGA than unstimulated control cells. Both doses of IGF-I significantly reduced the number of TUNEL-positive SGA cells observed at day 5 (Figure 4.7) to a similar degree.

4.3.5 Summary of investigations into apoptosis

SGA cells are more susceptible to apoptotic stimuli compared to control cells, as demonstrated by both the TUNEL and caspase-3 activity assays of apoptosis. The response to GH and IGF-I is comparable between SGA and control cells; however, the combination of GH and IGF-I were not investigated in this set of experiments.
Apoptosis is reduced by IGF-I in a similar manner in both SGA and control cells. Cells were plated out at $1 \times 10^5$ in 12 well-tissue culture plates and incubated in serum free media with 10ng/ml or 100ng/ml IGF-I and harvested every 24 hours for five days. Apoptotic cells were identified by TUNEL according to the Roche protocol and counterstained with DAPI. The apoptotic index was calculated as the number of TUNEL-positive cells relative to total cell number (determined by DAPI staining of nuclei). Data (mean ± SE) from four SGA fibroblast lines and three control cell lines performed on three separate occasions in triplicate are shown. * represents a significant ($p<0.05$) difference at day five between unstimulated controls (UC) and stimulated controls (SC), unstimulated SGA cells (USGA) and IGF-I stimulated SGA cells (SSGA), and SC versus SSGA by Mann Whitney.
4.4 ELISA of key molecules in apoptosis

In order to further investigate the process of apoptosis in the SGA cells, and perhaps unveil the mechanism(s) responsible for the increased level of apoptosis observed in these cells, specific molecules that are key to the complex process of apoptosis (Figure 4.8) were analysed using a commercially available ELISA kit.

4.4.1 Cleaved caspase-3 and PARP

Cleavage of caspase-3, which is a common mid-point of many of the pathways leading to apoptosis, exposes an active binding domain that enables the enzyme to cleave PARP, resulting in reduced cellular ATP and the initiation of DNA damage and apoptosis (Namura et al 1998). The data presented in Figure 4.9 demonstrates that, in accordance with the findings reported in section 4.3, the level of activated/cleaved caspase-3 is increased in SGA compared to control cells.

Interestingly, the level of PARP in SGA cells was comparable to that of control cells (Figure 4.9), despite increased caspase-3 activity in the former; this was expected as the levels of the two usually correspond. However, caspase-3 activates a number of pathways and may be initiating a non-PARP associated pathway in these SGA cells (Figure 4.8).

4.4.2 p53

p53 is a tumour suppressor, whose gene is mutated in 50% of tumours (Farnebo et al 2010). It is activated by numerous stress signals and inhibits proliferation by initiating cell cycle arrest and promoting apoptosis (Vousden and Prives 2009). Activation or phosphorylation of p53 indicates a shift towards apoptosis; thus, an increase in the level of phospho p53 in SGA cells would suggest that aberrant activation of p53 may contribute to the increased apoptosis observed in these cells.
**Figure 4.8 Schematic of key molecules and pathways involved in apoptosis.**

Cellular stress, cell cycle progression and environmental factors all contribute to a cell undergoing apoptosis. These stress signals activate caspases that induce DNA damage, cell shrinking and membrane blebbing or p53-mediated DNA truncation. Growth factors inhibit apoptosis, in this example, via Akt inhibition of Bad.
However, as shown in Figure 4.9, the levels of p53 and, importantly, phospho p53 in the SGA samples were comparable to those measured in control samples. Consequently, the percentage of p53 that was phosphorylated was no different between SGA and control cells. These data suggest that the increased apoptotic rate seen in SGA cells is not due to increased activation of pathways involving p53.

4.4.3 Bad

Bad is a protein downstream of many apoptotic stimuli (Zeestraten et al. 2013). Its activation initiates a number of separate modes of apoptosis by phosphorylation or dephosphorylation of key amino acid residues. The activation (phosphorylation) of the residue measured in this ELISA de-activates Bad and inhibits its pro-apoptotic actions. Consequently, a decrease in the phosphorylation of Bad is associated with an increase in apoptosis, presumably due to a reduction in pro-survival signals or an increase in apoptotic stimuli. If phosphorylation of Bad is increased, there is either an increase in cell survival signals or a decrease in apoptotic stimuli. The levels of total Bad and phospho Bad were significantly reduced (p=0.02) in the samples from SGA children compared to control cells (Figure 4.9); however, the percentage of Bad that was phosphorylated was not significantly different between the two cell types.

In order to confirm that the levels of Bad and phospho Bad were significantly reduced in SGA cells, a Western immunoblot analysis of these molecules was also performed (Figure 4.10). A band of appropriate molecular weight for Bad and phospho Bad was observed (Figure 4.10A), confirming the presence of these molecules in both SGA and control cells. However, in contrast to data obtained by ELISA, analysis of the blots by densitometry suggested that there was no significant difference in the level of Bad or phospho Bad between SGA and control cells.
**Figure 4.9 Analysis of key apoptotic proteins in SGA cells and control cells.** Cells were serum starved for five days then lysates were analysed using the pathscan apoptosis kit from Cell Signalling technology. Data (mean ± SE) from four SGA fibroblast cell lines and three control cell lines are shown. SGA cells had comparable levels of P53, phospho P53 and PARP but increased levels of cleaved caspase-3 activity, and reduced levels of Bad and phospho Bad. * indicates a significant difference (p<0.05) between control and SGA cells by Mann Whitney testing.
Figure 4.10 Levels of Bad and phospho Bad are comparable between SGA and control cells. A) Cells were lysed after five days of serum starvation and 15 µg total protein was run on a 10% acrylamide gel then transferred to nitrocellulose. Proteins were detected by antibodies specific to Bad and phosphor Bad according to the manufacturer’s protocol. A representative example of data from four SGA fibroblast lines and three control cells performed on three separate occasions in triplicate is shown. B) phospho Bad expression relative to total Bad from densometric analysis of Western blots.
4.4.4 Summary of investigations into apoptosis

Two assays of apoptosis – TUNEL and caspase-3 activity – indicated that apoptosis is significantly increased in cells from SGA children compared to control cells. This remained the same irrespective of GH and IGF-I treatment, despite responding to the hormones in a similar manner. Initial investigations into the key molecules involved in apoptotic pathways did not provide a clear explanation for these findings, however, Bad may play a role.

4.5 Key findings

This series of experiments sought to assess cell turnover in SGA and control cells, and investigate how the cells respond to GH, IGF-I and a combination of GH and IGF-I. The key findings are listed below:

- Basal proliferation is comparable between SGA and control cells.
- The proliferative response to GH is comparable between SGA and control cells. The proliferative response to IGF-I is significantly impaired in SGA cells compared to control cells.
- A combination of GH and IGF-I is more effective in promoting cell growth compared to either hormone alone in both SGA and control cells.
- Apoptosis is significantly increased in SGA cells compared to control cells.
- SGA cells respond in a similar manner to GH and IGF-I under apoptotic conditions.
- Levels of PARP, p53 and phospho p53 (measured by ELISA) are comparable between SGA and control cells, while the level of cleaved caspase-3 were raised in SGA cells and that of Bad and phospho Bad were decreased. The latter finding was not confirmed by Western blot.

- An increase in apoptosis and altered IGF-I signalling may contribute to the non catch-up phenotype in these individuals.
4.6 Discussion

Alterations in GH or IGF-I pathways are associated with post-natal growth failure in rare syndromes. With no clear endocrine cause for ~1500 SGA children failing to demonstrate post-natal catch-up growth each year, it is important that these growth promoting pathways are explored at a molecular level. Proliferation and apoptosis are normal cell processes; it can be assumed that reduced growth of an individual is reflected at the cellular level by reduced cell growth or turnover.

In the experiments presented in this chapter, proliferation was assessed by cell counting and BrdU incorporation. Both techniques demonstrated no significant difference in basal proliferation between SGA and control cells. Similar experiments were undertaken using \(^{3}\text{H}\)-thymidine incorporation to measure proliferation in fibroblasts isolated from children with ISS (Kamp et al 2002). In that study, the fibroblasts demonstrated a reduced level of \(^{3}\text{H}\)-thymidine incorporation under basal conditions. This suggests that the SGA cells do not have as severe an alteration in the IGF-I signalling pathway as the children with ISS in this study. Nonetheless, sequencing of the IGF-I receptor gene should be undertaken in the SGA cells to rule out a mutation.

GH-stimulated proliferation was comparable between SGA and control cells in this study, suggesting no abnormalities in GH-induced proliferation. Kamp et al (2002) also investigated GH-stimulated proliferation in fibroblasts from children with ISS. In their experiments, much higher levels of GH were used, from 1000-50,000ng/ml and no significant difference in \(^{3}\text{H}\)-thymidine thymidine incorporation was noted in either ISS or control cells. This is interesting as 20ng/ml of GH elicited a significant response on cell number and BrdU incorporation in both control and SGA cells in our experiments. It is possible that the levels of GH used by Kamp et al were so high that the GH signalling system was down regulated and, thus, no response was seen.

GH’s proliferative actions can be observed in patients with GH secreting tumours that result in acromegaly. As well as increased soft tissue growth, patients with acromegaly are at increased risk of developing colorectal cancer,
by direct GH actions ( Renehan et al 2003, Loeper and Ezzat 2008). It is interesting to note that SGA cells respond to GH in a similar manner to control cells as the response rate of SGA individuals to rhGH therapy is variable ( Ranke et al 2003).

IGF-I-induced proliferation was significantly decreased in SGA cell over five days compared to controls. In the study by Kamp et al (2002) discussed earlier, IGF-I (both 10 and 100ng/ml) did increase $^3$H-thymidine incorporation in both ISS and control cells, and the ISS cells appeared to have an increased response to IGF-I compared to control (Kamp et al 2002). The same concentrations of IGF-I were used in this study and both SGA and control cells also demonstrated a significant increase in proliferation. However, contrary to the ISS cells, the SGA cells responded significantly less well to IGF-I than control cells. ISS and SGA without post-natal catch-up growth are two different syndromes of short stature. ISS cells suggesting decreased IGF-I availability is responsible for their short stature (Kamp et al 2002) is clearly not the same defect as in the SGA cells. The decrease in IGF-I-induced proliferation in SGA cells suggests an alternation in the IGF-I signalling pathway.

A familial $IGF1R$ mutation Arg481Glu (R481Q), thought to be the cause for short stature in this study was investigated using NIH3T3 cells (Inagaki et al 2007). The mutant receptor was over expressed and a series of experiments were undertaken to define this mutation compared to WT receptor properties. IGF-I-stimulated proliferation was found to be decreased in these cells compared to cells not containing the mutant receptor. These data are comparable to the response to IGF-I in SGA cells; therefore, it is tempting to speculate that an aberration in the IGF-I signalling pathway may cause the short stature in the patients investigated in this study. The patients in the Inagaki study had a height at age 13.6 of -5.2 SDS, and other affected family members heights were -4.7 to -5.4 SDS. The children in our study at entry have an average height of -2.5 SDS; therefore, the clinical phenotype appears to be far more severe in patients with an $IGF-IR$ mutation, suggesting a more subtle alteration in IGF-I signalling may be the cause for the patients in our study’s short stature. Another $IGF-IR$ mutation (G1125A) has been documented in a family whose heights range from
-1.51 SDS to -3.93 (Kruis et al 2010) These individuals also presented with type II diabetes, delayed menarche (females) and clinodactyly. The children in this study do not have type II diabetes or clinodactyly, and as they are pre-pubertal we cannot comment on menarche.

In a study of ISS cells, Kamp et al (2002) used a combination of 10ng/ml IGF-I plus 5000ng/ml GH eliciting a significant response compared to no treatment, and the addition of GH enhanced the effect of IGF-I alone. Our concentrations of the combinations of hormones were different; however, a similar response was observed as GH and IGF-I together elicited a greater response than GH or IGF-I alone.

While SGA cells appeared to respond in a similar manner to GH as control cells, the response of SGA children without post-natal catch-up growth to GH remains highly variable (Ranke et al 2003). Either these experiments are not a fair model for response to GH in the SGA child, or GH itself is behaving normally and it is other downstream molecules and pathways that affected the efficacy of growth response in our patients.

To conclude, SGA cells appear to proliferate in a similar manner to control cells under basal and GH-stimulated conditions. IGF-I stimulated proliferation is reduced suggesting an alteration in the IGF-I pro-proliferative pathways. The addition of GH in the combination treatments appears to correct the aberration seen in the IGF-I response and this combination may be useful in treating SGA infants not responding well to GH. Detailed investigations of the IGF-I signalling pathways will need to be conducted in order to determine if an IGF-I signalling alteration could contribute to the post-natal growth delay observed in such SGA children.

In this study apoptosis was investigated by analysis of TUNEL and caspase-3 activity. Both techniques demonstrated increased rates of apoptosis in SGA cells independent of GH or IGF-I actions. Apoptosis is increased in placentas from pregnancies complicated with IUGR (Erel et al 2001). TUNEL staining was used in this paper on placental explants and a significant increase was
demonstrated. Another paper looking at placentas went further and determined the expression of Bcl-2 and Bax in them (Shen and Hua 2006). Bcl-2 is a pro-survival gene and Bax is pro-apoptotic. In pregnancies resulting in IUGR babies Bax expression was increased compared to controls (14% to 3%, respectively) and Bcl-2 expression was decreased compared to controls (18% to 24%, respectively). If increased apoptosis is present in the placenta (which is of fetal origin) of babies born SGA, it is possible that this abnormal cell turnover mechanism has been programmed in the foetus, and could contribute to the increased apoptosis found in SGA cells.

A recent study by Garcia-Garcia et al (2011) has demonstrated that in addition to stimulating proliferation, GH also plays a role in neuronal survival. Sleep deprivation is known to increase neuronal apoptosis. Rats were put under sleep deprivation conditions and half were given a GH infusion. BrdU incorporation was measured after two days and then after two weeks. Levels of BrdU incorporation over this period in rats treated with GH were significantly increased compared to controls, indicating GH acts as a cell survival factor in this model. In our experiments, GH reduced apoptosis in a similar manner compared to control cells.

We were unable to determine the molecular cause of the increased apoptosis in SGA cells, and we obtained some conflicting data on the levels of total Bad and phospho Bad in control and SGA cells. Cell cycle regulation is a complex process, much of which has been discovered by studying carcinoma cells in which the cell cycle has become unregulated. p53 is the most frequently mutated gene in lung cancer (Iggo et al 1990), and mice containing null mutations for p53 are prone to spontaneous neoplasms from the age of six months (Donehower et al 1992). The ablation of p53, or the presence of an inactivating mutation, results in unregulated increased proliferation. p53 levels are kept low in normal cells by MDM2 and other proteins. Stress or external insults to the cell block this down regulation and p53 accumulates and is activated by numerous kinases (Levine et al 2006). It is clear that disrupting the p53 results in increased proliferation and carcinomas. We found no significant alteration in levels of p53 or in p53 activation in SGA samples compared to
controls. This suggests that the increased apoptosis may not be due to molecules associated with p53 pathways.

Caspase-3 cleavage was increased in these experiments. Cleaved caspase-3 is known to induce apoptosis by activating PARP (Namura et al 1998) and is a mid point for many pro-apoptotic pathways (Figure 4.9). Total PARP was not increased in these experiments, suggesting the increase in caspase-3 activation is via a non-PARP mechanism. Decreased levels of Bad phosphorylation indicate increased apoptosis (Peruzzi et al 1999). Decreased levels of Bad and phospho Bad were found in SGA samples compared to controls; however, when comparing the percentage activation no significant difference was observed. Also, when aiming to confirm these decreased levels by Western blotting no difference was observed. Bad is primarily regulated by the IGF-I/Akt pathway. An alternation in the levels of phospho Bad and Bad may be the cause for increased apoptosis in SGA cells; however, more investigations into Bad, as well as other key molecules involved in apoptosis, are required to pinpoint a specific mechanism.

Bad is a pro-apoptotic protein that de-activates when phosphorylated by Akt via the PI-3K pathway, for example, inhibiting apoptosis. Decreased phosphorylation of Bad may indicate a decrease in the inhibitory pathway via Akt, which could be an explanation for the increased rate of apoptosis seen in SGA cells.

The stimuli for apoptosis are varied and pathways numerous. Inducing apoptosis via UV radiation, or etoposide may enable a specific pathway to be identified as altered in the SGA cells.

To conclude, apoptosis is increased in SGA cells compared to controls, irrespective of GH and IGF-I treatment. Bad may play a role in this; however, a more conclusive look at the cell cycle and apoptotic stimuli are required to uncover the mechanism behind this diminished cell survival in SGA cells compared to control cells.
In the GH, IGF-I and GH + IGF-I induced proliferation experiments (sections 4.2.3, 4.2.4 and 4.2.5) an unstimulated SGA control was not used. This was because at basal proliferation in both BrdU and cell counting experiments, results were comparable over ten days between SGA and control cells. However, this did not allow for assessing how the growth factors had an effect on SGA cells compared to unstimulated SGA cells. Whether or not this was significant could not be determined and, therefore, future work in this section should include the addition of unstimulated SGA cells. Also, IGF1R sequencing and assessment of copy number should be undertaken to determine if a mutation is the cause of the significantly reduced proliferative response in SGA cells to IGF-I.
Chapter 5  Growth hormone and insulin like growth factor I signalling in fibroblasts from SGA children in comparison to AGA children
5.1 Introduction

The GH/IGF-I axis is the main regulator of post-natal growth, therefore, aberrations in this axis have detrimental effects on growth (Walenkamp et al 2007). The complete ablation of GH or IGF-I results in severely restricted post-natal growth in both animal models and humans (Le Roith and Roberts 2003). GH and IGF-I levels in children born SGA are variable; however, it seems that normal GH levels and low or normal IGF-I levels are most common in this patient group (Clayton et al 2007). With no clear endocrine deficiency present, defects in GH and IGF-I signalling inside the cell may play a role in this growth disorder. The cellular actions of these hormones are complex, with many associated signalling molecules and target genes.

The aims of this series of experiments are to:

- determine the expression level of key signalling proteins downstream from the GHR and the IGF-IR in SGA cells compared to control cells, and
- define the activation pattern of these molecules in response to GH and IGF-I.

5.2 GH signalling

The GH signalling cascade was investigated in SGA cells by Western blotting of total and phospho forms of Stat5b and MAPK. Lysates were generated from four SGA cell lines and four control cell lines. Cells were lysed at baseline (without GH) and 15 and 30 minutes post GH stimulation (200ng/ml). pStat5b (phospho Stat5b) and pMAPK (phospho MAPK) are presented as a relative expression to the total expression level for each molecule.

5.2.1 Stat5b activation

Western blot analysis of lysates from SGA and control cells revealed a band of appropriate molecular weight (100kDa; Figure 5.1 A) for Stat5b. Statistical analysis (Mann Whitney test) of the densitometric data obtained from all blots
suggests that the expression of Stat5b by SGA cells and control cells is comparable (p=0.74).

Basal activation of Stat5b was observed in both control and SGA cell, though the expression of pStat5b was significantly less in SGA cells than control cells (Figure 5.1 A and B). In control cells, GH-stimulation of pStat5b was maximal at 15 minutes with over a two-fold increase (p=0.001) in pStat5b levels at this time point (Figure 5.1A and B). At 30 minutes, the level of pStat5b was comparable to that observed at baseline. Treatment of SGA cells with GH for 15 minutes also resulted in activation of Stat5b; however, the response was attenuated when compared to control cells (p<0.001; Figure 5.1A and B); at 30 minutes post GH-stimulation, the level of pStat5b was similar to that observed at baseline (p=0.412).

5.2.2 MAPK activation

Western blot analysis of control cell and SGA cell lysates demonstrated that both cell types express MAPK because bands representing both the 42kDa and the 44kDa isoforms were detected (Figure 5.2A) Densitometric analysis of the two bands, both individually or together, suggests that the expression of MAPK by SGA cells is similar to that of control cells (Figure 5.2B; p=0.984).

There was evidence of MAPK activation at baseline in both control and SGA cells (Figure 5.2); however, treatment of both cell types with GH for 15 or 30 minutes did cause a significant increase in pMAPK (p <0.05; Figure 5.2). Interestingly, the response of SGA cells was similar to that of control cells (p=0.468), with maximal activation at 15 minutes.

5.3 IGF-I signalling

The IGF-I signalling pathway was assessed using Western blotting to investigate expression of the IGF-IR and the insulin receptor (IR) and activation of Akt and MAPK. IGF-I-induced activation of Akt and MAPK was assessed using lysates
Figure 5.1 GH stimulated Stat5b activation in SGA cells and control cells

A) Western blot analysis of phospho (p)- and total-Stat5b (blot shown is representative of data obtained in four independent experiments). Cells were incubated in serum free media for 24 hours at 80-90% confluence before adding 200ng/ml GH. Cells were lysed at baseline, and at 15 and 30 minutes post-GH stimulation. Lysates were then run on a 10% acrylamide gel to separate the proteins that were then transferred to a nitrocellulose membrane and visualised using specific antibodies for total Stat5b and pStat5b.

B) All Western blots (n=4) were analysed by densitometry to determine the mean (±SEM) expression of pStat5b relative to total Stat5b. * represents p <0.001 by Mann Whitney analysis in expression of pStat5b in SGA cells compared to control cells.
Figure 5.2 GH-stimulated MAPK activation is comparable in SGA and control cells. A) Representative (n=4) Western blot analysis of total and phospho MAPK. Lysates generated from cells that were serum-starved for 24 hours prior to stimulation with 200ng/ml GH for 15 or 30 minutes were run on a 10% acrylamide gel and then proteins were transferred to nitrocellulose and probed with antibodies specific to MAPK and phospho MAPK. B) Expression (mean±SEM) of pMAPK relative to total MAPK determined by densitometric measurements of data from four separate experiments per SGA and control cell line.
generated at baseline and after 15 and 30 minute stimulation of cells with 100ng/ml IGF-I.

5.3.1 IGF-IR and IR expression

Western blot analysis revealed a band at the appropriate molecular weight for IGF-IR (100kDa) in both cell lines (Figure 5.3). Data obtained from three separate experiments using four SGA cell lines and three control lines suggests the two cell types have comparable expression of IGF-IR (p=0.463). In contrast, CAM1 cells have drastically reduced levels of the IGF-IR compared to control cells (p=0.000), which is expected as these cells carry only one copy of the IGF-IR gene.

IGF-I signals not only through the IGF-IR, but also through hybrid IGF-I / IR receptors and, at high concentrations, the IR (Rowzee et al 2009). The IR was detectable, at a comparable level, in all cell lines (p=0.0343; Figure 5.4). As the SGA cell lines express a similar level of both the IGF-IR and the IR as control cells, it is unlikely that an aberration at this point in the pathway is the explanation for the post-natal growth restriction observed in these children born SGA, whereas the defect in IGF-IR expression displayed by CAM1 cells is a major contributory factor to the short stature of this individual.
Figure 5.3 IGF-IR expression in CAM1 cells, but not SGA cells, is reduced in comparison to control cells. A) Cells were lysed under basal conditions and lysates were run on a 10% acrylamide gel. Proteins were then transferred to nitrocellulose and the IGF-IR receptor was visualised using antibodies specific to the IGF-I β subunit. B) IGF-IR expression (mean ±SEM), relative to actin, was determined using densitometric analysis of Western blots from four separate experiments performed in triplicate.
Figure 5.4 Both SGA and CAM1 cells have comparable IR expression to control cells. A) Cells were lysed under basal conditions and run on a 10% acrylamide gel then transferred to nitrocellulose. Proteins were then visualised using antibodies specific to the IR. B) Relative expression of the IR compared to actin (mean ± SEM) from densitometric analysis of Western blots from four separate lysis experiments, performed in triplicate.
5.3.2 Akt activation

Akt is a 60kDa protein that is expressed in comparable levels by SGA and control cells (p=0.984; Figure 5.5).

Full activation of Akt requires the phosphorylation of two amino acid residues – Serine473 (Ser473) and Threonine308 (Thr308) (Kanan et al. 2010). Consequently, antibodies that specifically recognise the phosphorylated form of each of these residues were used in Western blot analysis of lysates generated from cells treated with IGF-I (100ng/ml) for 15 minutes. The data presented in Figure 5.5 shows that in both control and SGA cells, the two residues show a similar pattern of activation.

At baseline, the band representing pAkt migrates to the same position as the 60kDa molecular weight marker; upon stimulation with IGF-I, a second band, at a slightly greater molecular weight, appears in the samples generated from SGA cells whereas in the control cell samples, the band at 60kDa disappears and only the band at slightly greater molecular weight (co-migrating with the second band observed in SGA cells) is apparent (Figure 5.5). No difference in Akt activation between control and SGA cells was observed, in three independent experiments, when either the pSer473 antibody or the pThr308 antibody is used to assess Akt phosphorylation. This suggests that the two separate stages of Akt activation occur, and do so in a comparable fashion in SGA and control cells; however, it does not determine why there appears to be a different pattern of Akt activation in SGA cells.

At both baseline and 15-minute post IGF-I stimulation, the pAkt levels were comparable between SGA and control cells using the Threonine308 antibody (p=0.686, p=0.114 respectively) and the Serine743 antibody (p=0.886 and p=0.114, respectively) (Figure 5.5). This demonstrates similar levels of pAkt at baseline and post IGF-I stimulation, and that SGA cells activate Akt at the same level as control cells.
Figure 5.5 IGF-I-stimulated Akt activation is comparable in SGA and control cells. A) Cells were lysed at baseline and at 15 minutes post stimulation with IGF-I (100ng/ml). Lysates were run on 8% acrylamide gels and separated proteins transferred to nitrocellulose. Proteins were visualised using antibodies specific to pAkt(Ser), pAkt and total Akt. Blots shown are representative of data obtained from four independent experiments. The expression (mean±SEM) of pAkt (B), pAkt(Ser) (C) and total Akt (D) expression, relative to actin, was determined using densitometric analysis of Western blots from four separate experiments performed in triplicate.
In regards to the different pattern of activation observed, there are three Akt isoforms, which have different molecular weight. It is possible we are seeing an additional isoform in the SGA cells or differential activation of one of the isoforms. The phospho antibodies used to detect Akt activation are pan phospho antibodies, consequently more specific methods to precisely define Akt activation are required to determine the significance of the extra band present in SGA samples upon stimulation with IGF-I.

5.3.3 Akt isoform expression

The three Akt isoforms have distinct functions as demonstrated by studies in knockout mice. The Akt1 knockout mouse is small at birth, with significantly reduced post-natal growth (Cho et al 2001b). The Akt2 knockout mouse demonstrates insulin resistance (Cho et al 2001a), while the Akt3 knockout mouse had reduced brain size (Easton et al 2005). Determining the presence or absence of these proteins in the SGA cells may enable the altered pattern of Akt activation in SGA cells to be further elucidated.

Western blot analysis using antibodies that are specific for each of the Akt isoforms revealed that both control and SGA cells express all three isoforms of Akt and that the level of expression is similar between the two cell types (Akt1 p=0.886, Akt2 p=1.000, Akt3 p=0.686; Figure 5.6). Akt1 appears as a doublet in these experiments. This is interesting because total Akt appeared as a single band, and pAkt in SGA cells, when treated with IGF-I, appear as a doublet in the previous experiments. This altered pattern may be therefore associated with Akt1.

Despite levels of Akt1, 2 and 3 appearing normal in SGA cells, the activation of these molecules may be altered and provide more insight into this pathway.
Figure 5.6 Comparable expression of Akt1, 2 and 3 in SGA and control cells. Lysates were generated from SGA and control cells, under basal conditions. Lysates were run on 8% acrylamide gels and transferred to nitrocellulose. These blots are representative of three separate experiments; per SGA line performed in triplicate. Proteins were visualised using antibodies specific to Akt1, 2 and 3. Anti-actin was used to detect actin. B) Data in graph form of the Akt isoform expression in SGA cells compared to controls. Akt expression was determined by densitometry, and shown as a percentage of relative expression compared to actin densitometric measurements.
5.3.4 Isoform specific activation of Akt

Analysis of the activation of the specific Akt isoforms was undertaken by immunoprecipitation followed by Western blotting. Samples from control and SGA cells generated at baseline (-) and after stimulation with IGF-I for 15 minutes (+) were incubated with or without (negative control; B) Akt isoform-specific antibodies. Immune complexes were later precipitated using Protein G beads and analysed by Western blotting using phospho pan Akt antibodies. Whole cell lysates were also probed to provide a positive control (C) for the presence of the Akt isoforms.

Figure 5.7 demonstrates the results obtained following immunoprecipitation of samples using an anti-Akt1 antibody. The first four lanes are samples from SGA cells whereas the last four lanes contain samples from control cells. In the first lane, which contains the baseline sample, there are no bands present, suggesting that in SGA cells Akt1 is not activated under basal conditions. The next lane, containing the sample obtained post IGF-I stimulation, depicts a band at 60kDa; this band co-migrates with the band observed in the next lane, the positive control, suggesting that IGF-I is able to stimulate the phosphorylation of Akt1 in SGA cells. Similar data were obtained upon analysis of Akt1 activation in control cells; indeed there was no significant difference in the IGF-I stimulated phosphorylation of pAkt1 between SGA and control cells (p=0.114).

The next figure (5.8) depicts the data obtained following immunoprecipitation of samples with an anti-Akt2 antibody. In both the SGA and control cells, Akt2 is not activated at baseline. However, following stimulation with IGF-I, a band representing pAkt2 is apparent in the samples from SGA cells but not the control cells. This suggests that the difference in IGF-I stimulated Akt activation between SGA and control cells observed upon analysis with a pan-AKT antibody (Figure 5.5A) may be due to the additional activation of Akt2 in SGA cells. IGF-I also stimulates phosphorylation of Akt3 (Figure 5.9) in SGA cells but in contrast to the activation of Akt2, a similar response to IGF-I is observed in control cells (p=0.886).
Figure 5.7 Akt1 activation is comparable in SGA cells and control cells. A) Lysates were generated from SGA and control cells at baseline and 15 minutes post IGF-I stimulation, and later incubated with an anti-Akt1 antibody. Samples were then incubated with protein G beads to precipitate immune complexes and the resulting precipitates were run on an 8% acrylamide gel and proteins were transferred to nitrocellulose. Proteins were visualised using a phospho pan Akt antibody. (–) depicts samples obtained at baseline, whereas (+) notes samples from cells treated with 100ng/ml IGF-I for 15 minutes. Lane C represents the positive control - whole cell protein lysate and lane B contains a sample that was generated by precipitation in the absence of antibody as a negative control. The blots were then stripped and re-probed with Akt1, to show its presence in all samples, and to act as a loading control. The blots shown are representative of data obtained from four independent experiments. (B) Relative expression (mean±SEM) of pAkt1 compared to Akt1 was determined by densitometric measurements of data obtained from experiments performed on four separate occasions on four SGA cell lines.
Figure 5.8 Activation of Akt2 is increased in SGA cells compared to control cells.

A) Lysates were generated from SGA and control cells at baseline and 15 minutes post IGF-I stimulation and incubated with an anti-Akt2 antibody. Samples were then incubated with protein G beads to precipitate immune complexes and the resulting precipitates were run on an 8% acrylamide gel and proteins were transferred to nitrocellulose. Proteins were visualised using a phospho pan Akt antibody. (–) depicts samples obtained at baseline, whereas (+) notes samples from cells treated with 100ng/ml IGF-I for 15 minutes. Lane C represents the positive control - whole cell protein lysate and lane B contains a sample that was generated by precipitation in the absence of antibody as a negative control. The blots were then stripped and re-probed with Akt2, to show its presence in all samples, and to act as a loading control. The blots shown are representative of data obtained from four independent experiments. * represents a significant increase in pAkt levels in SGA cells but not control cells and a significant difference in (+) lanes between SGA and control cells. B) Relative expression (mean±SEM) of pAkt2 compared to Akt2 was determined by densitometric measurements of data obtained from experiments performed on four separate occasions, on four SGA lines.
Figure 5.9 Akt3 activation is comparable in SGA cells and control cells. A) Lysates were generated from SGA and control cells at baseline and 15 minutes post IGF-I stimulation and incubated with an anti-Akt3 antibody. Samples were then incubated with protein G beads to precipitate immune complexes and the resulting precipitates were run on an 8% acrylamide gel and proteins were transferred to nitrocellulose. Proteins were visualised using a phospho pan Akt antibody. (−) depicts samples obtained at baseline, whereas (+) notes samples from cells treated with 100ng/ml IGF-I for 15 minutes. Lane C represents the positive control - whole cell protein lysate and lane B contains a sample that was generated by precipitation in the absence of antibody as a negative control. The blots were then stripped and re-probed with Akt3 to show its presence in all samples, and to act as a loading control. The blots shown are representative of data obtained from four independent experiments. B) Relative expression (mean±SEM) of pAkt3 to Akt3 was determined by densitometric measurements of data obtained from experiments performed on four separate occasions, on four SGA lines.
5.3.5 PHLPP1 and PHLPP2

Akt2 appears to be activated in SGA cells, but not in control cells, following stimulation with IGF-I. Phosphatases are responsible for removing phosphate groups from signalling molecules, either activating or, more commonly, de-activating the molecules/pathway (Brognard et al 2009). A phosphatase imbalance may be responsible for the difference in Akt activation seen in SGA cells.

PH domain and leucine rich repeat protein phosphatases (PHLPP1 and PHLPP2) ‘turn off’ Akt signalling by removing the phosphate group at the Serine473 residue. PHLPP1 specifically de-phosphorylates Akt2, and PHLPP2 is responsible for the de-phosphorylation of Akt1 (Andreozzi et al 2011, Nitsche et al 2012). In type II diabetes, Akt2 activation is decreased and PHLPP1 expression is increased (Cozzone et al 2008). In this study, Akt2 is activated in SGA but not control cells, thus it is possible that the level of PHLPP1 may differ between the two cell types. Although the activation of Akt1 in SGA cells appears to be comparable with that observed in control cells, due to its acknowledged role in growth, the level of PHLPP2 was also investigated (Cho et al 2001b).

A band at the appropriate molecular weight for PHLPP1 (180 kDa) was present in both SGA and control cells and there appeared to be no significant difference in the level of expression between the two cell types (Figure 5.10). PHLPP2 is also present in comparable levels between SGA and control cells (Figure 5.10). Normal levels of these phosphatases suggest they may not be the cause for the significant increase in Akt2 activation seen in SGA cells upon stimulation with IGF-I. However, the activity of these molecules was not investigated, which could have provided more insight into the signalling anomaly observed in these experiments.
Figure 5.10 Both PHLPP1 and PHLPP2 are expressed in comparable levels in SGA and control cells A) Lysates were generated from SGA and control cells (C) under basal conditions and run on a 10% acrylamide gel. Proteins were transferred onto nitrocellulose and visualised by antibodies specific for PHLPP1 and PHLPP2. Blots are representative of four separate experiments per SGA line performed in triplicate. B) Expression of phosphatases (relative to actin; mean±sem) by densitometric analysis of Western blots obtained from four separate experiments.
5.3.6 MAPK signalling

MAPK is present in both SGA and control cells, as demonstrated in a previous experiment (Figure 5.2). pMAPK is present at baseline, and at 15- and 30-minute post IGF-I stimulation, in both SGA and control cells (Figure 5.11). However, there was no significant change in the level of pMAPK following stimulation of either cell type, which suggests that IGF-I did not activate this signalling molecule in these experiments.

5.3.7 Summary

IGF-I signalling appears to be altered in SGA cells compared to control cells. There is a different pattern of Akt activation, and a significant increase in Akt2 activation in SGA cell compared to controls. This alteration in Akt activation could be associated with changes in other signalling molecules. Therefore, an array approach to identify potential outcomes for the altered IGF-I signalling seen in the SGA cells was used. Analysing the phosphorylation status of 21 proteins including, ERKs, JNKs, p38 isoforms, Akt, GSK and p70 S6 kinase in response to IGF-I in SGA and control cells may lead to a greater understanding of these alterations in SGA signalling (Figure 5.12).

5.4 Human Phospho-MAPK array

Array analysis of samples from both basal and IGF-I-stimulated conditions was undertaken. Samples from four individual experiments were pooled for each SGA and control cell line, and one array per cell line was performed (ten arrays in total). In each cell type, the effect of IGF-I was determined by subtracting the densitometric measurements obtained from the array of basal samples from those obtained from the array of samples from stimulated cells, then the resulting data from SGA cells was compared to that of control cells (Figure 5.12). A column above zero represents increased activation of a molecule compared to control cells, and a column below zero represents a decrease in
Figure 5.11 Comparable IGF-I stimulated activation of MAPK in SGA and control cells. A) Lysates were generated from SGA and control cells under basal conditions and at 15 and 30 minutes post 100ng/ml IGF-I stimulation. Lysates were run on a 10% acrylamide gel and transferred to nitrocellulose, proteins were visualised using antibodies specific to MAPK and pMAPK. B) pMAPK to total MAPK expression data was determined by densitometric analysis of Western blots from four separate lysis experiments.
Figure 5.12 Up- and down-regulated activity of key signalling molecules in SGA cell compared to control cells. Exemplar phospho-arrays from control and SGA cells under basal (A and C, respectively) and IGF-I stimulated (B and D, respectively) conditions. (E) Levels of phospho molecules upon stimulation with 100ng/ml IGF-I in four SGA fibroblast line and CAM1 compared to control cells levels. Bars above zero indicate an increase in phospho values in SGA cells compared to control cells, and bars under zero indicate a decrease in phospho values compared to control cells. Red lines above and below zero represent mean SD which is used as a cut off for significant values.
activation of molecules compared to control. The mean SD was determined using the values from each experiment for each molecule, and measurements above and below the mean SD were deemed significant. See Table 5.1 for increased and decreased activation pattern in the SGA cells compared to controls.

5.4.1 Phosphoarray analysis

Many molecules demonstrated increased or decreased activation across the four SGA samples, though there was considerable heterogeneity across the samples with a maximum of three SGA samples demonstrating similar changes for any one molecule. The proteins in which three SGA samples had comparable results were pursued. The three molecules that showed differences across three or more SGA patients were - ERK1, p38 alpha and JNK.

ERK1/MAPK had already been analysed by Western blotting (data presented in section 5.2) and there appeared to be no significant difference in the activation of ERK1 in SGA cells compared to controls. Activation of p38 and JNK was also assessed by Western blotting (Figures 5.13 and 5.14, respectively). The data suggest that there is no significant difference in the activation of either molecule upon treatment of cells with IGF-I. JNK showed significantly increased levels post IGF-I stimulation in both SGA and control cells, whereas p38 showed non-significant levels of activation post IGF-I stimulation in both SGA and control cells. Activation of these molecules by Western blot analysis demonstrates no significant difference in SGA cells compared to control cells.
Table 5.1 Table of increased and decreased activated proteins in SGA cells compared to controls. Highlighted rows represent molecules that were further investigated in this series of experiments.

<table>
<thead>
<tr>
<th></th>
<th>SGA1</th>
<th>SGA2</th>
<th>SGA3</th>
<th>SGA4</th>
<th>CAM1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1)</td>
<td>+ Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2)</td>
<td>+ Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3)</td>
<td>ERK1</td>
<td>↓</td>
<td></td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>4)</td>
<td>JNK1</td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>5)</td>
<td>JNK Pan</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>6)</td>
<td>P38</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7)</td>
<td>P38</td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>8)</td>
<td>RSK1</td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>9)</td>
<td>GSK-3</td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>10)</td>
<td>Akt1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11)</td>
<td>Akt2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12)</td>
<td>ERK2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13)</td>
<td>JNK2</td>
<td></td>
<td></td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>14)</td>
<td>P38</td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>15)</td>
<td>P38</td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>16)</td>
<td>RSK2</td>
<td></td>
<td></td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>17)</td>
<td>GSK</td>
<td></td>
<td></td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>18)</td>
<td>Akt3</td>
<td></td>
<td></td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>19)</td>
<td>Akt Pan</td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>20)</td>
<td>JNK3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21)</td>
<td>MSK2</td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>22)</td>
<td>HSP27</td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>23)</td>
<td>P70 S6 K</td>
<td></td>
<td></td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>24)</td>
<td>+ Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5.1 Table of increased and decreased activated proteins in SGA cells compared to controls. Highlighted rows represent molecules that were further investigated in this series of experiments.
Figure 5.13 Comparable pJNK activation in SGA cells compared to control cells. A) Lysates were generated from the SGA and control cells (C) under basal conditions and 15 minutes post 100ng/ml IGF-I stimulation. Samples were run on a 10% acrylamide gel and transferred to nitrocellulose, and protein visualised by antibodies specific to phospho JNK and Actin. * represents significant increase in pJNK in SGA and control cells compared to baseline levels. Blots are representative of four separate experiments on four SGA lines. B) pJNK to actin expression was determined by densitometric analysis from Western blots of four separate lysis experiments, performed on four SGA lines.
Figure 5.14 Comparable p38 activation in SGA cells and control cells. A) Lysates were generated from SGA cell and controls under basal conditions and 15 minutes post 100ng/ml IGF-I stimulation. Lysates were run on a 10% acrylamide gel and transferred onto nitrocellulose. Proteins were visualised by phospho p38 and actin specific antibodies. Blots are representative of four separate experiments on four SGA lines. B) Actin to p38 expression data was obtained by densomteric analysis from Western blots of four separate lysis experiments, in four SGA lines.
5.4.2 Summary

Signalling pathways are complex, and the same molecules can be activated in different pathways by different signals. Identifying and assessing key molecules, that are known to be disrupted in syndromes of abnormal growth, has enabled some anomalies in these pathways to be detected in cells from SGA children. These anomalies may be the cause of postnatal growth failure in these children, or a reaction/compensation of growth failure. Stat5b is the main promoter in the production of IGF-I, a key hormone in post-natal growth. The reduction in activation of Stat5b may be enough to reduce the amount of IGF-I produced, therefore reducing the drive for growth post-natally. IGF-I is reduced in some SGA children (Clayton et al 2007), and potentially a decrease in Stat5b activation may be the cause. A reduction in Stat5b activation may also be a compensatory mechanism. Stat5b inhibits more signalling molecules than it activates, and this reduction may be decreasing its inhibitory roles to increase activation of other growth promoting pathways (Cohen et al 2006). Akt2 is a pro-survival protein kinase and also has a metabolic role in facilitating the uptake of glucose; it has been demonstrated that a reduction in activation can lead to type II diabetes (Cozzone et al 2008). Increased activation of Akt2 is most likely an attempt at compensation for the increased apoptosis seen in SGA cell compared to controls reported in section 4. Also, it may be involved in ensuring cells have enough glucose stores to proliferate when the correct signals are present to optimise growth.

Key Findings:

- Stat5b, MAPK, IGF-IR, IR Akt1, 2 and 3 are all expressed at similar levels in SGA cells compared to control cells.
- GH signalling is altered in SGA children, evidenced by decreased activation of Stat5b.
- MAPK activity is normal when SGA cells are stimulated with either GH or IGF-I.
- Akt activation is altered in SGA children, with Akt2 being activated in response to 100ng/ml IGF-I in SGA, but not control cells.
• Phosphoarray analysis of key signalling molecules upon IGF-I stimulation of SGA cells demonstrated decreased p38 and JNK activity phosphorylation compared to control cell. However, this result could not be confirmed by Western blotting.

5.5 Discussion

The aims of this series of experiments were to assess the expression of key GH and IGF-I signalling molecules in cells from SGA children without catch-up growth and their activation patterns in response to GH and IGF-I compared to cells from AGA children.

Stat5b is present in comparable levels in SGA cells and control cells. Seven cases of STAT5B mutations resulting in no Stat5b protein are documented worldwide (Hwa et al 2005). Individuals with a STAT5B mutation are born small and have severe post-natal growth failure as well as chronic respiratory and immune system issues throughout life (Bernasconi et al 2006). STAT5B ablation results in no IGF-I production, which is believed to be responsible for restricted post-natal growth. (Hwa et al 2007). In this study, the SGA cells express Stat5b in normal levels; therefore, the post-natal growth failure seen in these individuals is not due to altered levels of Stat5b. Also, none of the participants in this study had a previous history of chronic immune issues, which also suggests that Stat5b levels are normal.

In this study, GH-induced activation of Stat5b was significantly reduced at 15 and 30 minutes in cells from SGA children compared to control cells. In fibroblasts from patients with Laron syndrome, GH cannot activate Stat5b which is thought to be one mechanism behind the growth failure observed in these individuals (Silva et al 2002). The reduction of Stat5b activation in SGA cells compared to control cells in this study, may demonstrate a mechanism by which post-natal growth has failed. Reduced Stat5b activation may in turn result in a reduction in IGF-I production and, consequently, its proliferative actions. IGF-I production by SGA fibroblasts should be investigated in the future to assess
whether this is the case; however, it is interesting to note that serum IGF-I levels in the SGA group were normal (section 3.7.1). GH binding to the GHR and transphosphorylation of the GHR sub units by JAK2 all need to occur for Stat5b to be activated. GH binding assays and JAK2 activity should be investigated as GH may not be signalling efficiently via this particular pathway.

The level of MAPK is comparable between control and SGA cells. Similarly, the activation of MAPK by GH, at both 15 and 30 minutes, is comparable in cells from SGA individuals and AGA controls. Fibroblasts from patients with Laron syndrome could not activate MAPK by GH (Silva et al 2002). This suggests that a mutation in the GHR may not be present in these individuals as Sta5b alone is affected in this study. Noonan’s syndrome is associated with increased MAPK activation due to mutations in SHP-2, SOS1 and Raf1 (Limal et al 2006, Razzaque et al 2007, Roberts et al 2007). As activation of MAPK appears to be normal in the SGA cells in this study, the mutation in SHP-2, SOS1 and Raf1 found in Noonan’s syndrome is unlikely to be the cause of the post-natal growth failure of the SGA participants in this study.

IGF-IR and IR expression is comparable between SGA and control cells. IGF1R mutations and, therefore, reduced IGF-IR expression are associated with post-natal growth restriction (Abuzzahab et al 2003). Comparable levels of IGF-IR expression suggest that the IGF1 gene is normal; however, this should be confirmed by sequencing of the gene in future studies. The level of IGF-I in individuals with IGF1R mutations is commonly high, thus it is interesting to note that serum levels of IGF-I in the SGA group in this study are at the high end of the normal range.

The Akt isoforms (Akt1, 2, and 3) were expressed in both SGA and control cells, with no difference observed in expression levels. IGF-I-induced Akt phosphorylation, of both the ser473 and thr308 residues, was comparable between SGA cells and control cells. However, a different pattern of activation was observed in SGA cells. Analysis of IGF-I induced Akt isoform activation demonstrated both SGA and control cells activate Akt1 and 3; however, only the SGA cells activate Akt2 in response to IGF-I stimulation.
Research into the Akt isoforms and their specific roles has recently been undertaken. Akt1 knockout mice are smaller than wild litter mates and show post-natal growth restriction. Akt2 knockout mice have a diabetic phenotype and Akt3 knockout mice have a smaller brain and neurological issues (Cho et al 2001a, Easton et al 2005). It is interesting to note Akt1 activation is not different between our two cell types as its role in growth is well documented (Cho et al 2001b). Decreased Akt2 activation, in response to insulin, has been demonstrated in muscle cells from individuals with type II diabetes (Cozzone et al 2008). Individuals born SGA are known to be at higher risk of developing insulin resistance and type II diabetes (Barker 1997). Decreased Akt2 activation has also been associated with low birth weight in type II diabetes (Friedrichsen et al 2010). The increased IGF-I induced activation of Akt2 observed in this study may be compensating for reduced activation by insulin of Akt2; however, an assessment of insulin-induced activation of Akt2 was not performed in these experiments, and could therefore be pursued in the future. Increased Akt2 activity has been associated with breast cancer and gliomas, and is known to accelerate cell cycle (Nicholson and Anderson 2002). In section 4.2.4, increased rates of apoptosis were observed in SGA cells compared to control cells; increased Akt2 activity may be attempting to compensate for this abnormality.

PHLPP1 and PHLPP 2 are phosphatases responsible for de-activating the Akt isoforms. As well as decreased Akt2 activation in individuals with type II diabetes, PHLPP1 expression was increased (Cozzone et al 2008). In this study, PHLPP1 and PHLPP2 were expressed in comparable levels between SGA and control cells. The increased activation of Akt2 appears not to be associated with any anomalies in PHLPP1 expression. However, the activation of these molecules was not assessed and so it remains a possibility that the function of these phosphatases is altered. Decreased activation of PHLPP1 would in turn increase the activation of Akt2. Polymorphisms are present in PHLPP2 which are associated with increased Akt3 activity and breast cancer, levels of PHLPP2 are normal, but its activation is reduced (Brognard et al 2009). Investigations focusing on PHLPP1 and PHLPP2 activities in SGA cells should
be undertaken in the future to determine whether these molecules could be contributing to the altered activity of Akt2 in SGA cells.

Western blot analysis of IGF-I-induced phosphorylation of MAPK in SGA cells was comparable to that in control cells.

Phosphoarray analysis of key molecules downstream of the IGF-I receptor demonstrated significant differences between SGA cells and control cells in MAPK (ERK1), p38 and JNK. p38α is a key signalling molecule responsive to stress stimuli such as inflammatory cytokines, TNFα, and environmental stresses. Stress signals are delivered to the p38 signalling cascade by Rho family small GTPases, and activation of transcription factors for cell cycle regulation occurs (Cohen 1997). Cell lines derived from a p38α knockout mouse have demonstrated enhanced activation of the MAPK signalling pathway and an increased resistance to apoptosis (Porras et al 2004). In the phosphoarray p38α activation is decreased in our SGA cell compared to controls upon stimulation with IGF-I; however, repeating this experiment by Western blotting did not see these significant differences. It is clear p38 plays a role in cell cycle regulation, and in the future investigating the p38 isoforms and their activation should be undertaken, especially as the cell cycle is disrupted but increased rates of apoptosis compared to control cells in SGA cells.

JNK is also involved in cell survival. JNK1- and JNK2-knockout mice show early embryonic death and decreased apoptosis (Davis 2000). Disruption of MKK4, which activates JNK, results in increased apoptosis (Tournier et al 2000). JNK showed decreased activation in our SGA cell compared to controls when stimulated with IGF-I in the phosphoarray experiment, but not by Western blotting. If JNK was decreased, a decrease in apoptosis in our SGA cells might have been expected as a result; however, as discussed previously, we see the opposite in our investigation into apoptosis.

The discrepancy between the data obtained by array and Western blotting analysis of p38, JNK and ERK1 must not be overlooked. Furthermore, the three Akt isoforms are present in this array but the activation of Akt2 was not found to
be different between SGA and control cells, which contrasts with the data presented in section 5.3.4. The reason may be the other antibodies on the phosphoarray competed with the Akt2, p38, JNK and ERK1 binding sites in a way that is artificial, rather than how these molecules bind *in vitro*. Also, a different lysis buffer was used to prepare the samples for the array and this may have contained insufficient phosphatase inhibitors compared to the Western blot lysis buffer. In addition, a higher level of protein was used in the array experiment and this may have enabled the array to pick up more differences compared to the Western blot. In order to confirm either of the results, another more sensitive technique could be used, such as immunoprecipitation.

On the array the three Akt isoforms were present. Extensive immunoprecipitation experiments to establish Akt1, 2 and 3 IGF-I induced activity demonstrated significantly increased Akt2 activation. This was not demonstrated using the phosphoarray. It may be the level of protein suggested for the phosphoarray was too great to pick up these signalling alterations.

Fibroblasts are a well established model for investigating the mechanism behind growth disorders. However, there are limitations to this model as described in section 4.7 Concentrations of GH and IGF-I were determined in the laboratory prior to this particular study being undertaken. The experiments could all be undertaken at lower and higher levels of hormone; however, the concentrations that provided the greatest response were chosen in this particular study. An increased time course may provide a more robust example of GH and IGF-I signalling with samples taken every 5 minutes for an hour.

Expanded time courses will enable greater understanding of GH and IGF-I signalling in fibroblasts alongside variations in IGF-I and GH dose. A combination of GH and IGF-I should also be used in signalling experiments as in section 4.2.3 this combination produced significantly increased cell number. The IGF-IR should be sequenced, because despite comparable levels of IGF-IR protein, there may be mutations present which alter IGF-IR function. PHLPP1 and PHLPP2 activation assay should be undertaken to establish the cause of the significant increase in Akt2 activation.
Chapter 6  A metabolomic approach to characterising post-natal growth restriction using cells from SGA infants and AGA infants
6.1 Introduction

Metabolomics is the quantification of a large number of small molecules, including metabolic intermediates, sugars, lipids and amino acids, in a biological system, with metabolites being measured in serum, urine, tissue samples or culture media (Griffin and Nicholls 2006). Metabolomics is used to compare disease states, or to identify the effects of treatment regimes, and is becoming widely used to provide mechanistic understanding of many disorders. Metabolomic analysis was undertaken on cultured fibroblast cells and the media they were cultured in, taken from SGA and control, healthy children.

The aims of this aspect of the work were:

- To define a metabolomic profile characteristic of SGA children with no catch-up growth and,

- Using this profile, to determine the metabolic pathways that are altered in cells from children with no catch-up growth.

Metabolomic analysis was undertaken on cellular extracts from fibroblasts derived from four SGA children without post-natal catch-up growth, and four normal, healthy children. This sample, from the cellular extracts is called the fingerprint, and allows a snapshot of cellular metabolism to be obtained. As well as this cellular sample, the growth media harvested after 24 hours in culture of these cells was analysed. This is called footprint sample and contains the molecules that are available for, and are the results of, cellular metabolism, or the average metabolic change within a time period (Chen et al 2011).

Using both these sample types will provide a comprehensive view of the metabolome in SGA and control cells. If SGA children prove to have a specific metabolome profile compared to controls, further profiling in blood and urine will be warranted, which may aid detection of post-natal growth failure and, perhaps, enable an earlier clinical intervention.
Once fingerprint and footprint samples were obtained using the method described in section 2.3.2, gas chromatography mass spectroscopy was used to determine the levels of small molecule metabolites in the samples. This was performed very kindly by Dr Warwick Dunn of the Manchester Innovation Building. Once all the samples had been measured, statistical analysis was kindly performed by Dr Marie Brown, University of Manchester, using Matlab® (http://www.mathworks.com).

Exploratory multivariate analysis was performed using principal components analysis (PCA), which is an unsupervised approach that transforms a large set of related variables into a new, smaller set of independent variables, termed principal components (PCs) (Jolliffe and Morgan 1992). Each PC represents an axis in multidimensional space and corresponds to the direction of maximum variation of the original data. PCA was performed on data normalised to zero mean and unit variance, so that results were not dominated by a small number of high intensity peaks but gave equal weighting to peaks of low intensity.

6.2 SGA profile compared to control

18 metabolites in the fingerprint and 21 in the footprint from SGA samples were found to be significantly different in comparison to control cells (Tables 6.1 and 6.2).

In the fingerprint metabolome of SGA samples, 13 molecules were increased and 6 molecules were decreased compared to control samples (Table 6.1). The majority of increased molecules were amino acids, though levels of fructose, citric acid and phosphate were also significantly raised. As the fingerprint represents a snapshot of cellular metabolism, it appears that sugar metabolism and amino acid biosynthesis, utilisation and catabolism were altered in the SGA cells.
In the footprint sample, the majority of the molecules (17) were decreased in SGA compared to control samples. Only four molecules were higher in SGA than control cells (Table 6.2).

The footprint is an example of the average metabolic change in relation to the fingerprint. This could suggest the cells have an increased requirement for the 17 metabolites, which include amino acids, glucose and organic acids. The four increased molecules include amino acids, sugar derivatives and phosphate. To analyse this amount of data, a comparison was made between the results of the fingerprint samples and the footprint. A mixture of amino acids (both essential and non essential), phospho-inositol and sugars are present in the fingerprint and footprint samples and some of these are common to both the finger and footprint (Figure 6.1). Most of the overlapping molecules change in opposite directions; however aspartic acid and ornithine are both present in significantly higher levels in the fingerprint and footprint samples from SGA children compared to controls. These differences in the SGA samples are very significant (Tables 6.1 and 6.2); an individual metabolomic profile of non catch-up growth appears to be present.

6.3 Metabolic pathways

In this and the following sections it will be discussed the alterations in the SGA metabolome compared to controls in relation to the pathways each metabolite is involved in. Many molecules overlap in function and contribute to more than one pathway. The clear alterations in the SGA versus control cellular metabolism will then be discussed in relation to other metabolomic data in the literature.
<table>
<thead>
<tr>
<th>Increased metabolites</th>
<th>Fold change compared to control samples</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic Acid</td>
<td>7.143</td>
<td>0.000100766</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.714</td>
<td>0.000162429</td>
</tr>
<tr>
<td>Proline</td>
<td>3.125</td>
<td>0.000316921</td>
</tr>
<tr>
<td>Serine</td>
<td>1.786</td>
<td>0.000605172</td>
</tr>
<tr>
<td>Creatinine</td>
<td>3.125</td>
<td>0.000962324</td>
</tr>
<tr>
<td>Threonine</td>
<td>1.389</td>
<td>0.004986864</td>
</tr>
<tr>
<td>Citric Acid</td>
<td>1.563</td>
<td>0.004986864</td>
</tr>
<tr>
<td>Fructose</td>
<td>1.695</td>
<td>0.13005369</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.428</td>
<td>0.021966365</td>
</tr>
<tr>
<td>Phosphate</td>
<td>2.5</td>
<td>0.022102891</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.34</td>
<td>0.026387678</td>
</tr>
<tr>
<td>Glutamine</td>
<td>1.96</td>
<td>0.026387678</td>
</tr>
<tr>
<td>Ornithine</td>
<td>1.34</td>
<td>0.044538616</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Decreased metabolites</th>
<th>Fold change compared to control samples</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myoinositol</td>
<td>0.145</td>
<td>2.50E-07</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.474</td>
<td>0.001455492</td>
</tr>
<tr>
<td>Inositol</td>
<td>0.521</td>
<td>0.006041843</td>
</tr>
<tr>
<td>Glycerol-3-Phosphate</td>
<td>0.361</td>
<td>0.012314688</td>
</tr>
<tr>
<td>Inositol-1-Phosphate</td>
<td>0.704</td>
<td>0.01653304</td>
</tr>
<tr>
<td>Lactic Acid</td>
<td>0.704</td>
<td>0.044538616</td>
</tr>
</tbody>
</table>

**Table 6.1 Metabolome data: Fingerprint of SGA cells compared to control cells.**
Fingerprint samples were taken from four SGA and four control cell lines and were analysed by gas chromatography mass spectroscopy (GCMS) and p values were determined by Mann Whitney testing. Molecules are ranked from highest to lowest significance and are separated into increased metabolites (upper section) and decreased metabolites (lower section) compared to control samples.
<table>
<thead>
<tr>
<th>Increased metabolites</th>
<th>Fold change compared to control samples</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate</td>
<td>1.493</td>
<td>0.000119485</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>3.246</td>
<td>0.000216323</td>
</tr>
<tr>
<td>Cystine</td>
<td>1.451</td>
<td>0.00054396</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>1.162</td>
<td>0.009497973</td>
</tr>
<tr>
<td>Ornithine</td>
<td>1.136</td>
<td>0.033365736</td>
</tr>
<tr>
<td><strong>Decreased metabolites</strong></td>
<td><strong>Fold change compared to control samples</strong></td>
<td><strong>p value</strong></td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.313</td>
<td>3.1E-06</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.256</td>
<td>0.00162367</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.934</td>
<td>0.000272505</td>
</tr>
<tr>
<td>Pantothenic Acid</td>
<td>0.934</td>
<td>0.000499787</td>
</tr>
<tr>
<td>3-methylpentanoic acid</td>
<td>0.7</td>
<td>0.001337261</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.88</td>
<td>0.001462717</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.9</td>
<td>0.001707806</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.95</td>
<td>0.003550313</td>
</tr>
<tr>
<td>2-methyl-3-hydroxybutanoic acid</td>
<td>0.438</td>
<td>0.005759468</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.95</td>
<td>0.00832857</td>
</tr>
<tr>
<td>Glutamine</td>
<td>0.793</td>
<td>0.009497973</td>
</tr>
<tr>
<td>Fructose</td>
<td>0.465</td>
<td>0.012473986</td>
</tr>
<tr>
<td>Aminomalonic Acid</td>
<td>0.73</td>
<td>0.016901996</td>
</tr>
<tr>
<td>Hexonic Acid</td>
<td>0.8</td>
<td>0.017022319</td>
</tr>
<tr>
<td>Valine</td>
<td>0.85</td>
<td>0.032470181</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.85</td>
<td>0.043736484</td>
</tr>
<tr>
<td>Lactic Acid</td>
<td>0.858</td>
<td>0.01771488</td>
</tr>
</tbody>
</table>

**Table 6.2 Footprint Metabolomic Data:** Footprint samples (media conditioned for 24 hours) were obtained from four SGA and four control cell lines and analysed by GCMS. p values were determined by Mann Whitney testing. Molecules rank from highest to lowest significance and separated into increased metabolites (upper section) and decreased metabolites (lower section) compared to control samples.
Figure 6.1 Significantly increased and decreased metabolites in SGA finger and footprint samples compared to control samples.
Molecules in red indicate increased levels compared to controls (+), and molecules in blue indicate decreased levels compared to controls (-). * are p<0.001 (Mann Whitney analysis). Molecules in the centre rectangle are present in both the finger and footprint.
6.3.1 Sugars and fats

Glucose levels are significantly reduced in the SGA footprint sample compared to control (0.85 fold change; \( p=0.043 \)). This suggests an increase in glucose requirement in SGA metabolism. Glucose levels in the fingerprint sample from SGA cells are comparable to controls, which suggest increased glucose utilisation in the SGA cells.

Fructose is increased in the fingerprint sample of SGA cells (1.65 fold change; \( p=0.130 \)) and reduced in the footprint (0.465 fold change \( p=0.016 \)). This suggests an increase in cellular fructose requirement in the SGA samples.

Pyruvate levels are significantly increased in the footprint sample of SGA cells (1.162 fold change; \( p=0.009 \)) compared to control cells, suggesting that aerobic respiration, in particular glycolysis, is up-regulated. Up-regulated glycolysis would result in reduced glucose and increased pyruvate levels, which was indeed observed in the SGA footprint sample.

These data suggest an increased glucose and fructose requirement in the cell and an increase in glycolysis reflected by increased pyruvate levels in the footprint samples. Pyruvate feeds into the citric acid cycle for the next stage of aerobic respiration; therefore, an accumulation of pyruvate may be due to ineffective respiration in the SGA cells.

Indicators of increased aerobic respiration and decreased anaerobic respiration are present in samples from SGA cells compared to controls. Lactic acid levels (a product of anaerobic respiration) are reduced in both the finger and footprint samples from SGA cells when compared to control cells (0.704 and 0.858 fold change respectively).
Figure 6.2 The Glycolysis Pathway. Glucose is converted into 2 molecules of pyruvate by a series of biochemical reactions. Pyruvate is available to enter the TCA cycle under aerobic conditions, or is converted into lactic acid in anaerobic conditions. Highlighted molecules are altered in the SGA metabolome when compared to that of control cells.
Glycerol-3-phosphate (G3P), which is involved in gluconeogenesis, is decreased in SGA fingerprint samples when compared to control samples (0.361 fold change; p=0.012). Fat is metabolised into glycerol and G3P, which are responsible for maintaining the level of electrons for transfer to the mitochondrial electron transport chain. Decreased levels of G3P may indicate that fat or glycerol metabolism is decreased in the SGA cells. G3P can also be converted into pyruvate and, as pyruvate levels are increased in the footprint samples from SGA cells, it may be that glycolysis is increased to compensate for a decrease in normal fatty acid metabolism in SGA cells.

6.3.2 Sugar and fat metabolism summary

Energy requirement in the form of sugars appears to be higher in SGA cells compared to control cells, which is demonstrated by up-regulated glycolysis, perhaps due to reduced fat metabolism. For example:

- Higher glucose requirement for aerobic respiration demonstrated by decreased glucose levels, increased pyruvate and decreased lactic acid levels;
- Reduced fatty acid metabolism demonstrated by decreased G3P and increased pyruvate levels.

6.4 Amino acids

Ten amino acids in the fingerprint, and eleven amino acids in the footprint samples of SGA cells are present in abnormal levels when compared to control samples. Five of these (lysine, alanine, tyrosine, methionine and ornithine) are in both the finger and footprint samples. Alanine and ornithine share the same pattern as the finger and footprint, with alanine significantly reduced and ornithine significantly increased in the SGA samples in comparison to control. In contrast lysine, tyrosine and methionine are increased in the fingerprint sample but decreased in the footprint (see Table 6.3). Lysine is the only essential amino acid of these five; therefore, the increased levels of lysine in the fingerprint and
decreased levels in the footprint may indicate that the SGA cells have an increased requirement for its metabolism.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Fingerprint</th>
<th>Footprint</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Ornithine</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Lysine</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Methionine</td>
<td>↑</td>
<td>↓</td>
</tr>
</tbody>
</table>

*Table 6.3 Levels of alanine, ornithine, lysine, tyrosine and methionine in SGA samples compared to control samples*

6.4.1 Lysine

Lysine is incorporated into many key proteins. One of its important roles is as a pre-cursor to the fatty acid transporter carnitine, which transports fatty acids to the mitochondria for oxidation (Tanphaichitr and Broquist 1973). An increased level of lysine in the fingerprint sample of SGA cells may suggest a decrease in the incorporation of lysine into proteins, and by inference a reduction in total protein synthesis. Lysine residues within proteins are also targeted in the ER or golgi apparatus for O-glycosylation and therefore cellular excretion of protein (Furfaro *et al* 2012). Lysine’s role in transport may be important, as decreased transport out of the cell leaves less metabolite available for metabolism. Although if this was the case, levels of lysine would have been expected to be higher in the footprint samples of SGA cells compared to controls, and this was not observed.

6.4.2 Methionine

Methionine is increased in the fingerprint sample of SGA cells (1.34 fold change; p = 0.026) and reduced in the footprint sample (0.9 fold change; p = 0.001). The biosynthesis of methionine requires aspartic acid, and pyruvate is produced as a byproduct (Figure 6.3). The increased pyruvate described in the previous
Figure 6.3 Methionine biosynthesis and catabolism. Methionine is synthesized from aspartate, and requires other amino acids present in the metabolome including cysteine. The catabolism of methionine by transulfation involves glycine and serine, both amino acids altered in the SGA metabolome.
section may be a result of increased methionine biosynthesis/requirement.

Aspartic acid is increased in both the finger and footprint SGA samples, which again may be in response to an increase in methionine biosynthesis. It is impossible to know which process the excess methionine found in the fingerprint sample from SGA comes from, or whether it is due to an increased requirement by the cells compared to control cells. Improper conversion of methionine can lead to atherosclerosis (Jakubowski 2006); and because low birth weight individuals have increased risk of atherosclerosis it is important to discover what role the increased methionine is playing in the SGA samples. (Oren et al 2004). Methionine is an intermediate in the production of several key molecules including cysteine and phospholipids discussed later in this section.

6.4.3 Alanine

Alanine is produced by glutamine and pyruvate (Figure 6.4), and alanine levels are proportionate to those of pyruvate. This is interesting as pyruvate is increased compared to control cells in the footprint sample, but not the fingerprint sample obtained from SGA cells; whereas alanine is decreased in both sample types. Reduced alanine in both the finger and footprint suggests it is readily metabolised and degraded. Increased pyruvate levels may be present in an attempt to drive increased alanine production. Reduced alanine may also point to inadequate conversion of pyruvate to alanine via alanine transferase. Glucose and pyruvate requirement is greater in SGA cells; therefore, conversion to alanine is reduced as a result. Alanine is also a precursor to urea, and the decreased levels of alanine may be associated with increased urea production by the cells.
Figure 6.4 Glucose-Alanine cycle. Glucose is converted into pyruvate by glycolysis. Pyruvate is then converted into alanine, the most abundant protein in the circulation, by alanine transferase. Alanine is then taken up by tissues around the body, mainly the muscle, and can be converted back into glucose.
6.4.4 Tyrosine

Tyrosine is produced from the oxidation of phenylalanine. In the footprint samples from SGA cells, both tyrosine and phenylalanine are reduced compared to control samples; however, tyrosine is increased in the fingerprint sample. This suggests a shift towards oxidation of phenylalanine to produce tyrosine, potentially reflecting an increased requirement for tyrosine in SGA cells compared to control cells. Tyrosine is a component of proteins that are part of intracellular signal transduction processes as it is a receiver of phosphate groups. In the central nervous system, tyrosine is a key component of the neurotransmitters adrenaline and noradrenalin. This increase in intracellular tyrosine may indicate an increase in tyrosine signalling activity in the SGA cells. This was demonstrated in Chapter 5, where it appears that SGA cells have increased flux through the Akt pathway, specifically the activation of Akt2 by IGF-1, which does not occur in control cells. Additionally, in Chapter 4, an increase in apoptosis was demonstrated in SGA cells compared to control cells, which translates to an increase in signalling in the pathways promoting apoptosis. Tyrosine phosphorylation occurs on numerous molecules in numerous signalling pathways; this increase cannot be related to a specific pathway. However, it may be a direct result of the aforementioned signalling alterations found in SGA cells compared to controls.

6.4.5 Threonine and serine

Threonine and serine are significantly decreased in the SGA fingerprint samples compared to control samples. They are both essential amino acids, are the building blocks of many proteins, and are important phosphorylation sites for many signalling molecules. The decreased levels of these amino acids in the SGA fingerprint samples may result in a decrease in the proteins these amino acids are incorporated into. The signalling pathways previously investigated (Chapter 5) did not demonstrate reduced levels of signalling molecules; however, only a minority of signalling molecules were analysed which may not have been affected by this reduction of threonine and serine levels. Noting that tyrosine is increased in SGA cells, an imbalance in signalling may be occurring,
with a shift towards pathways featuring tyrosine containing proteins or tyrosine phosphorylation.

6.4.6 The urea cycle

Ornithine is produced from glutamate and is a key component in the urea cycle. The urea cycle removes excess nitrogen from the cell (Figure 6.5), and as both the SGA finger and footprint samples have increased levels of ornithine compared to control samples, it appears that the urea cycle may be up-regulated in SGA cells. It can be speculated this may be due to increased levels of nitrogen in the SGA cells, due to an increase in general metabolism compared to control cells as demonstrated previously. Other components of the urea cycle, glutamic acid and aspartic acid, are also altered in the SGA samples.

Aspartic acid is a non-essential amino acid that is required for the excretion of ammonia via the urea cycle. It is produced by the conversion of citruline into arginosuccinic acid by argininosuccinate synthase in an ATP dependent step. Glutamic acid and ornithine are also increased in SGA fingerprint samples, which suggest an up-regulation of the urea cycle. Glutamine is the most abundant amino acid in the circulation and is responsible for transporting ammonia from the tissues to the kidneys. It is increased in the fingerprint sample from SGAs and this may be expected as the urea cycle appears to be up-regulated in these samples. However, glutamine is significantly reduced in the footprint sample of SGA cells. This could represent an increased requirement by the SGA cells compared to control cells.

As previously discussed, alanine (which is converted into urea, Figure 6.4) is decreased in the SGA finger and footprint samples, which provides another indication for an up-regulation of the urea cycle. Increased levels of glutamine in the footprint sample to represent the removal of nitrogen from the cell could be expected; however, in vitro the removal of substances from the cell into the media may not accurately represent tissues removing substances into the circulatory system.
Figure 6.5 The Urea Cycle. Diagram showing the production of urea in a mammalian cell. * Highlighted molecules are up-regulated in the fingerprint sample from SGA cells, and in addition glutamic acid is down-regulated in the footprint sample from SGA cells compared to controls.
6.4.7 Valine

Valine is an essential amino acid which is decreased in the SGA footprint sample compared to control cell samples. It belongs to the branched chain amino acid group which makes up 70% of amino acids in the body. Valine is known to be involved in the breakdown of ammonia, working alongside α-ketobutyrate. As levels of valine are reduced in the SGA footprint sample, there may be an increase in the cellular requirement for valine, potentially, as a direct result of the apparent up-regulation of the urea cycle in SGA cells.

6.4.8 Glycine

Glycine is significantly reduced in the SGA footprint sample compared to that of control cells. Glycine is synthesised from serine and is a precursor of porphyrins and a key part of all purines and therefore DNA (Figure 6.6). Glycine is also an inhibitory neurotransmitter. A reduction in glycine may suggest an increased requirement in the cell; however, this does not correspond with an increase in the fingerprint sample, suggesting increased metabolism of glycine in the cell.

Glycine is a product of methionine catabolism. Reduced glycine in SGA cells reflects a reduction in methionine catabolism, as demonstrated by increased methionine levels in SGA footprint samples compared to controls.

6.4.9 Cysteine

Cysteine is synthesised from serine and methionine and has an easily oxidised thiol group (Figure 6.6B). Cysteine is reduced in the SGA footprint sample when compared to control samples, which is interesting as serine levels are increased in the fingerprint samples. An increased demand for cysteine may be present in the SGA cells, which corresponds with the observed increase in methionine.
Figure 6.6 Glycine and cysteine biosynthesis. A) Glycine is synthesized from serine by the enzyme serine hydromethyltransferase. B) Cysteine is synthesized from serine and homocysteine (from methionine synthesis).
6.4.10 Amino acid summary

The synthesis, metabolism and catabolism of amino acids are altered in the SGA metabolome. For example:

- Increased aerobic respiration demonstrated by decreased alanine levels,
- Up-regulated urea cycle demonstrated by decreased alanine levels and increased ornithine, aspartic acid, glutamic acid, glutamine and valine levels,
- Increased methionine biosynthesis and reduced catabolism demonstrated by increased methionine, aspartic acid and pyruvate levels and decreased glycine levels,
- Altered fatty acid metabolism/transport demonstrated by decreased lysine levels and increased creatinine and decreased cysteine levels.

6.5 Inositols

Myoinositol is present in the SGA fingerprint sample in significantly lower levels than the control sample. Inositol-1-phosphate levels are also reduced compared to controls; however, inositol itself is higher in the SGA fingerprint samples. Inositol is a cyclic alcohol, with a stable structure, which readily crystallises and can form up to nine stereo-isomers. The most abundant of these is myoinositol, so named by its presence in muscle tissue. Myoinositol is synthesised from glucose. Glucose-6 phosphate is isomerised to inositol-1-phosphate which is dephosphorylated to give myoinositol. Inositols are involved in the IGF-I and insulin signalling pathways as they are incorporated into phosphatidylinositol-3-kinase, a key secondary messenger in Akt activation, and many other proliferative and anti-apoptotic pathways.

In Chapters 4 and 5, SGA cells were shown to have altered IGF-I actions, including a reduction in the proliferative response to IGF-I and activation of Akt2 by IGF-I. The reduced levels of myoinositol and inositol-1-phosphate, and the increase in inositol levels may be related to the changes in IGF-I signalling in SGA cells.
6.6 Acids

Aminomalonate was found to be significantly decreased in the footprint samples from SGA infants when compared to control samples. Aminomalonate imparts calcium binding properties to other proteins and is found in atherosclerotic plaques (Jakubowski 2006). It is also present when there are errors in protein synthesis. A pattern is emerging regarding fatty acid and glycerol metabolism in the SGA samples: decreased aminomalonate, alongside decreased myoinositol, glycine and increased methionine and lysine are a profile for altered/abnormal fat metabolism. Also, levels of pantothenic acid or vitamin B5 are significantly reduced in the SGA footprint sample compared to controls.

6.7 Summary

Samples from SGA infants without post-natal catch-up growth have a distinct metabolomic profile compared to control samples. This suggests that cells from SGA children have:

- Up-regulated urea cycle
- Higher glucose requirements
- Increased methionine biosynthesis and decreased catabolism
- Altered cell signalling
- Altered fatty acid metabolism

6.8 Discussion

Components of the metabolomic profile found to be altered in the SGA samples have been pinpointed in other metabolome studies. In a metabolomic analysis of urine in neonates born SGA, alterations were demonstrated in the urea cycle and in myoinositol levels (Dessi and Fanos 2013). Myoinositol was significantly increased in the urine of SGA babies. A study from Nissen et al (2010)
undertook metabolomic analysis of serum from IUGR piglets and found increased levels of myoinositol and d-chorio-inositol compared to non-IUGR piglets. These data are in contrast to the current findings; however, the sample type and species are different. An anomaly in inositol metabolism is present and further studies in SGA individuals need to be undertaken to determine serum and urine levels of inositol and myoinositol.

Metabolomic investigations in peroxisome proliferator-activated receptor-induced fatty acid metabolism in murine hepatocytes demonstrated reduced levels of vitamin B5 when compared to normal/non PPAR induced fatty acid metabolism (Patterson et al 2009). The reduction in vitamin B5 levels in the current experiment may reflect a shift towards PPAR induced fatty acid metabolism, which may reflect the suggested alteration in fatty acid synthesis. It is interesting to note PPAR can regulate amino acid synthesis and the urea cycle, both of which are increased in the SGA cells compared to control cells. It may be worth looking at the protein level of PPAR in this study to see if PPAR activity or level is increased; this would explain some alterations mentioned earlier.

A metabolomic study of fetal tissue samples from primates fed a high fat diet demonstrated increased levels of myoinsitol compared to controls (Cox et al 2009). It appears that myoinositol levels reflect nutrient/energy transfer across the placenta. Nutrient transfer across the placenta is known to affect fetal growth and potentially the SGA cohort in this study had a reduction in available nutrients/growth factors during gestation.

Reduced cysteine and increased valine and creatinine levels are all associated with the metabolomic profile of type II diabetes (Griffin and Nicholls 2006). In the SGA samples, creatinine levels are raised; however, both cysteine and valine levels are reduced. Although none of the SGA infants currently have diabetes, such children are known to be at higher risk of developing type II diabetes at a later date (Barker 1997). Sharing parts of a deleterious metabolomic pathway may reflect the increased risk of developing type II diabetes in SGA individuals.
A paper by Li et al (2010) recently demonstrated that mice with hepatic insulin resistance have a similar metabolomic profile to that of the SGA children demonstrated in this study. Intermediates of the urea cycle were increased, which they found to be consistent with increased de-amination of amino acids used for gluconeogenesis (Li et al 2010, Thorn et al 2009). Also, the pattern of methionine and myoinositol levels corresponds to those of the SGA group. Despite this study being conducted in obese mice (ob/ob), the similarities cannot be ignored. Hepatic insulin resistance in known to precede type II diabetes, and finding this pattern in the samples from SGA cells suggests a predisposition to insulin resistance.

Improper conversion of methionine can lead to atherosclerosis (Jakubowski 2006); therefore, it is important to discover the consequences of methionine levels in the SGA children as low birth weight individuals have increased risk of atherosclerosis (Oren et al 2004).

Recent metabolomic investigations (Halama et al 2011) of cancer cell lines associated changes in aspartate, methionine, alanine and glycine levels as indicators of apoptotic processes. In this experiment increased levels of aspirate, glutamate and methionine were demonstrated. In Chapter 4 increased levels of apoptosis were demonstrated in SGA cells compared to control cells, and the metabolomic profiling appears to reflect this.

It is important to note how different biological samples have different metabolomic profiles, even in disease states. Chen et al (2011) undertook a metabolomic analysis of urine and serum from patients with hepatocellular carcinomas. 43 molecules in the urine and 31 in the serum were found to be significantly different between affected and non-affected individuals. Only five out of these overlapped between serum and urine. Measuring urine and serum metabolome provides sample specific markers, but in more complex scenarios, a combination of markers in sample types may be also required.
Metabolomics as a technique is still relatively new and although currently there are no documented ‘rogue’ metabolites that are altered in every scenario, this is a possibility in the future.

Metabolomics has provided a profile of metabolism unique to an individual, disease and syndrome, which has not been previously undertaken in SGA children with no catch-up growth. In the future urine and serum samples can be utilised to obtain readily accessible profiles. Currently, post-natal growth restriction in the SGA infant cannot be predicted, and metabolomics may result in the identification of biomarkers that predict failure of catch-up growth and would allow earlier or more effective treatment of their growth failure.
Chapter 7  General discussion
Around 10,000 babies a year are born SGA, and out of those ~1500 will fail to
demonstrate post-natal catch-up growth and be eligible for rhGH. The reason for
pre- and post-natal growth failure are unknown and response to rhGH treatment
is variable (Ranke et al 2003). Insights into the molecular mechanisms involved
in growth in the SGA child are important to improve the final height and
metabolic outcome of these individuals.

In this set of experiments the aims were:

1) Asses cellular proliferation and apoptosis, and the affect of GH and IGF-I and
   a combination of GH and IGF-I on these processes.

2) Identify the activation of GH and IGF-I signalling pathways.

3) Investigate the metabolic status of SGA individuals and develop a
   metabolomic profile of post-natal growth restriction.

The following is a discussion of the results from the experiments performed to
address these aims.

1) Cell turnover was altered in SGA cell compared to controls. IGF-I stimulated
   proliferation was significantly reduced, apoptosis was significantly increased in
   both basal, GH and IGF-I treatment conditions. Altered or reduced cell turnover
   at the molecular level has not been documented to date in post-natal growth
   restriction; however, increases in cell turnover are documented in GH excess
   presented by increased bone and soft tissue growth (Renehan and Brennan
   2008). Reduced proliferation and increased apoptosis may be contributing
   factors to reduced post-natal growth. Investigations into key pathways
   controlling proliferation and apoptosis need to be undertaken to establish the
   pathway or molecules involved in this alteration in cell turnover in SGA cells.

2) Alterations in both GH and IGF-I signalling were observed in cells from
   SGANCU individuals compared to AGA controls. Reduced levels of GH

171
stimulated Stat5b activation was demonstrated, which is important to note as Stat5b is the main transcriptional activator of IGF-I, a key hormone in post-natal growth promotion. This reduction in Stat5b activation by GH may be a causative factor in the post-natal growth failure seen in these individuals. IGF-I production in these cells should be measured in the future to determine if this reduction in Stat5b activation in these cells is altering the production of IGF-I.

Alterations in the pattern of IGF-I stimulated Akt activity was observed, and Akt2 was phosphorylated in SGA cells and not at all in AGA cells. There is no data in the literature regarding IGF-I activation of Akt2. Decreased activation of Akt2 by insulin is present in muscle cells from type II diabetic individuals (Cozzone et al 2008). If SGA individuals are at increased risk of type II diabetes, it is possible our SGA also present this pattern. IGF-I may be activating Akt2 due to suboptimal insulin activation. Repeats of the immunoprecipitation experiments to assess activation of the Akt isoforms should be undertaken using Insulin stimulation. The majority of the literature portrays the Akts as anti-apoptotic and proliferative. One paper presents p21 binding to activated Akt2, and initiating cell cycle arrest (Heron-Milhavet et al 2006). The role of p21 is controversial, with some papers suggesting it initiates apoptosis and others cell cycle arrest (Gartel 2005). It is relevant to note that SGA cells have increased apoptosis, as this may be the result of increased activation of Akt2 as discussed earlier. Further studies investigating the interaction of Akt2 with p21 in these cells should be undertaken to determine if both alterations are linked.

3) Both SGACU and SGANCU infants had fasting glucose levels above the normal range indicating a degree of impaired glucose fasting. However, in the SGACU the levels of glucose were significantly higher. Higher fasting levels of glucose reflect the negative effect catch-up growth has on carbohydrate metabolism, and highlights a potential risk of insulin resistance in this group. Adiponectin results in this group reflected those published; adiponectin levels are inversely proportionate to catch-up growth. The significantly higher levels of adiponectin seen in the SGANCU group are a predisposing factor to central mass adiposity and obesity. These differences in the biochemical investigations in SGACU infants compared to SGANCU infants suggest the SGACU group are
already showing risk factors of developing metabolic syndrome later in life, similar to those documented in the literature (Polo Perucchin et al 2011).

SGA cells have a significantly different metabolomic profile compared to controls. Areas of the metabolism in SGA affected include: the urea cycle, fatty acid metabolism, aerobic and anaerobic respiration, inositol metabolism, amino acid utilisation and cell signalling mediators. The profile also carries components of profiles seen in type II diabetes.

The strikingly different profile in SGA cells is potentially a marker of post-natal growth restriction. Continuing these studies in urine and serum, and from as early as birth, may reveal a prediction model for catch-up growth which would be beneficial to the individual and their family.

The sets of experiments presented here have opened up many new avenues of research into the mechanism behind post-natal growth in children born small. The molecular insights into the post-natal growth failure of individuals born small could eventually aid in the prediction and treatment of these individuals.
Chapter 8  References


